THE ENDOCANNABINOID SYSTEM AS A NOVEL TARGET IN THE PATHOPHYSIOLOGY AND TREATMENT OF DEPRESSIVE ILLNESS

by

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B.Sc., The University of British Columbia, 2002
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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Psychology)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

August 2008

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ABSTRACT

The endocannabinoid system is a neuromodulatory system which has recently gained attention in the pathophysiology and treatment of depressive illness. However, to date, the research investigating these relationships has been sparse. This dissertation aimed to further this understanding by examining the extent to which the activity of the endocannabinoid system (1) responds to a variety of regimens which reduce depression, (2) responds in an animal model of depression, and (3) differs in humans diagnosed with major depression. In Chapter 2 it was demonstrated that the cannabinoid CB₁ receptor is upregulated in the hippocampus and hypothalamus following long-term treatment with the tricyclic antidepressant desipramine. In addition, it was also found that this increase in CB₁ receptor activity contributed to the stress-attenuating effects of desipramine, as pharmacological antagonism of the CB₁ receptor prevented the ability of desipramine treatment to suppress activation of the hypothalamic-pituitary-adrenal axis in response to stress. In Chapter 3, it was found that providing rodents with free access to a running wheel for 8 days resulted in a robust increase in hippocampal endocannabinoid signaling. This increase in endocannabinoid activity was, in turn, required for voluntary exercise to increase the proliferation of progenitor cells in the dentate gyrus of the hippocampus. In Chapter 4 it was revealed that repeated exposure of rodents to electroconvulsive shock evokes a coordinate sensitization of amygdalar CB₁ receptor signaling with a suppression of prefrontal cortical endocannabinoid activity. In Chapter 5 it was demonstrated that subcortical endocannabinoid activity is dampened in the chronic unpredictable stress model of depression, while prefrontal cortical CB₁ receptor binding is increased. Concurrent treatment with the antidepressant imipramine was capable of reversing some,
but not all, of these changes. In Chapter 6, it was found that the circulating content of the endocannabinoid 2-arachidonoylglycerol was significantly reduced in women diagnosed with major depression. These data collectively provide evidence for the hypothesis that deficient endocannabinoid signaling may be involved in the etiology of depression, and that pharmacological augmentation of endocannabinoid neurotransmission may be a suitable target for the development of novel antidepressants.
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<th>Description</th>
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<tbody>
<tr>
<td>2-AG</td>
<td>2-arachidonoylglycerol</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AEA</td>
<td>N-arachidonylethanolamine (anandamide)</td>
</tr>
<tr>
<td>AMPA</td>
<td>Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>B&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximal binding site density</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>Celsius</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CMS</td>
<td>Chronic mild stress</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CON</td>
<td>Control</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotropin releasing hormone</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CUS</td>
<td>Chronic unpredictable stress</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DES</td>
<td>Desipramine</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DSM-IV</td>
<td>Diagnostic and statistical manual of mental disorders-IV&lt;sup&gt;th&lt;/sup&gt; edition</td>
</tr>
<tr>
<td>ECS</td>
<td>Electroconvulsive shock</td>
</tr>
<tr>
<td>ECT</td>
<td>Electroconvulsive therapy</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>FAAH</td>
<td>Fatty acid amide hydrolase</td>
</tr>
<tr>
<td>FGF2</td>
<td>Fibroblast growth factor-2</td>
</tr>
<tr>
<td>G</td>
<td>Gram</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GCL</td>
<td>Granule cell layer</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptors</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>H</td>
<td>Hours</td>
</tr>
<tr>
<td>HAM-D</td>
<td>Hamilton rating scale for depression</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-pituitary-adrenal</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IMI</td>
<td>Imipramine</td>
</tr>
<tr>
<td>Ir</td>
<td>Immunoreactive like</td>
</tr>
<tr>
<td>K&lt;sub&gt;d&lt;/sub&gt;</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>KPBS</td>
<td>Potassium phosphate buffered saline</td>
</tr>
<tr>
<td>M</td>
<td>Molar units</td>
</tr>
<tr>
<td>MAG</td>
<td>Monoacylglycerol</td>
</tr>
<tr>
<td>MAP</td>
<td>Microtubule associated protein</td>
</tr>
<tr>
<td>MAO-I</td>
<td>Monoamine oxidase inhibitor</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium Chloride</td>
</tr>
<tr>
<td>Min</td>
<td>Minutes</td>
</tr>
<tr>
<td>MR</td>
<td>Mineralocorticoid receptors</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NRI</td>
<td>Norepinephrine reuptake inhibitor</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal cortex</td>
</tr>
<tr>
<td>PI3-K</td>
<td>Phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus of hypothalamus</td>
</tr>
<tr>
<td>S</td>
<td>Seconds</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SSRI</td>
<td>Selective serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricyclic antidepressant</td>
</tr>
<tr>
<td>THC</td>
<td>Tetrahydrocannabinol</td>
</tr>
<tr>
<td>TME</td>
<td>Tris-HCl- MgCl₂.Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vasoactive endothelial growth factor</td>
</tr>
<tr>
<td>VEH</td>
<td>Vehicle</td>
</tr>
<tr>
<td>VEx</td>
<td>Voluntary exercise</td>
</tr>
<tr>
<td>Vₘₐₓ</td>
<td>Maximal hydrolytic velocity</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

The current dissertation is the culmination of work that has been performed over
the past four years, to which the accomplishment of, could not have been completed
without the help of others. First and foremost, this dissertation would never have
culminated without the guidance and assistance of my “academic parents”. Unlike most
graduate students, I was fortunate enough to have direction and input from two
supervisors. I must acknowledge Dr. Boris Gorzalka, my graduate supervisor and friend
who has put me up for eight years and took me on as only a second year undergraduate.
Boris has given me guidance, trust, support and independence to allow me to discover my
niche in the research world and shape who I am as a researcher today. Equally as
important to my development has been another close friend and collaborator, Dr. Cecilia
Hillard, who has been my mentor through my entry into the world of cannabinoid
research. Cece took me under her wing, welcomed me into her laboratory and has
provided me with opportunities, knowledge, and scientific training. I know we have years
of collaborative work to continue, but I must reiterate my gratitude to both of these
individuals for everything they have given me and helped me with. In addition to my
supervisors, I must also thank Dr. Victor Viau and Dr. Greg Miller for the hours of time I
stole from them discussing research and their input to my ideas and thoughts. During the
years I have been a graduate student at UBC, I have also collaborated with several other
researchers who have both influenced my research and helped me get through the various
milestones of becoming a scientific researcher. Accordingly, I send out a thanks to Drs.
Liisa Galea, Joanne Weinberg, Cathy Rankin, Stan Floresco, Brian Christie and John
Pinel for all their assistance and discussion throughout the last six years. Within the
laboratory, there are so many people who have devoted their time and sweat to my research, and whom I am happy to call my friends; thanks for everything Ryan McLaughlin, Anna Morrish, Tiffany Lee, Jas Kambo, Stephanie Lieblich, Katia Sinopoli, Christine Mazzucco, Jane Sun, Sachin Patel, Erica Carrier, Vanessa Ho, Brenda Bingham, Martin Williamson, Andrea Titterness, Larissa Froese and Sarah Meier.

Outside the laboratory, many others in my life have helped me get to where I am and are due credit within these acknowledgements. I would not be here if not for my parents. All their support and kindness over the years have turned me into who I am today; my brother Dave, for his unending generosity (it doesn’t go unnoticed); and my ever supportive friends Nigel, John, Donnie, Keena, Jenny Jones and Josh. Last, but obviously not least, I have to thank my wife, Orsha Magyar, for her enduring love, support and devotion. You give me the confidence I need to push through and the friendship I need to survive.
CO-AUTHORSHIP STATEMENT

The biochemical protocols used throughout this thesis were provided by Dr. Cecilia Hillard, who also contributed ideas to the discussion of all the chapters encompassed within this thesis.

The protocol for immunohistochemical analysis of c-fos was provided by Dr. Victor Viau, and was performed with his assistance (Chapter 2).

The analysis of endocannabinoid content in extracted methanol fractions employing liquid chromatography/mass spectrometry was performed by Dr. Vanessa Ho (Chapters 2, 4 and 6) and Dr. Erica Carrier (Chapters 3 and 5) using a protocol designed by Dr. Sachin Patel (detailed in Chapter 2).

The activity assays for the hydrolysis of anandamide by fatty acid amide hydrolase were performed by Sarah Meier (Chapters 3, 4 and 5) and Ryan McLaughlin (Chapter 5).

For Chapters 2 and 5, Anna Morrish assisted with all behavioral testing and stress induction procedures.

For Chapter 3, Anna Morrish, Andrea Titterness and Tiffany Lee assisted with the immunohistochemical analysis of Ki-67+ cells, and Dr. Brian Christie assisted with employment of running wheels and contributed ideas to the discussion of this manuscript.

For Chapter 4, Dr. Alasdair Barr performed the electroconvulsive shock treatment on the rodents.

For Chapter 6, Dr. Greg Miller provided the serum samples for endocannabinoid analysis, assisted with the data analysis of these variables and contributed to ideas in the discussion of this manuscript.
CHAPTER I

GENERAL INTRODUCTION

Major depression is a devastating disease that affects approximately 8% of men and 15% of women (Kessler et al., 1994). In 75% of cases of depression, the disease course is recurrent, and is manifested as multiple cycles of remission and exacerbation (Frank and Thase, 1999). The major symptomatic disturbances in depression occur in the emotional, motivational, cognitive and neurovegetative realms. The typical emotional disturbances in depression include depressed mood, often associated with feelings of guilt, low self-esteem and worthlessness, and high anxiety (DSM-IV, 2000). A loss in pleasure for most, if not all, daily activities, or “anhedonia”, is considered a hallmark symptom of depressive illness; in fact, the presence of either depressed mood or anhedonia is a prerequisite for the diagnosis of a depressive episode (DSM-IV, 2000). Cognitively, impairments in memory and concentration are often seen, and most major homeostatic systems (such as feeding, sleeping and reproductive drive) exhibit some form of disturbance (DSM-IV, 2000). Albeit, while the symptomatic expression of depressive illness occurs within common facets, the manifestation of this disease can vary significantly between individuals. This variance of symptom patterns argues that despite common diagnostic descriptions, depression is not a unitary disease, but a very heterogeneous psychopathology that can take on very diverse phenotypes. At both a personal and financial level, depressive illness has become the mental disease which results in the greatest burden and disability in North America (McKenna et al., 2005).
1.1 CURRENT STATUS OF ANTIDEPRESSANT TREATMENTS: FOCUS ON MONOAMINERGIC NEUROTRANSMISSION

Much of the current understanding of the neurobiology of depression was obtained from serendipitous findings, such as the discovery that pharmacological agents which increase synaptic levels of monoamines improve mood. This finding lead to both the monoamine hypothesis of depression (Schildkraut, 1965; that depression was a disease characterized by reduced monoamine activity in the brain) and the development of several classes of antidepressant agents which enhance monoamine activity through inhibition of enzymatic degradation or presynaptic uptake. The first class that was clinically used was the monoamine oxidase inhibitors (MAO-I’s), which act to inhibit monoamine oxidase, an enzyme responsible for monoamine catabolism. While MAO-I’s did demonstrate efficacy as antidepressants, they are also toxic and poorly tolerated (Vida and Cooper, 1999). These drugs were largely replaced by the tricyclics (TCA’s), which act non-selectively to inhibit uptake of serotonin, norepinephrine and dopamine. These drugs also affect histaminergic and cholinergic systems and cardiac ion channels (Schatzberg, 2002), and, like the MAO-I’s, also lead to a constellation of toxicity problems and are lethal in overdose (Vida and Cooper, 1999). This was followed by the development of selective serotonin reuptake inhibitors (SSRI’s) and norepinephrine reuptake inhibitors (NRI’s) which are less toxic, yet exhibited other adverse side effects including sexual dysfunction, nausea and agitation (Meston and Gorzalka, 1992; Montgomery et al., 2002; Vida and Cooper, 1999). However, to date there is no class of antidepressant drug that leads to sustained remission in the majority of depressed
patients, and all of the currently available agents are limited by the fact that they take several weeks of administration to elicit clinical benefit (Thase, 2003).

In addition to pharmacotherapy, several somatic regimens have also been found to possess antidepressant properties, some even with a more rapid onset of action than chemical agents offer. In particular, electroconvulsive shock treatment (ECT) was the first therapeutic tool utilized by psychiatry to treat depression in the 1930's. While ECT has declined significantly in therapeutic use, due to the invasiveness of the procedure, it is still frequently employed to treat individuals with severe, treatment-resistant depression (Silverstone and Silverstone, 2004). Temporally, however, ECT begins to exert therapeutic benefit in as little as 5-7 days relative to the 2-8 weeks required by conventional antidepressants (Thase, 2003). Another very rapid somatic treatment for depression is sleep deprivation. Clinically, it has been found that total sleep deprivation for one night can result in a rapid improvement in depressive symptoms in between 40-60% of afflicted individuals (Giedke and Schwarzler, 2002). While this response only lasts on the order of days, it is the most rapid therapeutic option available for depression. A less invasive and more accessible therapeutic option has also been demonstrated through the clinical employment of physical activity and exercise (Blumenthal et al., 2007; Dunn et al., 2005). The mechanisms by which these somatic regimens elicit their antidepressant effects are not well characterized, but determination of these targets may aid in fostering rapid onset pharmacotherapeutic options for depressive illness.

Monoamine altering antidepressant agents induce an immediate elevation in synaptic monoamine levels, but take weeks to elicit behavioral changes, suggesting that an immediate enhancement of synaptic monoamine activity is not the direct mechanism
of action of antidepressants (for review see Hindmarch, 2001). If depression were exclusively due to a reduction in monoamines, one would expect to see a rapid and effective reversal to the pre-morbid state following pharmacotherapy, which is rarely, if ever, documented in clinical trials (Nelson et al., 2004). Consistent with this discrepancy, while depletion of the 5-HT precursor tryptophan has been shown to promote a depressive relapse in remitted individuals, tryptophan depletion in healthy individuals has not been demonstrated to evoke depressive shifts in mood (van der Does, 2001).

Thus, despite the fact that all conventional antidepressant drugs target the monoaminergic system, there is little evidence to support the argument that depression is a disease causally linked to a reduction in monoaminergic neurotransmission. In fact, the current dogma on the clinical efficacy of antidepressant drugs is that there is a neuroadaptive change that occurs, either at the cellular, intracellular or genetic level, in response to prolonged enhancement of monoaminergic signaling that is responsible for the antidepressant responses to these agents. Accordingly, determination of neural systems which are regulated by protracted antidepressant treatments is a commonly used strategy in preclinical research to establish which systems are responsible for contributing to the clinical efficacy of conventional antidepressants, and thus help to identify novel target systems that could be exploited for drug discovery. For example, prolonged administration of most classes of antidepressants downregulate 5-HT1A autoreceptors and cortical 5-HT2A receptors, suggesting that decreasing activity at these receptors may be functionally relevant for the actions of antidepressants (Blier, 2001; Marek et al., 2003). This hypothesis is supported by the finding that administration of either a 5-HT1A or 5-HT2A receptor antagonist as an adjunct to conventional antidepressant treatment...
augments the rate of remission (Artigas et al., 1996; Blier, 2001; Marek et al., 2003; Sokolski et al., 2004). Thus, examining systems that are regulated by long-term antidepressant treatment may aid in the development of faster acting, more selective antidepressant agents.

1.2 NEUROBIOLOGICAL THEORIES OF DEPRESSION: THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS, NEUROPLASTICITY AND THE HIPPOCAMPUS

The limited efficacy of current therapeutic options for depression has resulted in the search for novel, non-monoamine based targets. One system that has received particular attention is the hypothalamic-pituitary-adrenal (HPA) axis, the neuroendocrine bridge which converts stress-induced neuronal signaling to peripheral secretion of glucocorticoid hormones. Activation of the HPA axis is integrated in the paraventricular nucleus (PVN) of the hypothalamus, which contains a large body of corticotrophin releasing hormone (CRH) secretory neurons that project to the median eminence. Stimulation of these neurosecretory cells releases CRH into a local portal vessel that stimulates adrenocorticotropic hormone (ACTH) to be released from the anterior pituitary into the general circulation. ACTH then travels to the adrenal cortex and stimulates the synthesis and release of glucocorticoids, primarily cortisol in humans and corticosterone in rodents, which in turn both bind to mineralocorticoid (MR) and glucocorticoid receptors (GR).

The idea that HPA axis dysfunction may play a critical role in the manifestation of depression is based on several important findings. First, CRH immunoreactivity is up regulated in the CSF of depressed patients (Nemeroff et al., 1984; Widerlov et al., 1988)
as well as in the locus coeruleus (Bissette et al., 2003) and peripherally in the plasma (Catalan et al., 1998). Consistently, it has been found that CRHR-1 receptors in the prefrontal cortex of depressed patients are also down regulated, a predictable response to hypersecretion of CRH (Nemeroff et al., 1988). Elevated basal cortisol levels in both the CSF and the blood are found in approximately two thirds of depressed patients, more frequently in patients suffering from a severe form of depressive illness (reviewed in Holsboer, 2000; Murphy, 1991; Parker et al., 2003). Furthermore, diseases which result in hypercortisolemia, such as Cushing's disease, display many symptoms which are similar to those observed in major depression (Murphy, 1991; Starkman et al., 1981). While detailed investigations of central GR binding are lacking in depressed patients, one study has found that frontocortical levels of GR mRNA are significantly reduced in major depression (Webster et al., 2002).

Evidence that long term treatment with antidepressant agents alters HPA axis functioning further supports the functional relevance of HPA axis disturbance in depression (Barden et al., 1995; Budziszewska, 2002). Long term treatment with all pharmacological classes of antidepressants and electroconvulsive shock has been shown to upregulate GR mRNA, binding or immunoreactivity in the brain (Budziszewska et al., 1994; Pepin et al., 1989; Przegalinski et al., 1993; Seckl and Fink, 1992). Consistently, treatment with multiple classes of antidepressants has been shown to result in reductions in both basal and stress-induced CRH and glucocorticoid release (Brady et al., 1991, 1992; Connor et al., 2000; Fadda et al., 1995; Reul et al., 1993, 1994) and down regulation of CRHR-1 receptors (Aubry et al. 1999), suggesting that despite the mechanism by which they act, all classes of antidepressants target the HPA axis in some
manner. Because these effects occur only after repeated antidepressant administrations, and not immediately, they correlate well with the documented therapeutic lag characteristic of typical antidepressant agents (Brady et al., 1991, 1992; Connor et al., 2000). In line with these findings, CRHR-1 and GR antagonists have both been found to reduce depression in clinical trials (Murphy, 1997; Wolkowitz and Reus, 1999; Wolkowitz et al., 1999; Zobel et al., 2000) and in pre-clinical animal models of depression (Alonso et al., 2004; Healy et al., 1999; Korte et al., 1996; Overstreet and Griebel, 2004). These data argue that hyperactivity of the HPA axis may be a prognosticating factor for depression in some individuals, and that attenuation of HPA axis responsivity may be one of the long-term adaptations in response to chronic administered antidepressants that contributes to their therapeutic efficacy.

Mechanistically, hypersecretion of glucocorticoids may contribute to the etiology of depression via its modulation of structural and functional plasticity in the hippocampus (McEwen, 2005; Sheline et al., 1999). Several reports have demonstrated that individuals suffering from depression exhibit significant atrophy of the hippocampus, a phenomenon that is found to correlate strongly with the chronicity of the disease (Sheline et al., 1999). Preclinical studies have revealed that glucocorticoids have adverse effects on the hippocampus following prolonged exposure. Specifically, protracted secretion of glucocorticoids and/or stress can induce dendritic retraction, suppress proliferation of neural progenitor cells and inhibit cell survival, in part through an excitotoxic enhancement of glutamatergic signaling (Ambrogini et al., 2002; Czeh et al., 2002; Galea et al., 1997; McEwen, 2005; Wong and Herbert, 2004). Given that the hippocampus is an important site for both integration of emotional behavior and suppression of HPA axis
activity (Engin and Treit, 2007; Herman and Mueller, 2006; Phillips et al., 2003), these changes in hippocampal plasticity may be relevant for the pathophysiology of depression (Campbell and MacQueen, 2004; Pittenger and Duman, 2008).

In line with these data, every class of chemical antidepressant that has been examined, as well as both electroconvulsive shock and voluntary exercise, has been found to enhance expression of various signaling pathways or transcriptional regulators related to cellular resilience and synaptic plasticity in the hippocampus (D'Sa and Duman, 2002; Malberg and Blendy, 2005). In particular, all forms of conventional antidepressant treatment upregulate the cyclic adenosine monophosphate (cAMP) cascade, resulting in an enhanced production of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), as well as an increase in cell proliferation and neurogenesis, within the hippocampus (D'Sa and Duman, 2002; Nakagawa et al., 2002; Saarielainen et al., 2003; Tardito et al., 2006). These plastic changes also correspond temporally to the clinical onset of action of antidepressants as these effects are not seen following acute treatment with antidepressants, but require sustained administration before modifications can be detected (D'Sa and Duman, 2002; Malberg et al., 2000; Nakagawa et al., 2002). Moreover, the behavioral efficacy of many antidepressants in preclinical paradigms appears to be contingent upon increases in BDNF expression and neurogenesis, providing suggestive evidence that these modifications are functionally relevant changes elicited by antidepressants (Saarielainen et al., 2003; Santarelli et al., 2003). Accordingly, antidepressant treatment has been found to reverse alterations in hippocampal plasticity in preclinical models of depression. Furthermore, clinical studies indicate that individuals with major depression who are on long-term antidepressant maintenance treatment do not
exhibit the hippocampal atrophy seen in their unmedicated comparators (Alonso et al., 2004; Jayatissa et al., 2006; Keilhoff et al., 2006; Malberg and Duman, 2003; Sheline et al., 2003).

Thus, the current theories for the mechanism by which antidepressants exert their therapeutic effects involve a suppression of HPA axis sensitivity and an enhancement of neuroplasticity and cellular resilience within the hippocampus, which are evoked by prolonged enhancement in monoaminergic signaling. Given that conventional antidepressant treatments have limited efficacy, require a temporal lag to exert their effects and can elicit adverse and unwanted side effects, the determination of novel target systems which can regulate the same targets, but work faster and produce a more acceptable side effect profile is a worthwhile approach in psychopharmacological research.

1.3 THE ENDOCANNABINOID SYSTEM: PHYSIOLOGY AND BIOCHEMISTRY

One system that has recently garnered interest in the framework of depression is the endocannabinoid system. This system was first characterized as the neuronal system to which the psychoactive constituent of cannabis, delta-9-tetrahydrocannabinol (THC), interacted to exert its effects on humans. The endocannabinoid system is a relatively unique system, exerting modulatory actions in both central tissue and in the periphery. At the signaling level, two cannabinoid receptors have been characterized to date (Howlett, 2002). The cannabinoid CB₁ receptor is the receptor that is expressed almost ubiquitously throughout the brain (Herkenham et al., 1991; Moldrich and Wenger, 2000; Tsou et al., 1998); however, the CB₁ receptor is also known to exhibit some expression patterns in
peripheral tissue, such as immune cells, vascular tissue and adipocytes (Cota et al., 2003; Hillard, 2000; Parolaro, 1999). The CB₂ receptor is located predominately in peripheral immune tissue, such as macrophages, (Munro et al., 1993; Parolaro, 1999). There is some recent evidence indicating that cannabinoid CB₂ receptors may exhibit some limited neuronal expression (Gong et al., 2006; Van Sickle et al., 2005), but in central tissue the primary source of CB₂ receptors appears to be perivascular microglial cells (Nunez et al., 2004). Both are G-protein coupled receptors that are coupled to Go i/o proteins which function to inhibit adenylyl cyclase activity, activate potassium channels and inhibit voltage-gated calcium channels (Felder and Glass, 1998; Howlett and Mukhopadyhyay, 2000; Piomelli, 2003). The CB₁ receptor appears to be located predominately on presynaptic axon terminals, and is capable of regulating calcium influx, and hence neurotransmitter release. Evidence shows that the endocannabinoid system has the ability to inhibit glutamate, GABA, acetylcholine, serotonin and norepinephrine release (Nakazi et al., 2000; Ohno-Shosaku et al., 2001; Schlickler and Kathmann, 2001).

The endogenous ligands for cannabinoid receptors are the arachidonate derived lipophilic molecules N-arachidonylethanolamine (anandamide; AEA; Devane et al., 1992) and 2-arachidonylglycerol (2-AG; Sugiura et al., 1995). Several other lipid molecules, including virodhamine (Porter et al., 2002), noladin ether (Hanus et al., 2001) and N-acyl dopamines (Bisogno et al., 2000) have been identified as putative endocannabinoid ligands; however, it is premature to refer to these compounds as endocannabinoids until they are further characterized. Both AEA and 2-AG do not behave as typical neurotransmitters. It is currently believed that both AEA and 2-AG are formed post-synaptically by activity-dependent cleavage of phospholipids' head groups
by activation of specific enzymes. The biosynthesis of 2-AG is mediated by generation of diacylglycerol, via the actions of either phospholipase C (PLC) or phospholipase D (PLD), which is subsequently converted to 2-AG via the actions of DAG lipase (Hillard, 2000; Sugiura et al., 2002; Sun et al., 2004).

**Figure 1.1:** The synthetic and metabolic pathways of 2-arachidonoylglycerol

![Pathway Diagram]

The pathways mediating AEA synthesis are less well understood. To date, three distinct and independent mechanisms have been found to generate AEA (Liu et al., 2006; Okamoto et al., 2004; Simon and Cravatt, 2006; see Fig 1.2 for details); however, the pathway that is primarily responsible for neuronal AEA synthesis is not currently known (putative biosynthetic/metabolic pathways for AEA synthesis are outlined in Fig 1.2).
In terms of functional lifespan, the endocannabinoid molecules are believed to be formed "on demand" in post-synaptic cells by excitatory activity and are released into the synapse where they act in a retrograde manner to activate their presynaptically located receptor and inhibit neurotransmitter release (Ohno-Shosaku et al., 2001; Schlickler and Kathmann, 2001; Wilson et al., 2001). Termination of endocannabinoid signaling is maintained by degradative enzymes. Fatty acid amide hydrolase (FAAH) is the primary catabolic enzyme of AEA, and hydrolyzes AEA into ethanolamine and arachidonic acid (Deutsch et al., 2002; Ueda, 2002; see Fig 1.2). On the other hand, 2-AG is primarily metabolized by monoacylglyceride lipase (MAG lipase) to form glycerol and arachidonic acid (Deutsch et al., 2002; Dinh et al., 2002; Ueda, 2002; see Fig 1.1). While it is not known why there are two endogenous ligands for one receptor, these molecules do exhibit slight pharmacokinetic differences which could result in differential signaling patterns. Specifically, AEA exhibits a high affinity for the CB1 receptor (approximately 50-100 nM), but has poor efficacy as an agonist at inducing intracellular signal
transduction (Hillard, 2000). By contrast, 2-AG has less affinity for the CB₁ receptor (approximately 1-10 μM), but is very efficacious in that 2-AG induces a robust intracellular response as assessed by CB₁ receptor mediated guanonucleotide exchange (Hillard, 2000). Thus, it is possible that 2-AG induces a rapid and robust CB₁ receptor response, while AEA evokes more of a tonic, but mild, stimulation of the CB₁ receptor, as we have previously suggested (Gorzalka et al., 2008); however, this pattern of signaling is purely speculative at this point.

The net effect of endocannabinoid activity is in large part dependent on the neuronal population on which the receptor resides. For example, CB₁ receptors located on GABAergic neurons reduce inhibitory transmission and lead to a net enhancement of post-synaptic activity, as has been found in the hippocampus (Carlson et al., 2002; Katona et al., 1999). However, activation of CB₁ receptors on glutamatergic terminals would result in a net suppression of excitation, as has been found in the hypothalamus (Di et al., 2003). Thus the cannabinoid system in the brain is largely a neuromodulatory system that regulates activity of distinct neural pathways in specific anatomical structures.

1.4 THE ENDOCANNABINOID SYSTEM: EVIDENCE FOR A ROLE IN EMOTIONAL REGULATION AND AFFECTIVE DISEASE

The idea that the endocannabinoid system may be involved in emotional behavior, and in particular depression, is rooted in the fact that consumption of cannabis in humans has profound effects upon mood. For centuries, cultures around the world have used cannabis recreationally for its mood elevating and euphoric effects (Williamson and Evans, 2000). Adverse reactions to cannabis, such as panic and paranoia, have been
reliably documented; however, these responses tend to be specific to particular environmental variables such as context (Thomas, 1993). Additionally, there is a growing body of evidence indicating that protracted, heavy exposure to THC during adolescence may have adverse effects on mental health and cognitive function (Rubino and Parolaro, 2008). More often, however, it appears that moderate consumption of cannabis increases positive and reduces negative mood. Large scale epidemiological studies have found that frequent users of cannabis exhibit less depressed mood and more positive affect than non-consumers of cannabis (Denson and Earleywine, 2006), and case study reports have documented that cannabis use exerts antidepressant effects in clinically depressed individuals (Gruber et al., 1996). Given that the psychoactive effects of cannabis consumption are mediated by activation of the CB₁ receptor (Huestis et al., 2001), these findings would argue that facilitation of CB₁ receptor signaling would promote positive mood and possibly exert antidepressant effects.

These findings have been validated at the preclinical level as well. Both acute and chronic administration of synthetic CB₁ receptor ligands have been found to elicit antidepressant-like effects in the forced swim test (Bambico et al., 2007; Hill and Gorzalka, 2005a; Jiang et al., 2005; Rutkowska and Jachimczuk, 2004). Similarly, antidepressant responses are seen following local infusion of a CB₁ receptor agonist into either the ventromedial prefrontal cortex (Bambico et al., 2007) or the dorsal hippocampus (McLaughlin et al., 2007). With regards to the endocannabinoid system itself, a comparable phenomenon is seen; specifically, potentiation of endocannabinoid signaling via inhibition of AEA hydrolysis and/or endocannabinoid uptake reduces depression-like behavior in a variety of preclinical paradigms (Bortolato et al., 2007;
Filip et al., 2006; Gobbi et al., 2005; Hill and Gorzalka, 2005a; Hill et al., 2007b; reviewed in Mangieri and Piomelli, 2007). Thus, similar to the mood elevation seen in humans following cannabis consumption, pharmacological enhancement of CB$_1$ receptor signaling promotes active coping responses to stress and modulates emotional behavior in rodents. Given the consistency of these findings, it seems plausible to consider that a deficit in CB$_1$ receptor activation may promote negative mood and the development of depression.

There are several lines of evidence which suggest that the endocannabinoid system may be functionally involved in the neurobiology of depression. Importantly, both endocannabinoid ligands and the CB$_1$ receptor are localized throughout neuroanatomical structures and circuits that are implicated in depression, such as frontocortical regions, the limbic system (particularly the hippocampus, hypothalamus and amygdala), the classical reward circuit (the ventral striatum and ventral tegmental area) and midbrain monoaminergic nuclei (particularly the raphe nuclei and locus coeruleus; Bisogno et al., 1999; Cadas et al., 1997; Herkenham et al., 1991; Moldrich and Wenger, 2000). The functional relevance of these expression patterns for depressive illness is highlighted by behavioral changes that are documented following disruption of endocannabinoid signaling. Genetic or pharmacological inhibition of endocannabinoid neurotransmission results in a phenotype that is strikingly reminiscent of the symptom profile of typical (or melancholic) depression (reviewed in Hill and Gorzalka, 2005b), with reductions in feeding and body weight (Cota et al., 2003; Ravinet Trillou et al., 2004), increased vigilance and time spent in wakefulness (Santucci et al., 1996), relative insensitivity to rewarding stimuli (anhedonia; Martin et al., 2002; Sanchis-Segura et al., 2004), impaired
stress coping behaviors (Martin et al., 2002; Steiner et al., 2008), increased anxiety-like behavior (Rodgers et al., 2005; Uriguen et al., 2004) and impairments in the clearance of aversive memories (Kamprath et al., 2006; Marsicano et al., 2002). Thus, it would appear that a deficit in endocannabinoid signaling may result in behavioral changes that are akin to depression.

From a neurobiological perspective, the endocannabinoid system exerts regulation of all of the systems that appear to be disturbed in depression. With regards to monoaminergic neurotransmission, CB1 receptors are found directly within the midbrain nuclei that are responsible for serotonergic, noradrenergic and dopaminergic innervation of the forebrain (Haring et al., 2007; Matyas et al., 2008; Oropeza et al., 2001). In line with these findings, administration of direct or indirect (via inhibition of FAAH and subsequent accumulation of AEA) CB1 receptor agonists increases the firing activity of these monoaminergic nuclei and/or efflux of all of 5-HT, norepinephrine and dopamine in terminal regions such as the nucleus accumbens and frontocortical regions (Bambico et al., 2007; Gobbi et al., 2005; Pillolla et al., 2007; Oropeza et al., 2005; Solinas et al., 2006). Similarly, there is evidence that cannabinoid receptor ligands also possess the ability to inhibit monoamine reuptake, similar to the properties of conventional antidepressants (Banerjee et al., 1975; Steffens and Feuerstein, 2004). In addition to enhancing monoaminergic transmission, endocannabinoid signaling also appears to possess the ability to antagonize activation of the 5-HT2A receptor (Boger et al., 1998; Gorzalka et al., 2005; Kimura et al., 1998), a trait common to many adjunctive treatments for depression (Marek et al., 2003). Thus, potentiation of endocannabinoid signaling
results in many of the current pharmacological signatures of both conventional antidepressants and adjunctive treatments.

In addition to modulating monoamine activity, endocannabinoid signaling is also a potent regulator of HPA axis activity. CB₁ receptors are located throughout the limbic structures which subserve regulation of the HPA axis (Herkenham et al., 1991; Moldrich and Wenger, 2000), and importantly, CB₁ receptors are localized to glutamatergic terminals impinging upon CRH neurosecretory cells within the PVN of the hypothalamus (Di et al., 2003). Accordingly, enhancement of endocannabinoid signaling can inhibit stress-induced activation of the HPA axis (Patel et al., 2004). Conversely, disruption of CB₁ receptor signaling results in hyperactive HPA axis activity and increased adrenocortical secretion. Specifically, genetic or pharmacological blockade of the CB₁ receptor can increase CRH mRNA expression in the PVN, increase basal corticosterone secretion, potentiate stress-induced corticosterone secretion, downregulate GR expression in hippocampal subfields and impair glucocorticoid mediated negative feedback (Barna et al., 2004; Cota et al., 2007; Patel et al., 2004). Thus, reductions in endocannabinoid activity can promote an increase in HPA axis function similar to that which is seen in some cases of depression, and enhancement of endocannabinoid activity can reduce HPA axis activity, similar to actions observed following conventional antidepressant treatments.

Additionally, endocannabinoid signaling can also promote neurogenic and neuroplastic processes within the hippocampus. Similar to the actions of conventional antidepressants, long-term enhancement of endocannabinoid signaling, through either administration of direct CB₁ receptor agonists, or via genetic deletion of FAAH (which
results in a tonic increase in AEA content) has been shown to increase cell proliferation and neurogenesis within the dentate gyrus of the hippocampus (Aguado et al., 2005; Jiang et al., 2005). Moreover, the ability of long-term administration of a CB₁ receptor agonist to evoke an antidepressant-like response is contingent upon this increase in neurogenesis, as focal irradiation treatment to the hippocampus (to prevent the induction of neurogenesis) abrogated this behavioral response (Jiang et al., 2005). Consistently, deletion of the CB₁ receptor reduces cell proliferation, neurogenesis and BDNF expression within the hippocampus (Aguado et al., 2005, 2006; Aso et al., 2008; Jin et al., 2004; Kim et al., 2006; Steiner et al., 2008), similar to what is seen in many animal models of depression (Alonso et al., 2004; Gronli et al., 2006; Keilhoff et al., 2006). Furthermore, the impairments in stress-related coping behavior that are seen in CB₁ receptor knockout mice are reversible following local administration of BDNF to the hippocampus, suggesting that this deficiency in neurotrophic activity is relevant to the changes in emotional behavior following disruption of endocannabinoid signaling (Aso et al., 2008).

These data would appear to suggest that deficiencies in endocannabinoid signaling may be associated with depressive illness. In support of this argument, employment of the chronic unpredictable (or mild) stress model of depression to rodents has demonstrated that both CB₁ receptor expression and endocannabinoid ligand content within the hippocampus are decreased in this preclinical paradigm (Hill et al., 2005). This finding has been validated, to some degree, as preliminary reports from some, but not all (see Bortolato et al., 2007) laboratories have demonstrated that chronic stress decreases CB₁ receptor mRNA, protein, binding and G-protein signaling in the hippocampus.
Collectively, these data demonstrate that the endocannabinoid system regulates many of the behavioral and physiological processes that are disrupted in depression. Furthermore, disruption of endocannabinoid function promotes behavioral, neuroendocrine and neuroplastic alterations that are strikingly reminiscent of those that are seen in preclinical animal models of depression, as well as the general symptom profile of typical depression. Within these same facets, potentiation of endocannabinoid neurotransmission results in many of the same behavioral and neuroadaptive changes that are seen following conventional antidepressant treatment. Moreover, endocannabinoid signaling within the hippocampus appears to be disrupted in an animal model of depression, supporting the idea that a deficiency in endocannabinoid activity may be involved in the pathophysiology of depression.

1.5 OVERVIEW AND OBJECTIVES

The experiments described within this dissertation were designed to investigate whether endocannabinoid signaling is regulated by various modalities of antidepressant treatments, whether these changes are functionally relevant to neuroadaptive changes elicited by these antidepressant regimens, whether endocannabinoid activity is altered in an animal model of depression and whether circulating endocannabinoid content is altered in women suffering from depressive disorders. The objectives of the present dissertation are as follows:

1. To determine if chronic treatment with a conventional antidepressant modulates the endocannabinoid system, and if these changes in endocannabinoid function are
relevant to the stress-attenuating effects elicited by this treatment regimen (Chapter 2). The experiments described in this chapter were designed to investigate if the endocannabinoid system at neuroanatomical sites implicated in depression (namely the prefrontal cortex, hippocampus, hypothalamus and amygdala) is modulated by chronic treatment with the tricyclic antidepressant, desipramine. This analysis was performed by examining how three weeks of treatment with desipramine modulates both the binding site densities of the CB₁ receptor, as well as tissue content of the endocannabinoid ligands AEA and 2-AG. Chronic desipramine treatment is also known to attenuate stress-induced activation of the HPA axis (Connor et al., 2000; Duncan et al., 1996), similar to an enhancement of endocannabinoid signaling (Patel et al., 2004); thus, the second aim of this chapter was to determine if the endocannabinoid system is involved in the stress-attenuating response evoked by chronic desipramine treatment. This will be examined by determining if pharmacological antagonism of the CB₁ receptor can reverse the ability of desipramine to suppress both neuronal activation within the PVN and adrenocortical secretion of the glucocorticoid hormone corticosterone. The specific hypothesis being tested is that protracted antidepressant treatment will up-regulate endocannabinoid activity in brain structures regulating HPA axis responsivity, and that this up-regulation will contribute to the ability of desipramine to reduce HPA axis responsivity.

2. To determine if voluntary exercise regulates endocannabinoid signaling in the hippocampus, and whether these changes contribute to exercise-induced increase in progenitor cell proliferation in the dentate gyrus of the hippocampus (Chapter 3). The experiments described within this chapter were designed to determine if endocannabinoid signaling in the hippocampus, as well as the prefrontal cortex, is
modulated by engagement in voluntary exercise provided by free access to running wheels. This was assessed by examining the effects of exercise on the binding site densities of the CB₁ receptor, the ability of the CB₁ receptor to stimulate intracellular guanoneucleotide exchange, the hydrolytic activity of the enzyme FAAH and the tissue content of the endocannabinoid ligands AEA and 2-AG. Voluntary exercise is also known to promote cell proliferation of progenitor cells within the dentate gyrus of the hippocampus (Eadie et al., 2005; Pereira et al., 2007; van Praag et al., 1999), a process that is also facilitated by endocannabinoid neurotransmission (Aguado et al., 2005); thus, a second aim of the experiments in this chapter was to determine if endocannabinoid signaling within the hippocampus is recruited by voluntary exercise to increase progenitor cell proliferation. This was assessed by determining if pharmacological antagonism of the CB₁ receptor can abrogate the increase in expression of the cell cycle protein Ki67 within the subgranular zone of the dentate gyrus following voluntary exercise. The specific hypothesis investigated was that voluntary exercise would enhance endocannabinoid signaling within the hippocampus, which in turn would drive the increase in cell proliferation within the dentate gyrus.

3. **To determine if the endocannabinoid system is regulated by electroconvulsive shock treatment (Chapter 4).** The experiments described in this chapter were designed to examine if the endocannabinoid system is regulated by electroconvulsive shock, one of the most efficacious treatments for depression available (Silverstone and Silverstone, 2004). This was assessed by determining if either a single session or repeated sessions of electroconvulsive shock modulate the binding site density of the CB₁ receptor, the ability of the CB₁ receptor to stimulate intracellular guanoneucleotide exchange, the hydrolytic
activity of the enzyme FAAH and the tissue content of the endocannabinoid ligands AEA and 2-AG. The specific hypothesis tested was that electroconvulsive shock treatment would increase endocannabinoid signaling in subcortical structures, such as was seen with the treatment regimens employed in Chapters 2 and 3.

4. To analyze the endocannabinoid system in an animal model of depression and to determine if these alterations are reversible by concurrent antidepressant treatment (Chapter 5). The experiments described in this chapter were initially designed to validate the chronic unpredictable stress (CUS) model of depression in our laboratory by determining if CUS exposure modulates sexual motivation in male rats. After we established that this model was valid within our laboratory, we then examined if the endocannabinoid system was altered in this model of depression and if antidepressants could reverse any of these changes. To this extent, animals were exposed to CUS, with or without daily injections of the antidepressant imipramine, for three weeks after which endocannabinoid activity was assessed in all of the aforementioned brain structures (prefrontal cortex, hippocampus, hypothalamus, amygdala), as well as those involved in reward circuitry (namely the ventral striatum and the midbrain). This included examining the binding site density of the CB1 receptor, the hydrolytic activity of the enzyme FAAH and the tissue content of the endocannabinoid ligands AEA and 2-AG. Given that in the CUS model of depression, endocannabinoid activity within the hippocampus is reduced (Hill et al., 2005; Reich et al., 2007), and that antidepressant regimens examined in Chapters 2, 3 and 4 all increased endocannabinoid activity in subcortical structures, the specific hypothesis tested was that subcortical endocannabinoid activity would be
reduced in this model of depression and that antidepressant treatment would abrogate some, if not all, of these effects.

5. To determine if circulating endocannabinoid content is altered in women diagnosed with depressive disorders (Chapter 6). The experiments described in this chapter were designed to determine the serum content of the endocannabinoid ligands AEA and 2-AG in a population of medication-free, ambulatory women diagnosed with either major or minor depression, and their healthy, matched controls. Given that the data generated throughout this thesis (Chapters 2-5), in conjunction with other relevant literature in the area (reviewed in Hill and Gorzalka, 2005b), suggest the possibility of deficient endocannabinoid activity in depression, the specific hypothesis tested was that circulating endocannabinoid content would be reduced in women diagnosed with depression.

Chapters 2-6 will describe experimental data that have been collected to address these objectives. All experimental data have either been published (Chapter 2-Hill et al., 2006; Chapter 4-Hill et al., 2007a; Chapter 6-Hill et al., 2008) or have been submitted for publication and are currently under review (Chapter 3-Hill et al, under review-a; Chapter 5-Hill et al., under review-b) and are in manuscript form. The General Discussion (Chapter 7) discusses how these data can aid in the formulation of a theoretical role of the endocannabinoid system in the pathophysiology of depression and subsequently, the potential value of this system in the development of a novel class of antidepressant agents that function to enhance endocannabinoid neurotransmission.
1.6 REFERENCES


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CHAPTER 2

INVOLVEMENT OF THE ENDOCANNABINOID SYSTEM IN THE ABILITY OF LONG-TERM TRICYCLIC ANTIDEPRESSANT TREATMENT TO SUPPRESS STRESS-INDUCED ACTIVATION OF THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

2.1 INTRODUCTION

Major depression is a psychiatric disease that results in dramatic alterations in emotional, neurovegetative and cognitive processes. The neurobiology of depression is not well understood; however, a large body of evidence convincingly demonstrates a critical role of the hypothalamic-pituitary-adrenal (HPA) axis (Holsboer, 2000). Specifically, both corticotrophin releasing hormone (CRH) and cortisol are reported to be increased in the cerebrospinal fluid and plasma of depressed patients (Arborelius et al., 1999; Holsboer, 2000; Parker et al., 2003). Furthermore, the ability of glucocorticoid hormones to exert negative feedback on HPA axis activity appears to be deficient in depression, resulting in a feed-forward hyperactivation of this system (Holsboer, 2000; Parker et al., 2003). This enhanced output of the HPA axis appears functionally relevant to depression as long-term antidepressant treatment attenuates this phenomenon in humans (De Bellis et al., 1993; Greden et al., 1983; Michelson et al., 1997; Pariante et al., 2004), and suppresses stress-induced activation of the HPA axis in other species.

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(Butterweck et al., 2001; Connor et al., 2000; de Medieros et al., 2005; Holsboer and Barden, 1996; Reul et al., 1993; Stout et al., 2002). The ability of antidepressants to suppress HPA axis hyperactivity has been shown to be tightly coupled to their clinical efficacy. Specifically, normalization of glucocorticoid feedback and hypersecretion is associated with clinical remission and patients who do not exhibit normalization of this system exhibit a significantly higher tendency to experience depressive relapse and have a poorer long-term prognosis (Greden et al., 1983; Ribiero et al., 1993; Zobel et al., 2001). These data demonstrate that the ability of antidepressants to regulate the HPA axis could be integral to the remission of depressive symptoms; however, the mechanism by which antidepressants exert this effect is currently not well understood.

Given the role of the HPA axis in depression, it is interesting to note that recent work has suggested a critical role for the endocannabinoid system in regulating HPA axis activation. Specifically, electrophysiological studies have demonstrated that CB₁ cannabinoid receptors in the paraventricular nucleus of the hypothalamus (PVN) are located on glutamatergic terminals and gate excitatory activation of the CRH neurosecretory cells (Di et al., 2003). These data predict that activation of CB₁ receptors in the PVN would result in a suppression of HPA axis activity, whereas a disruption in endocannabinoid signaling would result in hyperactivity of the HPA axis. This hypothesis has received substantial support in vivo, as genetic or pharmacological disruption of endocannabinoid signaling results in exaggerated endocrine responses to stress, and conversely, inhibition of endocannabinoid uptake or metabolism attenuates stress-induced activation of the HPA axis (Barna et al., 2004; Cota et al., 2007; Patel et al., 2004; Steiner et al., 2008a).
Given the aforementioned role of the endocannabinoid system in regulating HPA axis activity, as well as an increasing interest in the potential role of the endocannabinoid system in both the pathophysiology and treatment of depression (Bortolato et al., 2007; Gobbi et al., 2005; Hill and Gorzalka, 2005a,b; Hill et al., 2007; Mangieri and Piomelli, 2007; Witkin et al., 2005), the present study was designed to examine whether chronic treatment with the tricyclic antidepressant desipramine, regulates endocannabinoid activity. Furthermore, we explored the functional relevance of antidepressant-mediated changes in endocannabinoid signaling with regards to adaptive changes in the HPA axis elicited by long-term antidepressant treatment.

2.2 METHODS

2.2.1 Animals

Seventy day old male Sprague-Dawley rats (approx. 285 g at the onset of the study) housed in groups of three in triple wire mesh caging were used in this study. Colony rooms were maintained at 21 °C, and on a 12 h light/dark cycle, with lights on at 0700 h. All rats were given ad libitum access to Purina Rat Chow and tap water. All treatments performed in this study were approved by the Animal Ethics Committee of the University of British Columbia and were consistent with the standards of the Canadian Council on Animal Care.

2.2.2 Treatment Procedure

For the biochemical studies, animals were divided into two treatment groups; one received 10 mg/kg desipramine (Sigma, Canada) in saline and the other an equivalent amount of saline alone. All subjects received daily intraperitoneal injections for 21 days; 18 hours following the last injection, all subjects were rapidly decapitated. Prefrontal
cortex (composed of medial prefrontal cortex and anterior cingulate), amygdala
(composed of central, basolateral and medial nuclei), hippocampus and hypothalamus
were dissected out on ice, immediately frozen in liquid nitrogen and stored at -80°C until
analysis.

For neuroendocrine studies, animals were divided into four treatment conditions:
1) saline-vehicle (1:1:8 Tween 80: dimethyl sulfoxide: 0.9% saline); 2) 10 mg/kg
desipramine-vehicle; 3) saline-1 mg/kg AM251 (a CB₁ receptor antagonist; Tocris-
Cookson, USA); 4) 10 mg/kg desipramine-1 mg/kg AM251. All injections were
performed intraperitoneally at a volume of 1 ml/kg using 26 1/2" gauge needles. In this
study, rats were administered vehicle or 10 mg/kg desipramine injections for 21 days, and
on the 22nd day, animals were given their final injection of vehicle or desipramine which
was immediately preceded by an injection of 1 mg/kg AM251 or vehicle. Two cohorts of
animals were prepared in these treatment conditions, one group to be exposed to swim
stress to permit examination of the effects of these treatment regimens on hormonal and
cellular responses to stress. The second cohort of animals was not exposed to swim stress
to permit examination the effects of these treatment conditions on basal activity of the
HPA axis. One h after the final injections, subjects were exposed to a 5 min swim stress
session, which was performed in a cylindrical plexiglass container, filled to a height of 30
cm with water at 21°C. Forty five min following stress, subjects were subjected to a brief
tail bleed to obtain blood for analysis of plasma corticosterone. One h following the tail
bleed all subjects were overdosed with sodium pentobarbital (120 mg/kg) and trans-
cardially perfused with 4% paraformaldehyde, the brains were then fixed in
paraformaldehyde overnight and stored in phosphatebuffered saline until sectioned for
Immunohistochemical analysis. These time points were based on previous studies in which the peak corticosterone secretion and expression of c-fos following exposure to the swim stress were determined (Connor et al., 2000; Duncan et al., 1996). Animals that were not exposed to swim stress were given injections, bled and perfused at comparable time points to assess any effects of these treatments on basal activity of the HPA axis. This paradigm allowed the investigation of whether the ability of chronic desipramine administration to suppress stress-induced activation of the HPA axis could be blocked by acute administration of the cannabinoid CB\(_1\) receptor antagonist AM251. This paradigm was chosen because in rats, chronic, but not acute, administration of antidepressants is required to elicit the suppression of corticosterone and reduction in c-fos expression in the PVN (Connor et al., 1998, 2000; Duncan et al., 1996).

2.2.3 Membrane Preparation

Dissected brain sections were homogenized in 10 volumes of 0.32 M sucrose containing 3 mM HEPES (pH 7.5) and 1 mM EDTA. The homogenates were initially centrifuged at 3,000 x g for 10 min, after which the supernatant was rapidly decanted, the remaining pellet was resuspended in the sucrose buffer and centrifuged at 18,000 x g for 20 min after which the supernatant was rapidly decanted. The remaining pellet, which is the membrane fraction, was resuspended in 1-2 ml TME buffer (50 mM Tris HCl, pH 7.4; 1 mM EDTA and 3 mM MgCl\(_2\)) containing 1 mM sodium orthovanadate. Protein concentrations were determined by the Bradford method (Bio-Rad, Hercules, CA, USA).

2.2.4 CB\(_1\) Receptor Binding Assay

CB\(_1\) receptor binding assays were performed using a Multiscreen Filtration System with Durapore 1.2-\(\mu\)M filters (Millipore, Bedford, MA) as described previously
(Hillard et al., 1995). Incubations (total volume = 0.2 mL) were carried out using TME buffer containing 1 mg/mL bovine serum albumin (TME/BSA). Membranes (10 μg protein per incubate) were added to the wells containing 0.25, 0.5, 1.0, or 2.5 nM [3H]CP 55,940. Ten μM Δ⁸-tetrahydrocannabinol was used to determine non-specific binding. Kᵦ and Bmax values were determined by nonlinear curve fitting to the single site binding equation using GraphPad Prism (San Diego, CA, USA).

2.2.5 Endocannabinoid Extraction and Analysis

For analysis of endocannabinoid content, brain regions were subjected to a previously validated lipid extraction process (Patel et al., 2003). Briefly, tissue samples were weighed and placed into borosilicate glass culture tubes containing 2 ml of acetonitrile with 84 pmol of [²H₈]anandamide and 186 pmol of [²H₈]2-AG for extraction. Tissue was homogenized with a glass rod and sonicated for 30 min. Samples were incubated overnight at -10°C to precipitate proteins, and subsequently centrifuged at 1,500 x g. The supernatants were removed to a new glass tube and evaporated to dryness under N₂ gas. The samples were resuspended in 300 μl of methanol to recapture any lipids adhering to the glass tube, and dried again under N₂ gas. Finally, lipid extracts were suspended in 20 μl of methanol, and stored at -80°C until analysis.

Analysis of endocannabinoid content in methanol fractions was performed as detailed by Patel and colleagues (2003). Specifically, the amounts of anandamide and 2-AG were determined by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (1100 LC-MSD, SL model; Agilent Technologies Inc., Wilmington, DE). Samples (5 μl) were separated on a reverse-phase C₁₈ column (Kromasil, 250 x 2 mm, 5-μm diameter) using mobile phase A (deionized water, 1 mM
ammonium acetate, and 0.005% acetic acid) and mobile phase B (methanol, 1 mM ammonium acetate, and 0.005% acetic acid). Samples were eluted at a flow rate of 300 μl/min by a linear gradient. The percentage of solvent B increased linearly from 85% solvent B to 100% solvent B in 25 min then held at 100% solvent B for 10 min. Over the next 10 min, solvent B decreased linearly from 100 to 85% and was held at 85% for an additional 10 min. Detection was made in a positive ion mode. Selective ion monitoring was used to detect [²H₈]anandamide (m/z 356; retention time = 13.7 min.), anandamide (m/z 348; retention time = 13.9 min), [²H₈]2-AG and 1(3)-AG (m/z 387; retention times = 14.3 and 15.1 min, respectively), and 2-AG and 1(3)-AG (m/z 379; retention times = 14.5 and 15.3 min, respectively). 2-AG is usually observed as a doublet because it isomerizes to 1(3)-AG during extraction (Stella et al., 1997), the area of both peaks were combined to yield total 2-AG. Endocannabinoid contents were normalized to wet tissue weight.

2.2.6 Radioimmunoassay

Blood was allowed to coagulate overnight at 4°C. The following morning, plasma was harvested by centrifuging blood samples at 1500 x g for 20 min, and was stored at -80°C until analysis. Plasma corticosterone levels were determined by employing a standard radioimmunoassay kit (MP Biomedicals, Solon, OH, USA), running each sample in duplicate.

2.2.7 Immunohistochemical Analysis

Fixed brains were sliced coronally into 35μm coronal sections using a vibratome. Sections were washed in potassium phosphate buffered saline (KPBS), incubated in a 0.4% peroxide bath, and thoroughly washed again in KPBS. Sections were then briefly exposed to 0.1% sodium borohydride solution, washed in KPBS and incubated for 48 h at
4°C in KPBS with 2% goat serum and 0.3% Triton X-100 (loaded KPBS) containing polyclonal rabbit antisera against residues 4–17 of human fos protein (Oncogene Labs, Cambridge, MA, USA at 1:26000). Sections were subsequently washed in KPBS and incubated in biotinylated, goat anti-rabbit secondary antibody (1:222) for 60 min, washed again in KPBS and transferred to a avidin biotin complex solution (Vector Laboratories, Burlingame, CA, USA) for 60 min. Tissue was then washed in KPBS, transferred to 1.0 M sodium acetate and developed using a diaminobenzidine reaction driven by glucose oxidase. Tissue was subsequently mounted, dehydrated and coverslipped. Light-level images were captured using a Hamamatsu optical system coupled to a Macintosh computer running Open Lab imaging and measuring software (Quorum Technologies, Guelph, Ontario, Canada). Fos-ir cell counts were taken by an observer blind to animal status in regularly spaced (150-μm) intervals through the rostrocaudal extent of the paraventricular cell group. Positive cells were identified as those expressing a black nuclear reaction product. Discrete localization of Fos-ir profiles to the medial parvocellular (neuroendocrine anterior pituitary-regulating) population of the PVN was accomplished by limiting the region of interest to the area medial to the magnocellular population and ventral to the dorsal cap, ensuring a high density of CRH-neurosecretory cells. Total cell number estimates were generated by counting bilaterally the number of Fos-positive cells through the medial parvocellular cell population, averaged by dividing cell counts by slice number, and corrected for sampling frequency (one in five sections, 150-μm intervals) by multiplying this product by a factor of five. Furthermore, to ensure that any determinations were not artifacts of PVN area, we also performed density analysis, determining how many fos-ir cells were present per mm² of the medial
parvocellular population of the PVN. Results thus represent estimates of the total number of Fos-positive cells per medial parvocellular region as well as number of Fos-ir cells per mm².

2.2.8 Statistics

Cannabinoid CB₁ receptor binding parameters and endocannabinoid contents were analyzed by a t-test comparing vehicle-treated animals with desipramine-treated animals. Analysis of the stress-induced hormonal and cellular effects was performed using a univariate analysis of variance, with drug treatment and swim exposure as fixed factors. Post-hoc tests were performed using a Tukey’s HSD test. Significance was established against an alpha value of 0.05.

2.3 RESULTS

2.3.1 Chronic Treatment with the Tricyclic Antidepressant Desipramine Up-regulates the CB₁ Receptor in Key Regions of the Stress Axis

Animals that had been treated with 21 days of desipramine exhibited significant increases in the binding site density (Bₘₐₓ) of the cannabinoid CB₁ receptor in the hippocampus [t (5) = 4.43, p < 0.01] and the hypothalamus [t (6) = 3.76, p < 0.01]. There was no significant effect of desipramine treatment on the Bₘₐₓ of the CB₁ receptor in the prefrontal cortex [t (6) = 2.20, p > 0.05] or amygdala [t (5) = 0.31, p > 0.05]. Data regarding the effects of desipramine treatment on the Bₘₐₓ of the cannabinoid CB₁ receptor can be seen in Fig 2.1. There was no significant effect of chronic desipramine treatment upon the affinity (Kₐ) of [³H]CP 55,940 for the CB₁ receptor in the prefrontal cortex [t (6) = -0.16, p > 0.05; vehicle: 0.27 +/- 0.04 nM vs. desipramine: 0.28 +/- 0.07 nM], the hippocampus [t (5) = 1.55, p > 0.05; vehicle: 1.14 +/- 0.14 nM vs. desipramine
1.67 +/- 0.28 nM], the hypothalamus [t (6) = 0.76, p > 0.05; vehicle: 2.21 +/- 1.24 nM vs. desipramine: 2.87 +/- 0.85 nM] or the amygdala [t (5) = 0.31, p > 0.05; vehicle: 1.71 +/- 0.86 nM vs. desipramine: 0.82 +/- 0.27 nM].

Figure 2.1: The effect of chronic desipramine (DES; 10 mg/kg) treatment on the maximal binding (B_max) of the cannabinoid CB_1 receptor as measured by [3H]CP55940 binding in the a) prefrontal cortex; b) hippocampus; c) hypothalamus; and d) amygdala, relative to vehicle (VEH) treated rats. Data are presented as mean values +/- SEM (n = 3-4 subjects/group). Significant differences (p < 0.05) denoted by *.

Animals that had been treated with desipramine for 21 days did not exhibit any significant changes in prefrontal cortical AEA [t (14) = 0.10, p > .05] or 2-AG content [t (13) = 1.70, p > .05]; hippocampal AEA [t (14) = 0.52, p > .05] or 2-AG content [t (13) = 0.31, p > .05]; hypothalamic AEA [t (11) = 0.95, p > .05] or 2-AG content [t (11) = 0.15, p > .05]; or amygdalar AEA [t (14) = 1.11, p > .05] or 2-AG content [t (14) = 0.99, p > .05].
. Data regarding the effects of chronic desipramine treatment on endocannabinoid content in these brain structures can be seen in Table 2.1.

Table 2.1 Effect of chronic desipramine treatment (10 mg/kg) on brain regional endocannabinoid content.
Desipramine treatment for 21 days did not change the content of either anandamide (AEA) or 2-arachidonoylglycerol (2-AG) in any brain region examined.
Data are presented as means +/- SEM (n = 6-8 subjects/group).

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Desipramine</th>
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</thead>
<tbody>
<tr>
<td><strong>Prefrontal Cortex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEA (pmol/g tissue)</td>
<td>9.99 +/- 0.56</td>
<td>10.09 +/- 0.85</td>
</tr>
<tr>
<td>2-AG (nmol/g tissue)</td>
<td>4.58 +/- 0.43</td>
<td>5.66 +/- 0.46</td>
</tr>
<tr>
<td><strong>Hippocampus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEA (pmol/g tissue)</td>
<td>23.76 +/- 0.55</td>
<td>23.25 +/- 0.84</td>
</tr>
<tr>
<td>2-AG (nmol/g tissue)</td>
<td>8.27 +/- 0.22</td>
<td>8.41 +/- 0.37</td>
</tr>
<tr>
<td><strong>Hypothalamus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEA (pmol/g tissue)</td>
<td>2.79 +/- 0.26</td>
<td>2.42 +/- 0.31</td>
</tr>
<tr>
<td>2-AG (nmol/g tissue)</td>
<td>7.81 +/- 0.52</td>
<td>7.93 +/- 0.71</td>
</tr>
<tr>
<td><strong>Amygdala</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEA (pmol/g tissue)</td>
<td>8.03 +/- 0.76</td>
<td>6.82 +/- 0.78</td>
</tr>
<tr>
<td>2-AG (nmol/g tissue)</td>
<td>7.56 +/- 0.81</td>
<td>8.68 +/- 0.79</td>
</tr>
</tbody>
</table>

2.3.2 Up-regulation of the Endocannabinoid System Mediates the Suppression of Stress-induced Activation of the HPA Axis Elicited by Chronic Desipramine Treatment.

To examine whether the up-regulation of the CB1 receptor following chronic desipramine treatment plays a functional role in the neuroendocrine effects of this treatment, we determined whether acute blockade of the CB1 receptor affected these parameters. There was a significant interaction between drug treatment and exposure to the stress on plasma corticosterone concentration $[F (3, 40) = 3.14, p < 0.05]$, with a
significant main effect of exposure to stress \( [F(1, 40) = 469.26, p < 0.01] \), but no main effect of drug treatment \( [F(3, 40) = 1.45, p > 0.05] \). Post hoc analysis revealed that exposure to swim stress increased plasma corticosterone \( (p < 0.01 \text{ for all treatment conditions}) \); however, chronic pretreatment with desipramine resulted in a significant reduction in plasma corticosterone following stress exposure \( (p < 0.04) \). Acute treatment with AM251 completely occluded the desipramine-induced reduction in plasma corticosterone \( (p < 0.05) \), while AM251 administration alone had no effect on the stress-induced increase in plasma corticosterone \( (p > 0.05) \). There was no effect of either desipramine or AM251 treatment on plasma corticosterone levels in animals that had not been exposed to the stressor \( (\text{vehicle vs. desipramine, } p > 0.05; \text{vehicle vs. AM251, } p > 0.05; \text{vehicle vs. desipramine and AM251, } p > 0.05) \). These data can be seen in Table 2.2.

**Table 2.2** Effect of chronic desipramine treatment (10 mg/kg) and acute pharmacological blockade of the cannabinoid CB1 receptor, using the CB1 receptor antagonist AM251 (1 mg/kg) on plasma corticosterone levels under basal conditions and following exposure to swim stress.

Antagonism of the CB1 receptor abrogated the ability of chronic desipramine treatment to suppress stress-induced corticosterone secretion. Data are presented as mean values +/- SEM. Significant differences between stress and no stress groups for each respective treatment \( (p < 0.05) \) are denoted by *; significant differences between desipramine-vehicle swim stress group and all other swim stress conditions \( (p < 0.05) \) are denoted by ‡.

<table>
<thead>
<tr>
<th>Plasma corticosterone (ng/ml):</th>
<th>No Stress</th>
<th>Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline-Vehicle</td>
<td>92.0 +/- 22.1</td>
<td>537.4 +/- 19.3*</td>
</tr>
<tr>
<td>Desipramine-Vehicle</td>
<td>138.2 +/- 31.8</td>
<td>417.7 +/- 11.9*‡</td>
</tr>
<tr>
<td>Saline-AM251</td>
<td>119.4 +/- 45.6</td>
<td>541.0 +/- 16.7*</td>
</tr>
<tr>
<td>Desipramine-AM251</td>
<td>137.2 +/- 13.9</td>
<td>536.9 +/- 32.4*</td>
</tr>
</tbody>
</table>

With respect to number of fos-ir cells present in the PVN, results paralleled the hormonal data. There was a significant interaction between exposure to stress and drug
treatment \([F (3, 25) = 5.00, p < 0.01]\), with significant main effects of both stress exposure \([F (1, 25) = 235.71, p < 0.01]\) and drug treatment \([F (3, 25) = 8.98, p < 0.01]\). Post-hoc analysis revealed that exposure to swim stress significantly increased fos expression in the PVN in all treatment groups (all p's < 0.01); however, animals that had been pretreated with desipramine exhibited significantly lower levels of c-fos expression in the PVN than all other groups exposed to stress (all p's < 0.01). However, acute treatment with AM251 prevented this reduction in fos-ir in the PVN in desipramine treated animals (p < 0.01). These data can be seen in Figure 2.2 and photomicrographs illustrating the changes in c-fos expression in the PVN can be seen in Figure 2.3.

**Figure 2.2:** The effect of chronic administration of desipramine (DES; 10 mg/kg), and the influence of acute cannabinoid CB1 receptor blockade through administration of AM251 (AM; 1 mg/kg), on both basal and stress-induced elevations in total number of fos immunoreactive-like (fos-ir) cells in the medial parvocellular population of the paraventricular nucleus (PVN) of the hypothalamus. Data are presented as mean values +/- SEM (n = 4-5 subjects / group). Significant differences (p < 0.05) denoted by *. 

![Graph showing fos-ir cells in the PVN](image)
**Figure 2.3:** Representative photomicrographs of fos immunoreactivity in the paraventricular nucleus of the hypothalamus under both basal conditions (left panel) and in response to swim stress exposure (right panel) [V=vehicle; D=desipramine; A=AM251].
A comparable trend was seen in density measurements of fos-ir cells per mm² of the PVN. Density analysis revealed that there was a significant interaction between exposure to stress and drug treatment \(F(3, 25) = 7.36, p < 0.01\); data not shown], with significant main effects of both stress exposure \(F(1, 25) = 316.01, p < 0.01\) and drug treatment \(F(3, 25) = 14.30, p < 0.01\). Again, as with total fos-ir cells in the PVN, density of fos-ir cells showed that all animals exposed to the swim stress exhibited a significant increase in fos-ir (all p's < 0.01); however, those that had been pretreated with desipramine exhibited a significantly lower density of fos-ir cells (p < 0.01). As with total fos-ir cells, the reduction in the density of fos-ir cells elicited by chronic desipramine treatment was prevented by acute treatment with AM251 (p < 0.01).

2.4 DISCUSSION

This study provides the first demonstration to date that chronic treatment with the tricyclic antidepressant desipramine produces an up-regulation of the maximal binding of the cannabinoid CB₁ receptor in the hippocampus and hypothalamus while not affecting the binding affinity of the agonist \(^3\text{H}\)-CP 55,940 for the CB₁ receptor or the content of the two major endocannabinoids in any brain structure examined. These data reveal that chronic desipramine treatment increases activity in the endocannabinoid system in several brain structures involved in processing and regulating responses to stress. These findings are intriguing given that chronic unpredictable stress, an animal model of depression, results in a significant reduction in CB₁ receptor binding and a down-regulation of the endocannabinoid 2-AG in the hippocampus (Hill et al., 2005). This demonstrates bidirectional regulation of hippocampal CB₁ receptors by stress and
antidepressants, suggesting that the endocannabinoid system in the hippocampus could be relevant for the development and treatment of depression. It is surprising that long-term desipramine treatment did not affect endocannabinoid content in any brain structure examined, given that these molecules are sensitive to stress exposure (Hill et al., 2005; Patel et al., 2004, 2005). However, it remains unknown whether chronic antidepressant treatment alters the responsiveness of the endocannabinoid system to chronic stress.

The mechanism by which chronic tricyclic antidepressant treatment regulates CB₁ receptor expression is currently unknown; however, previous studies have demonstrated that antidepressant treatment can increase receptor trafficking and up-regulate membrane expression of receptors, such as the AMPA receptor (Martinez-Turrillas et al., 2002). The CB₁ receptor is known to exist at both the membrane level and in intracellular endosomic stores, with the vast majority (~85%) of the receptor population typically existing in intracellular vesicles (Leterrier et al., 2004). Thus, the increase in CB₁ receptor binding sites following tricyclic antidepressant treatment may be due to an increase in receptor trafficking such that a higher proportion of CB₁ receptors are active at the membrane site. This increase in active expression of the CB₁ receptor may be an adaptive response elicited by treatment with desipramine. The primary pharmacological property of desipramine is its ability to inhibit norepinephrine reuptake and thus potentiate the synaptic action of norepinephrine (Frazer, 1997; Wong et al., 2000). Both in vivo and ex vivo work in rodent and human tissue has demonstrated that CB₁ receptors in the hippocampus and hypothalamus negatively regulate noradrenergic neurotransmission (Schlicker et al., 1997; Tzavara et al., 2001). Thus, the up-regulation of CB₁ receptors in the hippocampus and hypothalamus seen in this study may be an adaptive response.
launched by the central nervous system to decrease noradrenergic transmission by increasing the density of presynaptic CB₁ receptors, which in turn would reduce norepinephrine release and normalize the increased synaptic availability induced by desipramine treatment.

One common functional response to chronic antidepressant treatment, especially tricyclic antidepressants, is an attenuation of stress-induced activation of the HPA axis (Butterweck et al., 2001; Connor et al., 2000; de Medeiros et al., 2005; Duncan et al., 1996). To examine the functional relevance of the changes in the endocannabinoid system induced by chronic desipramine treatment, we examined the effects of acute blockade of the CB₁ receptor with AM251 on the hormonal and cellular responses to stress following this antidepressant regimen. Chronic treatment with desipramine produced a significant reduction in both stress-induced increases in neuronal activation within the medial parvocellular neurons of the PVN and stress-induced increases in plasma corticosterone concentrations, as has been previously shown with desipramine (Connor et al., 2000; Duncan et al., 1996). Acute treatment with AM251 completely occluded the effect of desipramine to reduce activation of the HPA axis. These data reveal that engagement of the endocannabinoid system is necessary for tricyclic antidepressants to suppress stress-induced activation of the HPA axis. This interaction between antidepressants and the endocannabinoid system is likely occurring at the level of the hypothalamus, a region in which desipramine increased CB₁ receptor binding. Specifically, recent data have demonstrated that CB₁ receptors in the PVN of the hypothalamus gate glutamatergic fibers which activate the HPA axis (Di et al., 2003), thus an up-regulation of the CB₁ receptor in this region could trigger the increased
suppression of HPA axis activity seen following desipramine treatment. Given that the suppression of corticosterone secretion following exposure to stress was matched by a selective reduction in activation of the medial parvocellular region of the PVN of the hypothalamus, an area rich in CRH-neurosecretory cells, the ability of this antidepressant regimen to attenuate HPA axis activity is likely through attenuation of CRH neurosecretory cells. As such, the current data suggest that the ability of desipramine to suppress HPA axis activation is through an up-regulation of CB₁ receptors in the PVN, which in turn lead to an increased suppression of excitatory activation of CRH neurosecretory cells. However, given that CB₁ receptors were also increased in the hippocampus, and the hippocampus is known to exert a potent role in regulation and feedback of the HPA axis (Herman and Mueller, 2006; Herman et al., 1998; Jacobsen and Sapolsky, 1991), the possibility does exist that changes in the endocannabinoid system upstream of the PVN could elicit a net reduction in activation of incoming afferents to the neurosecretory cells of the PVN. Regardless of the locus of action, the current data demonstrate that the endocannabinoid system mediates the neuroendocrine effects of chronic desipramine administration.

These data also support our recently proposed hypothesis that the endocannabinoid system acts as a buffer against the effects of stress in the brain (Patel et al., 2005). Specifically, chronic, homotypic stress results in a habituation of the stress response that is accompanied by up-regulation of endocannabinoid ligands in the limbic system, suggesting that increased endocannabinoid activity serves to dampen the stress axis (Patel et al., 2005). This hypothesis is supported by evidence that acute treatment with a CB₁ receptor antagonist can reverse habituation to chronic homotypic stress,
indicating that the endocannabinoid system acts to modulate or dampen activation of the neural stress axis (Patel et al., 2005). We suggest that chronic exposure to desipramine also up-regulates the endocannabinoid system, which, in turn, dampens the stress axis in a similar fashion. Preclinical animal data support this contention as transgenic mice which lack the CB₁ receptor exhibit an increased susceptibility to the anhedonic effects of chronic stress, suggesting that this system may be integral to the development and maintenance of effective coping strategies to stress (Gorzalka et al., 2008; Martin et al., 2002; Steiner et al., 2008b).

Given that the ability of antidepressants to regulate the HPA axis is tightly coupled to their clinical efficacy (Greden et al., 1983; Holsboer and Barden, 1996; Ribiero et al., 1993; Zobel et al., 2001), these data suggest that up-regulation of the endocannabinoid system may be a critical step in the normalization of hypercortisolemia that accompanies remission of depression. These data also support the suggestion that the endocannabinoid system could serve as a suitable target for the development of novel antidepressants (Gobbi et al., 2005; Hill and Gorzalka, 2005a; Jiang et al., 2005), especially for melancholic depression, (Hill and Gorzalka, 2005b) which exhibits a preferential response to tricyclic antidepressants and reliably exhibits hyperactivity of the HPA axis (Bielski and Friedel, 1976; Gold and Chrousos, 2002; Rush and Weissenberger, 1994).
2.5 REFERENCES


CHAPTER 3

ENDOGENOUS CANNABINOID SIGNALING IS REQUIRED FOR
VOLUNTARY EXERCISE-INDUCED ENHANCEMENT OF PROGENITOR
CELL PROLIFERATION IN THE HIPPOCAMPUS²

3.1 INTRODUCTION

There is growing awareness that the brain is a dynamic structure, capable of extensive synaptic and dendritic structural changes, even in adulthood. Some brain regions, including the subventricular zone and the subgranular zone of the dentate gyrus, can exhibit substantial neurogenesis in the adult brain (Kempermann et al., 2000; Marrone and Petit, 2002; Pascual-Leone et al., 2005). The development of new neurons, or neurogenesis, occurs via proliferation of quiescent progenitor cells into a population which differentiate primarily into neurons. As these new neurons mature, they extend axons and dendrites into the established cytoarchitecture of the existing neuronal networks, where they develop appropriate bioelectrical properties and connections (Lledo et al., 2006; Schmidt-Hieber et al., 2004; Toni et al., 2007; van Praag et al., 2002).

Cell proliferation and neurogenesis in the hippocampus are sensitive to regulation by environmental factors, such as stress, enrichment or exercise (Mirescu and Gould, 2006; Olson et al., 2006). Voluntary exercise (VEx) is a particularly robust way to enhance progenitor cell proliferation and neurogenesis, possibly because it also increases

²A version of this chapter has been submitted for publication and is under review. Hill MN, Titterness AK, Morrish AC, Carrier EJ, Lee TT, Gorzalka BB, Hillard CJ, Christie BR. Endocannabinoid signaling is required for voluntary exercise-induced enhancement of progenitor cell proliferation in the hippocampus.
a myriad of physiological responses that include neurotrophin expression, dendritic
length and complexity, spine density, angiogenesis and cerebral blood flow, within the
dentate gyrus (Eadie et al., 2005; Fabel et al., 2003; Farmer et al., 2004; Neeper et al.,
1996; Pereira et al., 2007; Redila and Christie, 2006; Soya et al., 2007; Stranahan et al.,
2007; van Praag et al., 1999). As a result of these changes, animals that exercise show
enhanced long-term potentiation, as well as observable improvements in performance on
cognitive tasks (van Praag et al., 1999; Vaynman et al., 2004, 2007). It has been
suggested that these mechanisms underlie the ability of exercise to facilitate recovery
following stroke as well as retard the functional declines associated with
neurodegenerative disorders such as Alzheimer’s, Huntington’s and Parkinson’s disease
(Cotman et al., 2007; Friedland et al., 2001; Kramer and Erickson, 2007; Pereira et al.,
2007; Rabadi, 2007; Stevens and Killeen, 2006).

Converging lines of evidence also support a role of the endocannabinoid system
in the structural and functional plasticity of the brain, particularly within the
hippocampus (Chevaleyre and Castillo, 2004; Hashimotodani et al., 2007; Zhu, 2006).
The endocannabinoid system is a neuromodulatory system composed of at least two
receptors (CB₁ and CB₂) and two arachidonate-derived endogenous ligands, N-
arachidonylethanolamide (anandamide; AEA) and 2-arachidonoylglycerol (2-AG)
(Hillard, 2000; Howlett et al., 2004). Both in vitro and in vivo studies have revealed that
neural progenitor cells express both CB₁ and CB₂ receptors and synthesize AEA and 2-
AG (Aguado et al., 2005, 2006; Jiang et al., 2005; Molina-Holgado et al., 2007;
Palazuelos et al., 2006). Genetic deletion of the CB₁ receptor suppresses progenitor cell
proliferation (Aguado et al., 2005, 2006; Jin et al., 2004; Kim et al., 2006), while genetic
deletion of the enzyme responsible for AEA hydrolysis (fatty acid amide hydrolase; FAAH) results in a profound increase in cell proliferation within the dentate gyrus (Aguado et al., 2005, 2006). In addition to these effects on progenitor cell proliferation, the endocannabinoid system interacts with several neurotrophic systems. Specifically, the endocannabinoid system can mediate increases in brain-derived neurotrophic factor (BDNF) under conditions of neurological insult (Khaspekov et al., 2004) and in vitro, fibroblast growth factor 2 (FGF-2) recruits endocannabinoid signaling to promote axonal growth (Williams et al., 2003). The regulation of neurogenic and neurotrophic processes within the hippocampus by the endocannabinoid system makes this system an ideal candidate for mediating the effects of VEx on hippocampal cell proliferation. In support of this suggestion, there is evidence that the protective and beneficial effects of VEx and enhanced endocannabinoid signaling are similar. For example, increased CB₁ receptor activation reduces cellular damage following a closed head injury or excitotoxic insult, similar to the neuroprotective effect seen following exercise (Aguado et al., 2007; Gobbo and O’Mara, 2005; Griesbach et al., 2004; Luo et al., 2007; Marsicano et al., 2003; Panikashvili et al., 2001). Further, in addition to the ability of VEx to increase circulating growth factors (such as VEGF and IGF-1; Schobersberger et al., 2000; Schwarz et al., 1996; Trejo et al., 2001), serum content of AEA increases in humans following a sustained period of physical exercise (Sparling et al., 2003). Therefore, the present experiments were designed to explore the hypothesis that VEx enhances endocannabinoid signaling within the hippocampus, and whether this is associated with changes in the rate of progenitor cell proliferation.
3.2 METHODS

3.2.1 Subjects

Seventy day old male Sprague-Dawley rats (300 g; Charles River Laboratories, Montreal, Canada) were housed in groups of three in triple mesh wire caging for a ten day acclimation period following arrival to the institution. Following acclimation, half of the rats were randomly assigned to either standard caging or caging that contained a running wheel connected to a PC computer (Mini-Mitter Systems Inc., WA, USA). Animals in the VEx condition were individually housed in the cages with running wheels for eight days while control animals were housed individually in equivalent caging that did not include a functional running wheel for the same period of time. Colony rooms were maintained at 21 °C, and on a 12 h light/dark cycle, with lights on at 0900 h. All rats were given ad libitum access to Purina Rat Chow and tap water. All protocols were approved by the Canadian Council for Animal Care and the standards of the Animal Care Committee of the University of British Columbia.

3.2.2 Experiment 1: The Effects of VEx on the Endocannabinoid System in the Hippocampus and Prefrontal Cortex

3.2.2.1 Treatment Procedure

Animals were housed and maintained as described above. VEx animals and sedentary controls were sacrificed between 0900-1100 h on the morning following the eighth day of access to running wheels. The hippocampus and prefrontal cortex (consisting of medial prefrontal cortex and anterior cingulate cortex) were sectioned out, frozen in liquid nitrogen within 5 min of decapitation and stored at -80 °C until analysis. Two cohorts of tissue were collected. One cohort of tissue (n = 7) was used for lipid
extraction to determine endocannabinoid ligand content. The other cohort of tissue (n = 5) was used to create membrane fractions to examine CB₁ receptor binding, CB₁ receptor-mediated GTPγS binding and FAAH activity.

3.2.2.2 Membrane Preparation

Dissected brain sections were homogenized in 10 volumes of TME buffer (50 mM Tris HCl, pH 7.4; 1 mM EDTA and 3 mM MgCl₂). The homogenates were centrifuged at 18,000 x g for 20 min after which the supernatant was rapidly decanted. The remaining pellet, which is the membrane fraction, was resuspended in 1-2 ml TME buffer. Protein concentrations were determined by the Bradford method (Bio-Rad, Hercules, CA, USA).

3.2.2.3 CB₁ Receptor Binding

Cannabinoid CB₁ receptor binding assays were performed identically as described in Chapter 2.

3.2.2.4 CB₁ Receptor-mediated GTPγS Binding Assay

The assay for [³⁵S]GTPγS binding was performed as previously described by Kearn et al. (1999). Briefly, membranes (final concentration, 5 µg of protein per incubation mixture) were added to TME buffer containing 0.1% fatty acid-free bovine serum albumin, 10 µmol/L GDP, and 150 mmol/L NaCl. [³⁵S]GTPγS (final concentration, 0.65 nmol/L) was added, and the incubation was continued for 30 min at 37°C using the Multiscreen Filtration System with Durapore filters (pore size, 1.2 µm; Millipore, Bedford, MA, USA). Non-specific binding was determined in the presence of 10 µmol/L Gpp(NH)p and accounted for <15% of the total binding. Bound [³⁵S]GTPγS was separated from free [³⁵S]GTPγS by filtration followed by washing the filters four
times with cold TME buffer containing NaCl and GDP. The cannabinoid CB₁ receptor agonist WIN 55,212 was added in 1 µL of dimethyl sulfoxide at concentrations of 0, 0.1, 0.3, 0.6, 1, 2, 3, 6, 10, 20 and 30 µmol/L. In each experiment, the agonist-dependent [³⁵S]GTPγS binding was divided by agonist-independent binding and multiplied by 100 to convert to a percentage. The EC₅₀ values and maximal agonist-induced increase in binding (Eₘₐₓ) of [³⁵S]GTPγS were determined by fitting the data to a sigmoidal concentration-response curve using nonlinear regression (Prism; GraphPad, San Diego, CA, USA).

3.2.2.5 Fatty Acid Amide Hydrolase Activity Assay

FAAH activity was measured as the conversion of AEA to arachidonic acid and ethanolamine by membrane preparations (Hillard et al., 1995). AEA labeled with [³H] in the ethanolamine portion of the molecule ([³H]AEA; Omeir et al., 1995) was the radiolabeled substrate. Membranes were incubated in a final volume of 0.5 ml of TME buffer (50 mM Tris-HCl, 3.0 mM MgCl₂, and 1.0 mM EDTA, pH 7.4) containing 1.0 mg/ml fatty acid-free bovine serum albumin and 0.2 nM [³H]AEA. Isotherms were constructed using eight concentrations of AEA at concentrations between 10 nM and 10 µM. Incubations were carried out at 37°C and were stopped with the addition of 2 ml of chloroform/methanol (1:2). After standing at ambient temperature for 30 min, 0.67 ml of chloroform and 0.6 ml of water were added. Aqueous and organic phases were separated by centrifugation at 1,000 rpm for 10 min. The amount of [³H] in 1 ml each of the aqueous and organic phases was determined by liquid scintillation counting and the conversion of [³H]AEA to [³H]ethanolamine was calculated. The Kᵢ and Vₘₐₓ values for this conversion were determined by fitting the data to a single site competition
equation using Prism. The $r^2$ value for the goodness of fit of the data to the single site, hyperbolic equation was always greater than 0.9 and typically closer to 0.98.

3.2.2.6 Endocannabinoid Extraction and Analysis

Extraction and analysis details are identical to those detailed in Chapter 2.

3.2.3 Experiment 2: The Role of the Endocannabinoid System in Exercise-induced Increases in Cell Proliferation in the Dentate Gyrus

3.2.3.1 Treatment Procedure

Subjects were acclimated and assigned to groups as described above. Once in their respective housing conditions, half of the VEx and sedentary controls received daily intraperitoneal injections of the cannabinoid CB$_1$ receptor antagonist AM251 (1 mg/kg; Tocris Biosciences, Ellisville, MO, USA), while the other half received daily injections of vehicle (1:1:8 solution of DMSO: Tween 80: 0.9% saline). Injections began the morning following the initial transfer to the new cages and were performed using 26 gauge 1/2" needles at a volume of 1 ml/kg. To minimize any disruption in activity levels, daily injections were given at the onset of the light cycle (between 0900-1100h), when the animals were the least active.

Following the morning of the eighth consecutive day of VEx, all subjects were overdosed with sodium pentobarbital (120 mg/kg) and perfused transcardially with 60 ml of 0.9% saline, followed by 60 ml of 4% paraformaldehyde. Brains were removed and stored in paraformaldehyde for 24 hours before being transferred to 30% sucrose until saturated. Coronal sections (40 μm) were obtained throughout the extent of the hippocampus with a Leica VT1000 vibratome.
3.2.3.2 Ki67 Immunohistochemistry and Volumetric Analysis of the Dentate Gyrus

For Ki67 immunohistochemistry, the tissue was rinsed in 0.1 M TBS, followed by 0.3% H₂O₂ and then transferred to the primary antibody solution contained 0.5% Triton X, 1% normal horse serum and 1:1000 rabbit anti-Ki67 (Vector, Burlington ON, Canada) in 0.1 M TBS. Tissue was incubated in the primary solution for 20 h at room temperature (approximately 21 °C) and then rinsed in 0.1 M TBS. Tissue was then incubated in Biotinylated goat anti-rabbit IgG (Vector, Burlington ON, Canada) diluted 1:1000 in 0.1 M TBS for 1 h at room temperature and was then washed in 0.1 M TBS. An avidin-biotin peroxidase was applied for 60 min (ABC elite kit; Vector, Burlington ON, Canada). Labeling was visualized by incubating tissue in 5 mg/ml 3,3′-diaminobenzidine (DAB; Sigma-Aldrich, Oakville ON, Canada). Finally, the tissue was mounted on glass slides, counterstained with cresyl violet and coverslipped.

We utilized a modified stereological approach to determine the expression of Ki67-labeled cells across the rostral-caudal extent of the hippocampus. Ki67-labeled cells were counted in every 10th section (400 μm apart) throughout the granule cell layer (including the subgranular zone) and the hilus to obtain an estimate of the total number of labeled cells in each region. Counting was performed using a 100× oil immersion objective and a Nikon E600 light microscope. Corresponding area measurements were made of the dentate gyrus (granule cell layer and hilus calculated separately) using the software program Image J. Volume estimations of the dentate gyrus were then calculated using Cavalieri’s principle (Gundersen and Jensen, 1987) by multiplying the aggregated areas by the distance between sections (400 μm). Total cell counts were calculated by multiplying the number of Ki67-labeled cells per animal by 10. Densities of Ki67-labeled
cells per cubic millimeter in each region (granule cell layer or hilus) were also calculated by dividing the total number of Ki67-labeled cells by the volume of the region (granule cell layer or hilus).

3.2.4 Statistical Analysis

For Experiment 1, the effects of VEx on the endocannabinoid system were analyzed by comparing VEx and sedentary control animals using an independent t-test. For Experiment 2, a univariate analysis of variance (ANOVA) was used with exercise and AM251 as fixed factors. Post hoc analysis was performed using a Tukey’s test. Significance was established against an alpha level equal to 0.05.

3.3 RESULTS

3.3.1 Exercise Increases Endocannabinoid Signaling in the Hippocampus

Animals with free access to a running wheel for eight days exhibited a significant increase in the maximal binding ($B_{max}$) of $[^3]$HCP55940 to the CB$_1$ receptor in the hippocampus [t (8) = 5.29, p < 0.005; Fig. 3.1]. This increase in the $B_{max}$ of the CB$_1$ receptor was accompanied by a significant reduction in the affinity ($K_d$) of $[^3]$H CP55940 for the CB$_1$ receptor [t (8) = 2.84, p < 0.05; Table 3.1]. There was no effect of VEx on the $B_{max}$ [t (8) = 0.48, p > 0.05; Fig. 3.1] or the $K_d$ [t (8) = 0.43, p > 0.05; Table 3.1] of the $[^3]$HCP55940 to bind to the CB$_1$ receptor in the prefrontal cortex.

Table 3.1: The effects of voluntary exercise (VEx) on the dissociation constant ($K_d$) of $[^3]$H-CP55940, a cannabinoid CB$_1$ receptor agonist, from the CB$_1$ receptor. Animals which engaged in VEx exhibited a significant increase in the $K_d$ of the CB$_1$ receptor in the hippocampus, but not prefrontal cortex. Significant differences from cage control (CON) animals (p < 0.05) denoted by *. For both treatment conditions, n = 5. Data are presented as mean nM of $[^3]$H-CP55940 +/- SEM.

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<td>Hippocampus</td>
<td>0.54 +/- 0.08</td>
<td>0.82 +/- 0.06*</td>
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<tr>
<td>Prefrontal Cortex</td>
<td>0.51 +/- 0.06</td>
<td>0.60 +/- 0.09</td>
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**Figure 3.1:** The effect of engagement in voluntary exercise (VEx) on the maximal binding ($B_{\text{max}}$) of the cannabinoid $CB_1$ receptor in the hippocampus (left panel) and the prefrontal cortex (right panel). Values denoted are means ± SEM. * denotes significant differences ($p < 0.05$) between VEx animals and the control group (CON).

VEx also increased $CB_1$ receptor mediated GTPγS binding within the hippocampus [$t (8) = 3.63$, $p < 0.02$; Fig. 3.2]. The EC50 for the $CB_1$ receptor agonist, WIN55,212-2 was dramatically reduced in animals allowed VEx. Vex evoked a trend toward an increase in the maximal stimulation of GTPγS binding ($E_{\text{max}}$) elicited by the $CB_1$ receptor agonist WIN-55212,2 in the hippocampus [$t (8) = 1.94$, $p = 0.10$; Fig. 3.2].

**Figure 3.2:** The effect of engagement in voluntary exercise (VEx) on the EC50 (left panel) and the maximal stimulation ($E_{\text{max}}$; right panel) of cannabinoid $CB_1$ receptor-mediated $^{35}$S- GTPγS binding in the hippocampus, elicited by the $CB_1$ receptor agonist WIN 55,212-2. Values denoted are means ± SEM. * denotes significant differences ($p < 0.05$) between VEx animals and the control group (CON).
The tissue content of AEA was significantly increased in the hippocampus following VEx \[ t(12) = 2.93, p < 0.02; \text{Fig. } 3.3 \]. This change was not associated with a decrease in the hydrolysis of AEA by FAAH because VEx had no effect on the maximal hydrolytic activity \( (V_{\text{max}}) \) of FAAH \[ t(8) = 0.55, p > 0.05; \text{Table } 3.2 \] or the binding affinity \( (K_{m}) \) of AEA for FAAH \[ t(8) = 0.66, p > 0.05; \text{Table } 3.2 \]. There was also a trend toward an increase in 2-AG content within the hippocampus \[ t(12) = 1.91, p = 0.08; \text{Fig. } 3.3 \]. Neither AEA \[ t(12) = 0.31, p > 0.05; \text{Fig. } 3.3 \] nor 2-AG \[ t(12) = 0.90, p > 0.05; \text{Fig. } 3.3 \] content was altered in samples from prefrontal cortex.

**Figure 3.3:** The effect of engagement in voluntary exercise (VEx) on the tissue content of the endocannabinoid ligands anandamide (AEA) and 2-arachidonoylglycerol (2-AG) in the a) hippocampus; and b) the prefrontal cortex. Values denoted are means \( \pm \) SEM. * denotes significant differences \( p < .05 \) between VEx animals and the control group (CON).
Table 3.2: The effects of voluntary exercise (VEx) on the maximal hydrolytic activity ($V_{\text{max}}$) and the binding affinity ($K_m$) of fatty acid amide hydrolase for anandamide.

There was no effect of VEx on either the $V_{\text{max}}$ or the $K_m$ of FAAH in the hippocampus. For both treatment conditions, $n = 5$. Data are presented as means +/- SEM.

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<td><strong>Hippocampus</strong></td>
<td></td>
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</tr>
<tr>
<td>$V_{\text{max}}$ (pmol/mg protein)</td>
<td>619.4 +/- 56.9</td>
<td>556.7 +/- 99.4</td>
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<tr>
<td>$K_m$ (nM)</td>
<td>0.53 +/- 0.09</td>
<td>0.61 +/- 0.09</td>
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3.3.2 Exercise-induced Increase of Cell Proliferation in the Dentate Gyrus requires the Cannabinoid CB$_1$ Receptor

Since VEx increases endocannabinoid signaling in the hippocampus and both endocannabinoid and physical activity can promote cell proliferation (Aguado et al., 2005, 2006; Eadie et al., 2005; Pereira et al., 2007), we examined if the exercise-induced increase in hippocampal endocannabinoid signaling contributes to enhanced cell proliferation in the dentate gyrus following VEx. Using the expression of an endogenous cell cycle protein, Ki67, as a marker of progenitor cell proliferation, we found that there was a significant interaction between voluntary exercise and administration of AM251 on the total number of Ki67 positive (Ki67+) cells in the granule cell layer of the dentate gyrus [$F(1, 19) = 7.40, p < 0.02$; Fig. 3.4]. Post hoc analysis revealed that this interaction was due to an increase in Ki67+ cells in the granule cell layer of animals that had engaged in VEx ($p < 0.04$); animals administered AM251, alone or in conjunction with VEx, did not exhibit any significant changes in Ki67 expression in the dentate gyrus relative to vehicle treated controls ($p > 0.05$). This increased expression of Ki67+ cells following VEx was not an artifact of volumetric changes within the dentate gyrus as there was neither a significant interaction [$F(1, 19) = 0.25, p > 0.05$; Table 3.3] nor main effect
of either VEx \[ F(1, 19) = 0.35, \ p > 0.05 \] or AM251 administration \[ F(1, 19) = 1.90, \ p > 0.05 \] on the volume of the granule cell layer of the dentate gyrus.

**Figure 3.4**: The effect of engagement in voluntary exercise (VEx), administration of the cannabinoid CB1 receptor antagonist AM251 (AM; 1 mg/kg), or both treatments combined, on the total estimated number of proliferating cells expressing the endogenous cell cycle protein Ki67 within the a) granule cell layer (GCL) of the dentate gyrus; or b) hilus of the dentate gyrus. Values denoted are means ± SEM. * denotes significant differences (\( p < .05 \)) between a treatment condition and the control group (CON) receiving vehicle injections (VEH).
Table 3.3: The effects of voluntary exercise (VEx) and administration of the cannabinoid CB1 receptor antagonist AM251 (1 mg/kg) on the volume of the granule cell layer (GCL) and hilus of the dentate gyrus.

There was no effect of VEx or AM251 on the volume of the GCL or the hilus of the dentate gyrus. For all treatment conditions, n = 5-6. Data are presented as means +/- SEM.

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<td><strong>GCL (mm³)</strong></td>
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<tr>
<td>Vehicle</td>
<td>3.08 +/- 0.12</td>
<td>3.19 +/- 0.13</td>
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<tr>
<td>AM251</td>
<td>3.14 +/- 0.18</td>
<td>3.28 +/- 0.29</td>
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<tr>
<td><strong>Hilus (mm³)</strong></td>
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<tr>
<td>Vehicle</td>
<td>7.16 +/- 0.29</td>
<td>7.27 +/- 0.58</td>
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<tr>
<td>AM251</td>
<td>7.81 +/- 0.57</td>
<td>7.12 +/- 0.12</td>
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When the expression of Ki67+ cells was examined as a density measurement of the number of Ki67+cells/mm³ of the granule cell layer, an interaction was found between VEx and AM251 administration [F (1, 19) = 6.90, p < 0.02; data not shown]. Post hoc analyses revealed that VEx increased the density of Ki67+ cells within the dentate gyrus (p < 0.04), which was abolished by administration of AM251. The effect of VEx and CB1 receptor antagonism on cell proliferation was specific to the granule cell layer of the dentate gyrus as there was no significant interaction between exercise and AM251 [F (1, 19) = 1.39, p > 0.05; Fig. 3.4] nor main effects of either exercise [F (1, 19) = 1.14, p > 0.05] or AM251 administration [F (1, 19) = 0.00, p > 0.05] on the total number of Ki67+cells in the hilus of the dentate gyrus. Similarly, there was no effect of exercise and AM251 administration on the volume of the hilus of the dentate gyrus [F (1, 19) = 3.23, p > 0.05; Table 3.3] or the density of Ki67+cells within the hilus [F (1, 19) = 0.40, p > 0.05; data not shown]. Representative photomicrographs of the expression of Ki67+proliferating cells in the dentate gyrus following VEx, AM251 administration, or
both treatments combined, can be seen in Fig. 3.5; representative Ki67+ cells can be seen in the photomicrograph in Fig. 3.6.

**Figure 3.5:** Representative photomicrographs of voluntary exercise, AM251 administration, or both treatments combined on the immunoreactivity of Ki-67 in the dentate gyrus. C=control; Ex=Exercise; V=vehicle; AM=AM251.
Figure 3.6: Representative photomicrograph of Ki67 immunoreactive cells in the subgranular zone of the dentate gyrus.

It is important to note that there was no effect of drug treatment on the total distance run during the eight day running period \( t(10) = 1.41, p > 0.05; \) data not shown] indicating that the reduction of proliferation following AM251 administration was not due to a reduction in VEx.

3.4 DISCUSSION

These data demonstrate that VEx significantly increases endocannabinoid signaling within the hippocampus, and endocannabinoid signaling is required for VEx to increase proliferation of progenitor cells within the dentate gyrus. It is our hypothesis that these events are mechanistically linked such that the effects of VEx on hippocampal endocannabinoid signaling drive the increase in progenitor cell proliferation.

Rats with free access to a running wheel for eight days exhibited a significant increase in the agonist binding site density of the cannabinoid CB\(_1\) receptor; a significant
increase in CB₁ receptor agonist potency to induce GTPγS binding; and a significant increase in the hippocampal total tissue concentration of the endocannabinoid anandamide. None of these effects were observed in the prefrontal cortex, indicating some degree of specificity to this phenomenon. VEx increased the number of Ki67+ cells in the granule cell layer of the dentate gyrus of the hippocampus, consistent with previous reports (Eadie et al., 2005; Fabel et al., 2003; Pereira et al., 2007; van Praag et al., 1999). However, the VEx-induced increase in cell proliferation did not occur in rats receiving daily administration of the cannabinoid CB₁ receptor antagonist, AM251 (1 mg/kg) throughout the eight day exercise period. Taken together, these data suggest a role for increased endocannabinoid signaling within the hippocampus by VEx in increasing mitotic activity within the granule cell layer of the dentate gyrus.

Interestingly, we recently reported that administration of the endocannabinoid uptake inhibitor/FAAH inhibitor, AM404, prevented stress-induced suppression of cell proliferation in the dentate gyrus (Hill et al., 2006). Given that acute exposure to stress reduces AEA content within the hippocampus (C.J. Hillard and S. Patel, unpublished data), these data suggest that reductions in AEA/CB₁ receptor signaling contribute to the effect of stress to suppress cell proliferation. In light of the current study, these data, collectively, indicate that the endocannabinoid system, and particularly AEA/CB₁ receptor signaling, is a critical mediator of experience-induced plasticity within the hippocampus, being both sensitive to environmental stimuli and a potent regulator of neuroplastic processes.

The mechanism(s) by which VEx increases endocannabinoid signaling remain to be determined. VEx increases cAMP response element (CRE) transcription activation
within the hippocampus (Shen et al., 2001). Phosphorylation of cAMP response element binding protein (CREB) is doubled following one night of physical activity and CRE binding within the hippocampus is increased following three days of VEx (Shen et al., 2001). It is plausible that the CB1 receptor is one of the many genes to possess a CRE, suggesting that the exercise-induced increase in CRE transcriptional activity, may promote the production of the CB1 receptor. Alternately, it is possible that the changes in receptor binding may be a result of changes in ligand availability. Previous studies have indicated that the regulation of the cannabinoid CB1 receptor and its endogenous ligands are not coupled in the typical negative regulation relationship, in which the ligand down-regulates its own receptor (Hill et al., 2005). In fact, direct infusion of 2-AG into the brain up-regulates CB1 receptor mRNA transcription (Kola et al., 2005) and 2-AG stimulates membrane expression of CB1 receptors in striatal tissue slices (Maccarone et al., 2008), indicating that increased endocannabinoid signaling can promote both the genetic expression and surface recycling of the CB1 receptor. Therefore, the VEx-induced increase of endocannabinoid content in the hippocampus observed in the current study could precede, and drive, the increase in the CB1 receptor pool.

The increase in AEA content observed here was not due to a reduction in FAAH activity, so it is not likely that the mechanism involves decreased catabolism but more so, is due to an enhancement in biosynthesis. Currently, there are three biochemical pathways that have been defined through which AEA synthesis can occur (Liu et al., 2006; Okamoto et al., 2004; Simon and Cravatt, 2006) and it is not known which of these is the predominant pathway in determining neuronal AEA synthesis, making it difficult to ascertain the enzymatic cascade responsible for mediating the increase in AEA following
VEx. However, the synthesis of AEA is tightly coupled to calcium signaling and neuronal activation and is positively regulated by excitatory neurotransmission in the hippocampus (Jung et al., 2005; Marsicano et al., 2003; Ohno-Shosaku et al., 2002). Any type of movement, particularly running, would be expected to enhance network activity (i.e. theta activity) in the hippocampus (e.g., Keleman et al., 2005) and this may further enhance endocannabinoid synthesis and transmission. Alternately, enhancement of cAMP signaling has been found to promote AEA synthesis in neuronal cultures and slice preparations (Azad et al., 2004; Cadas et al., 1996; Vellani et al., 2008) and voluntary exercise is known to increase hippocampal cAMP signaling (Shen et al., 2001); thus, it is also possible that changes in intracellular cAMP signaling, and subsequent protein kinase activity, could drive the up-regulation of hippocampal AEA content.

Neural progenitor cells in the hippocampus express CB1 and CB2 receptors and synthesize both AEA and 2-AG (Aguado et al., 2005, 2006; Jiang et al., 2005; Molina-Holgado et al., 2007; Palazuelos et al., 2006). Activation of these receptors, presumably on the progenitor cells themselves, pushes these precursor cells into a mitogenic state to produce a progeny population. Several in vitro and in vivo studies have demonstrated that the CB1 receptor can activate the phosphatidylinositol-3 kinase (PI3K)/Akt pathway (Galve-Roperh et al., 2002; Gomez del Pulgar et al., 2000; Molina-Holgado et al., 2002, 2005, 2007; Ozaita et al., 2007) and indicate that this pathway is instrumental for cannabinoid-induced proliferation (Molina-Holgado et al., 2007). Activation of the PI3K/Akt pathway within progenitor cells is sufficient to induce proliferation and is a common pathway through which many growth factors also induce proliferation (Aberg et al., 2003; Jin et al., 2005; Peltier et al., 2007). VEx can activate the PI3K/Akt pathway
(Chen and Russo-Neustadt, 2005) although it remains to be determined if this signaling pathway is required for VEx-induced proliferation.

Because trophic factors, such as VEGF and IGF-1, are essential for the proliferative effects of VEx (Fabel et al., 2003; Trejo et al., 2001), it is possible that endocannabinoids and neurotrophic factors act in concert to regulate VEx-induced cell proliferation. Endocannabinoids and many neurotrophic factors utilize the PI3/Akt pathway to promote cell proliferation; thus, these systems may converge at the signal transduction level (Aberg et al., 2003; Galve-Roperh et al., 2002; Gomez del Pulgar et al., 2000; Jin et al., 2005; Molina-Holgado et al., 2002, 2005, 2007; Ozaita et al., 2007; Peltier et al., 2007). Additionally, there is evidence that the CB1 receptor and the IGF-1 receptor also interact at the G protein level (Bouaboula et al., 1997). In particular, a CB1 receptor antagonist/inverse agonist, similar to AM251, can prevent IGF-1 induced phosphorylation of MAP kinase (Bouaboula et al., 1997). Phosphorylation of MAP kinase by IGF-1 is necessary for this growth factor to stimulate progenitor cell proliferation (Aberg et al., 2003); therefore, AM251 could reverse VEx-induced increases in proliferation by impairing IGF-1 signal transduction.

At the clinical level, exercise has demonstrated therapeutic benefit for an array of psychiatric and neurological conditions, such as Alzheimer's disease and depressive illness (Cotman et al., 2007, Dunn et al., 2005; Ernst et al., 2006). We have previously demonstrated that endocannabinoid signaling is dampened in the hippocampus in an animal model of depression and increased following treatment with a conventional antidepressant (Hill et al., 2005; Chapter 2), whereas local infusions of a CB1 receptor agonist directly into the dentate gyrus evoked an antidepressant-like response.
(McLaughlin et al., 2007). It has been suggested that induction of neural progenitor proliferation contributes to the antidepressant effects of exercise (Bjornebekk et al., 2005; Ernst et al., 2006). Therefore, the VEx-induced increase in hippocampal endocannabinoid activity could contribute to the antidepressant effects of this regimen, potentially via its contribution to changes in cell proliferation. Additionally, the neuroprotective effects of exercise might be afforded by increased endocannabinoid signaling. Both exercise and endocannabinoid signaling can dampen excitotoxic damage within the hippocampus and improve long-term outcomes following a neurological insult, indicating the possibility of functional overlap (Gobbo and O’Mara, 2005; Griesbach et al., 2004; Luo et al., 2007; Marsicano et al., 2003; Panikashvili et al., 2001). Future research should examine the extent to which endocannabinoid signaling contributes to the neuroprotective and antidepressant effects of exercise. In conclusion, these data demonstrate that VEx enhances endocannabinoid signaling in the hippocampus through effects on both ligand availability and receptor sensitivity to agonist. Furthermore, this enhanced endocannabinoid signaling appears to contribute to VEx-induced increases in cell proliferation in the dentate gyrus, as treatment with a CB1 receptor antagonist attenuated this effect. These data add to an increasing body of evidence supporting the global hypothesis that the endocannabinoid system mediates experience-induced alterations in plasticity within the hippocampus.
3.5 REFERENCES


cannabinoid receptors and on-demand defense against excitotoxicity. Science 302: 84-88.


CHAPTER 4
ELECTROCONVULSIVE SHOCK TREATMENT DIFFERENTIALLY MODULATES CORTICAL AND SUBCORTICAL ENDOCANNABINOID ACTIVITY

4.1 INTRODUCTION

The endocannabinoid system in the brain is a neuromodulatory system composed of at least two endogenous ligands, N-arachidonylethanolamine (anandamide; AEA) and 2-arachidonylglycerol (2-AG) and at least one receptor, the CB1 cannabinoid receptor (Howlett, 2002). The CB1 receptor is a G-protein coupled receptor (GPCR) which signals through G proteins of the alpha1 and alpha0 classes to inhibit adenylyl cyclase activity and reduce cellular calcium influx (Howlett, 2002). The CB1 receptor is believed to exhibit the highest density of expression for all GPCR's in the brain; with significant levels of expression within limbic structures such as the amygdala, and hypothalamus, and higher levels of expression in the frontal cortex and hippocampus (Herkenham et al., 1991; Moldrich and Wenger, 2000).

A recent increase in preclinical and clinical research has suggested that the endocannabinoid system could have potential relevance for the etiology or treatment of depression; however, the nature of this role has been somewhat controversial (Hill and Gorzalka, 2005a; Mangieri and Piomelli, 2007; Vinod and Hungund, 2006; Witkin et al.,

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Deficiencies in endocannabinoid signaling are associated with a behavioral phenotype similar to the symptom profile of severe depression, suggesting that impairments in endocannabinoid signaling may be involved in the etiology of depressive illness (reviewed in Hill and Gorzalka, 2005a). In line with this hypothesis, pharmacological activation of the endocannabinoid system elicits an antidepressant response in animal models (Bortolato et al., 2007; Gobbi et al., 2005; Hill and Gorzalka, 2005b; Hill et al., 2007). Preclinical studies have revealed that CB₁ receptor density and tissue content of the endocannabinoid ligand 2-AG are reduced in the hippocampus following chronic unpredictable stress, an animal model of depression (Hill et al., 2005). Further, clinical trials employing the CB₁ receptor antagonist rimonabant for the treatment of obesity demonstrated that the induction of depressive and anxious symptoms were the most prevalent adverse response following drug treatment (van Gaal et al., 2005). There is also evidence that the endocannabinoid system is involved in the mechanism of action of antidepressant medications. For example, chronic desipramine treatment increases CB₁ receptor binding in the hippocampus and hypothalamus (Chapter 2), voluntary exercise increases CB₁ receptor signaling and endocannabinoid ligand content in the hippocampus (Chapter 3) and sleep deprivation is associated with increased 2-AG content in the hippocampus (Chen and Bazan, 2005).

However, there is also evidence for the alternative hypothesis that CB₁ receptor activity is hyperfunctional in depression. For example, CB₁ receptor antagonists also produce the characteristic effects of antidepressant agents in preclinical tests (Griebel et al., 2005; Shearman et al., 2003; Tzavara et al., 2003), and a post-mortem study revealed that depressed individuals who committed suicide exhibit increased prefrontal cortical
CB₁ receptor expression, binding site density, and CB₁ receptor-dependent GTPγS binding (Hungund et al., 2004). In light of the complexity of the limbic circuitry, the wide distribution of the CB₁ receptor in the limbic system and the evidence that CB₁ receptor activity regulates several neurochemical systems (i.e., glutamatergic, GABAergic, serotonergic; Freund et al., 2003), it is possible that both increased and decreased CB₁ receptor activity could be involved in limbic dysfunction in the development of depression and during its treatment.

Electroconvulsive shock (ECS) treatment represents the most effective therapeutic option for depression, benefiting a higher proportion of patients than chemical antidepressants and requiring substantially less time to elicit a clinical response (Silverstone and Silverstone, 2004). As such, determination of the changes elicited by repeated ECS sessions could be relevant to its mechanism of action and by the same argument, systems regulated by ECS could represent potential targets for therapeutic development (Hyman and Nestler, 1996; Newman et al., 1998). Given that the endocannabinoid system appears to be engaged by chemical antidepressant treatment (Chapter 2), and that several other modes of convulsive stimuli result in dramatic alterations in endocannabinoid activity (Lutz, 2004; Marsicano et al., 2003; Wallace et al., 2003), we have tested the hypothesis that ECS modulates endocannabinoid signaling. We examined the effects of a single and ten ECS sessions on endocannabinoid content, CB₁ receptor binding and CB₁ receptor-mediated GTPγS binding in the prefrontal cortex (PFC), hippocampus, hypothalamus and amygdala.
4.2 METHODS

4.2.1 Subjects

Seventy day old male Sprague-Dawley rats (approx. 285 g at the onset of the study; Charles River, Montreal) housed in groups of three in triple wire mesh cages were used in this study. Colony rooms were maintained at 21°C, and were on a 12 h light/dark cycle, with lights on at 0700 h. All rats were given ad libitum access to Purina Rat Chow and tap water. All treatments performed in this study were approved by the Animal Ethics Committee of the University of British Columbia and were consistent with the standards of the Canadian Council on Animal Care.

4.2.2 Treatment Procedure

Three cohorts of animals were generated for all biochemical studies: a) sham stimulation; b) single ECS session; c) ten ECS sessions. Separate groups of animals were generated for analysis of endocannabinoid content and for analysis of parameters that were assessed in membrane fractions (i.e., CB₁ receptor binding, CB₁ receptor signaling and FAAH assays). For ECS sessions, animals were removed from their cages individually, and ECS was applied as described previously, based on a protocol that was effective in reversing behavioral anhedonia in an animal model of depression (Barr et al 2002a,b). ECS was applied bilaterally, with earclips that were coated in electroconductive gel. Treatments were delivered using a constant current UGO Basile (Varese, Italy) apparatus for small mammals; this device delivers a unidirectional brief pulse stimulus. Treatment parameters were set at 90 mA, 70 Hz for 2-4 s (the minimal values necessary for seizure induction), or sham ECS (no current applied). Animals in the ECS groups were required to exhibit a full tonicoclonic seizures for approximately 20–25 s. Eighteen
h following the last stimulation or sham stimulation, all animals were rapidly decapitated. This time point was chosen as previous work has demonstrated that at 18 h, residual effects of ECS treatment are still present without the confounding effect of immediate changes elicited by ECS itself (Nibuya et al., 1995). Prefrontal cortex (composed of medial prefrontal cortex and anterior cingulate), amygdala (composed of central, basolateral and medial nuclei), hippocampus, and hypothalamus were dissected out on ice, immediately frozen in liquid nitrogen and stored at -80°C until analysis.

4.2.3 Biochemical Measurements

One cohort of tissue was converted to membrane fraction as described in Chapter 3. These membranes were subsequently used for CB₁ receptor binding (see Chapter 2), FAAH activity assays (Chapter 3) and CB₁ receptor mediated GTPγS binding (see Chapter 3). A second cohort of tissue was generated for the extraction and analysis of endocannabinoid ligand content (see Chapter 2).

4.2.4 Statistics

Comparison of the effects of single and repeated ECS sessions on parameters of CB₁ receptor binding, [³⁵S]GTPγS binding and endocannabinoid content were analyzed with a one way analyses of variance (ANOVA). Subsequently, analysis of the data was also performed using a two tailed Dunnett's test to determine if either treatment condition differed from sham treated animals. To examine whether regional differences existed with regards to the binding and signaling properties of the CB₁ receptor, we used a one way ANOVA to compare the maximal binding, the binding affinity and the sensitivity of signal transduction of the CB₁ receptors in all four brain regions employing only animals that had only received sham treatment. Post hoc analysis was done with this analysis.
using a Tukey’s test. For CB₁ receptor binding and CB₁ receptor-mediated GTPγS
binding, 4-5 subjects/group were used; for tissue analysis of endocannabinoid content, 7-
9 subjects/group were used. Significance was established against an alpha level of .05.

4.3 RESULTS

4.3.1 Prefrontal Cortex

Within the PFC, there was a significant effect of ECS treatment on the maximal
binding (B_max) of [³H]CP,55940 to the cannabinoid CB₁ receptor [F (2, 10) = 13.30, p <
0.01; Fig. 4.1]. Analysis with a Dunnett’s test revealed that repeated ECS sessions
resulted in a significant reduction in the B_max of the CB₁ receptor (p < 0.01); a single ECS
session did not alter the B_max of the CB₁ receptor (p > 0.05).

Figure 4.1: The effects of sham stimulation (CON), a single electroconvulsive shock
stimulation (ECS-1) or ten electroconvulsive shock stimulations (ECS-10) on the
maximal binding (B_max) of [³H]CP55940 to the cannabinoid CB₁ receptor
in the (a) prefrontal cortex, (b) hippocampus, (c) amygdala and (d) hypothalamus.
Values are denoted as means ± SEM. * denotes values which are significantly different
from control (p < .05).
There was also a significant effect of ECS treatment on the affinity (K_D) of 
[^3]H]CP,55940 for the CB_1 receptor [F (2, 10) = 6.72, p < 0.02; Fig. 4.2]. A Dunnett's test 
revealed that the K_D of [^3]HCP 55,940 in PFC from rats exposed to a single ECS session 
is significantly greater than the sham exposed group (p < 0.05).

**Figure 4.2:** The effects of sham stimulation (CON), a single electroconvulsive shock 
stimulation (ECS-1) or ten electroconvulsive shock stimulations (ECS-10) on the binding 
affinity (K_d) of [^3]HCP55940 to the cannabinoid CB_1 receptor in the a) prefrontal cortex; 
b) amygdala; c) hippocampus; d) hypothalamus. Values are denoted as means ± SEM. * 
denotes values which are significantly different from control (p < .05).

Surprisingly, ECS treatment did not affect WIN 55212-2-dependent [^35]S]GTPγS 
binding; specifically, ECS treatment had no affect on the E_max [F (2, 9) = 0.62, p > 0.05; 
Table 4.1] or the EC50 [F (2, 9) = 0.76, p > 0.05; Table 4.1] of WIN 55,212-dependent 
[^35]S]GTPγS binding in the PFC.
Table 4.1: The effects of electroconvulsive shock treatment on CB$_1$ receptor-mediated $^{35}$S-GTP$_{y}$S binding in discrete brain regions.

$^{35}$S-GTP$_{y}$S binding was induced by the CB$_1$ receptor agonist WIN55,212 at concentrations ranging from 0 nM to 30000nM. Maximal $^{35}$S-GTP$_{y}$S binding ($E_{max}$) was determined relative to 0 nM concentration, and EC50 values represent the concentration of WIN55,212 required to induce 50% maximal $^{35}$S-GTP$_{y}$S binding.

A single electroconvulsive shock stimulation (ECS-1) did not affect CB$_1$ receptor-mediated $^{35}$S-GTP$_{y}$S binding in any brain region examined, whereas ten electroconvulsive shock stimulations (ECS-10) resulted in a sensitization of CB$_1$ receptor-mediated $^{35}$S-GTP$_{y}$S binding in the amygdala exclusively.

Significant differences from sham stimulated (CON) animals (p < 0.05) denoted by *.

Data are presented as mean values +/- SEM.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>CON Emax (% of vehicle)</th>
<th>ECS-1 Emax (% of vehicle)</th>
<th>ECS-10 Emax (% of vehicle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prefrontal Cortex</td>
<td>284.7 +/- 28.3</td>
<td>339 +/- 47.9</td>
<td>311.3 +/- 23.2</td>
</tr>
<tr>
<td></td>
<td>1367 +/- 69.4</td>
<td>1034.7 +/- 270.5</td>
<td>1039 +/- 213.9</td>
</tr>
<tr>
<td>Amygdala</td>
<td>219.7 +/- 12.4</td>
<td>215.7 +/- 12.2</td>
<td>211.3 +/- 23.3</td>
</tr>
<tr>
<td></td>
<td>673.7 +/- 65.3</td>
<td>434 +/- 96.6</td>
<td>390.3 +/- 48.1*</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>308.8 +/- 13.9</td>
<td>298 +/- 8.9</td>
<td>349.3 +/- 68.6</td>
</tr>
<tr>
<td></td>
<td>493 +/- 129.3</td>
<td>730 +/- 203.7</td>
<td>378.5 +/- 74.2</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>224.8 +/- 36.1</td>
<td>231 +/- 18.2</td>
<td>226.8 +/- 26.4</td>
</tr>
<tr>
<td></td>
<td>1511.3 +/- 93.3</td>
<td>1580.3 +/- 386.5</td>
<td>1652 +/- 413.2</td>
</tr>
</tbody>
</table>

There was no effect of ECS treatment on 2-AG content in the PFC [F (2, 23) = 0.51, p > 0.05; Table 4.2]. ECS treatment resulted in a significant reduction in AEA content of the PFC [F (2, 23) = 11.16, p < 0.001; Table 4.2], with a Dunnett’s test demonstrating that both a single session (p < 0.01) and repeated (p < 0.001) ECS sessions caused AEA content to decrease compared to sham. However, ECS treatment did not increase, but rather elicited a significant reduction in the maximal activity ($V_{max}$) of the FAAH enzyme in hydrolyzing AEA [F (2, 13) = 4.45, p < 0.04; Fig. 4.3], with a
Dunnett's test demonstrating that both a single session (p < 0.04) and repeated ECS sessions (p < 0.05) reduced FAAH V_max. Additionally though, ECS treatment also significantly increased the binding affinity of FAAH for AEA [F (2, 13) = 7.86, p < 0.01; Fig. 4.3], which again was due to an increase in the binding affinity of FAAH following both a single sessions (p < 0.02) or repeated ECS sessions (p < 0.01).

Table 4.2: The effects of electroconvulsive shock treatment on the tissue content of the two primary endocannabinoid ligands anandamide (AEA) and 2-arachidonylglycerol (2-AG).

A single electroconvulsive shock stimulation (ECS-1) resulted in reduced AEA content in both the prefrontal cortex and the hippocampus, whereas ten electroconvulsive shock stimulations (ECS-10) caused a dramatic reduction in prefrontal cortical AEA content exclusively. 2-AG content was not influenced by electroconvulsive shock treatment. Significant differences from sham stimulated (CON) animals (p < 0.05) denoted by *. Data are presented as mean values +/- SEM.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>CON</th>
<th>ECS-1</th>
<th>ECS-10</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Prefrontal Cortex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEA content (pmol/g tissue)</td>
<td>53.8 +/- 5.9</td>
<td>37.4 +/- 2.0*</td>
<td>30.3 +/- 2.3*</td>
</tr>
<tr>
<td>2-AG content (nmol/g tissue)</td>
<td>21.8 +/- 1.6</td>
<td>19.9 +/- 0.9</td>
<td>20.8 +/- 1.3</td>
</tr>
<tr>
<td><strong>Amygdala</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEA content (pmol/g tissue)</td>
<td>23.3 +/- 1.5</td>
<td>21.6 +/- 2.0</td>
<td>18.5 +/- 1.1</td>
</tr>
<tr>
<td>2-AG content (nmol/g tissue)</td>
<td>13.3 +/- 1.1</td>
<td>14.3 +/- 0.9</td>
<td>13.8 +/- 0.9</td>
</tr>
<tr>
<td><strong>Hippocampus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEA content (pmol/g tissue)</td>
<td>56.1 +/- 3.7</td>
<td>38.5 +/- 2.9*</td>
<td>49.5 +/- 4.7</td>
</tr>
<tr>
<td>2-AG content (nmol/g tissue)</td>
<td>13.2 +/- 0.5</td>
<td>12.6 +/- 0.6</td>
<td>13.4 +/- 0.5</td>
</tr>
<tr>
<td><strong>Hypothalamus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEA content (pmol/g tissue)</td>
<td>10.7 +/- 1.2</td>
<td>9.1 +/- 1.4</td>
<td>11.0 +/- 1.2</td>
</tr>
<tr>
<td>2-AG content (nmol/g tissue)</td>
<td>16.5 +/- 0.8</td>
<td>16.2 +/- 1.2</td>
<td>15.9 +/- 0.8</td>
</tr>
</tbody>
</table>
**Figure 4.3:** The effects of sham stimulation (CON), a single electroconvulsive shock stimulation (ECS-1) or ten electroconvulsive shock stimulations (ECS-10) on the maximal hydrolysis of anandamide (AEA) by fatty acid amide hydrolase ($V_{max}$; upper panel) or the affinity of FAAH for AEA ($K_m$; lower panel) in the prefrontal cortex. Values are denoted as means ± SEM.

* denotes values which are significantly different from control ($p < .05$).

4.3.2 Amygdala

In parallel with the results obtained in the PFC, ECS produced a significant effect on the $K_D$ for $[^3]H$CP55,940 binding in the amygdala [F (2, 12) = 9.82, $p < 0.01$; Fig. 4.2]; specifically, a single exposure to ECS produced a significant increase compared to sham exposed rats ($p < 0.02$). The $K_D$ for $[^3]H$CP55,940 was not different between sham and repeated ECS treated rats. There was also a significant effect of ECS treatment on the $B_{max}$ of $[^3]H$CP55,940 binding in the amygdala [F (2, 12) = 5.87, $p < 0.03$; Fig. 4.1], with analysis with a Dunnett’s test demonstrating that this effect was due to an increase in the $B_{max}$ of the CB$_1$ receptor following a single ECS session compared to sham ($p <$
0.03); the $B_{\text{max}}$ values determined in sham treated rats and repeated ECS treated rats were not different.

There was no effect of ECS treatment on the $E_{\text{max}}$ of $[^{35}\text{S}]$GTP$\gamma$S in the amygdala [F (2, 8) = 0.06, p > 0.05; Table 4.1]; however, there was a near significant effect of ECS treatment on the EC50 of $[^{35}\text{S}]$GTP$\gamma$S binding in the amygdala [F (2, 8) = 4.39, p < 0.07; Table 4.1]. Dunnett's test demonstrated that, relative to sham treated animals, repeated ECS resulted in a significant reduction in the EC50 (p < 0.03), whereas a single stimulation did not induce a significant change.

There was no significant effect of ECS treatment on either AEA content [F (2, 23) = 2.82, p > 0.05; Table 4.2] or 2-AG content [F (2, 23) = 0.25, p > 0.05; Table 4.2] in the amygdala.

### 4.3.3 Hippocampus

As was seen in the PFC and amygdala, there was a significant effect of ECS treatment on the $K_D$ of $[^{3}\text{H}]$CP55,940 [F (2, 11) = 15.38, p < 0.01; Fig. 4.2]. This effect of ECS was due to an increase in the $K_D$ of $[^{3}\text{H}]$CP55,940 for the CB$_1$ receptor following a single stimulation compared to sham (p < 0.01). Within the hippocampus, there was no effect of ECS treatment on the $B_{\text{max}}$ of $[^{3}\text{H}]-\text{CP}55,940$ [F (2, 11) = 0.55, p > 0.05; Fig. 4.1] for the CB$_1$ receptor.

There was no effect of ECS treatment on the $E_{\text{max}}$ [F (2, 11) = 0.44, p > 0.05; Table 4.1] or the EC50 [F (2, 11) = 1.51, p > 0.05; Table 4.1] of $[^{35}\text{S}]$GTP$\gamma$S binding in the hippocampus.

There was a significant effect of ECS treatment on AEA content in the hippocampus [F (2, 21) = 4.89, p < 0.02; Table 4.2]. Dunnett's test demonstrated that this
was due to a reduction in AEA content following a single ECS session (p < 0.02), but not following repeated ECS sessions compared to sham treatment. There was no effect of ECS treatment on 2-AG content in the hippocampus [F (2, 21) = 0.64, p > 0.05; Table 4.2].

4.3.4 Hypothalamus

There was a significant effect of ECS treatment on the K_D of [^3H]-CP 55,940 [F (2, 11) = 8.03, p < 0.01; Fig. 4.2], which was attributable to an increase in the K_D of [^3H]CP55,940 for the CB1 receptor following a single ECS session (p < 0.02), but not repeated ECS sessions compared to sham treated. ECS treatment did not affect the B_max of the CB1 receptor in the hypothalamus [F (2, 11) = 2.69, p > 0.05; Fig. 4.1].

There was no effect of ECS treatment on the E_max [F (2, 11) = 0.01, p > 0.05; Table 4.1] or the EC50 [F (2, 11) = 0.05, p > 0.05; Table 4.1] of [^35S]GTPγS binding in the hypothalamus.

There was no effect of ECS treatment on the hypothalamic contents of either AEA [F (2, 22) = 0.60, p > 0.05; Table 4.2] or 2-AG [F (2, 22) = 0.11, p > 0.05; Table 4.2].

4.3.5 Comparison Across Brain Regions of CB1 Receptor Binding and Activation

We compared the binding site densities (B_max) of the CB1 receptor and the binding affinity (K_d) for[^3H]CP55,940 across brain regions in the sham treated rats, as well as examined the EC50 for WIN55,212 mediated[^35S]GTPγS binding in these same areas, to determine if there were regional differences in CB1 receptor signaling that may help to interpret our current data. A one way ANOVA demonstrated that there was a significant effect of brain region on the B_max of the CB1 receptor [F (3, 15) = 4.14, p < 0.04;
prefrontal cortex: 0.45 +/- 0.07 pmol/mg protein vs. hippocampus: 0.26 +/- 0.02 pmol/mg protein vs. hypothalamus: 0.21 +/- 0.05 pmol/mg protein vs. amygdala: 0.37 +/- 0.07 pmol/mg protein]. Post hoc analysis employing a Tukey's test revealed that this effect was due to a significant difference between the prefrontal cortex and the hypothalamus (p < 0.05), such that the hypothalamus exhibited a significantly lower maximal binding of the CB1 receptor relative to the prefrontal cortex. One way ANOVA of the $K_d$ values for $[^3H]$CP55,940 also resulted in a significant difference across brain regions [F (3, 15) = 4.98, p < 0.03; prefrontal cortex: 0.58 +/- 0.10 nM vs. hippocampus: 0.28 +/- 0.07 nM vs. hypothalamus: 0.51 +/- 0.05 nM vs. amygdala: 0.24 +/- 0.05 nM], with post-hoc analysis demonstrating that this effect was due to the fact that the CB1 receptors in the prefrontal cortex exhibited a significantly weaker binding affinity relative to the hippocampus and amygdala (p < 0.05). Similarly, there was a significant effect of brain region of the EC$_{50}$ of CB1 receptor mediated $[^35S]$GTP$_{Y}$S binding [F (3, 15) = 25.94, p > 0.001; prefrontal cortex: 1367 +/- 69.4 nM vs. hippocampus: 493 +/- 193.3 nM vs. hypothalamus: 1511.3 +/- 93.3 nM vs. amygdala: 673.7 +/- 65.3 nM]. Tukey's analysis demonstrated that this was due to significantly higher sensitivity of the CB1 receptor to induce guanine nucleotide exchange in the hippocampus and amygdala relative to the prefrontal cortex and hypothalamus (all p's < 0.001).

**4.4 DISCUSSION**

Electroconvulsive shock treatment is currently the most efficacious treatment for severe and antidepressant-resistant depression; however the mechanism by which ECS treatment elicits clinical benefit is not well understood. Our data demonstrate that ECS treatments in rats produce several changes in endocannabinoid/CB1 receptor parameters.
First, repeated exposure to ECS results in a 60% reduction in the density of
[^3H]CP55,940 binding sites in the PFC in the absence of an effect on binding in
amygdala, hippocampus or hypothalamus. In spite of the large reduction in CB₁ receptor
binding site density, CB₁ receptor-mediated[^35S]GTPγS binding was not different in PFC
membranes from rats exposed to repeated ECS and sham. On the other hand, in the
amygdala, repeated ECS produced a significant reduction in the EC₅₀ for CB₁ receptor-
mediated guanine nucleotide exchange without an effect on CB₁ receptor binding
parameters. Second, ECS treatments did not affect 2-AG content in any brain region
examined; however, AEA content in the PFC was reduced 18 hours after the first and
tenth ECS treatment and AEA content was reduced in hippocampus after the first
exposure. Third, a single ECS exposure results in a pronounced and regionally non-
selective reduction in the affinity of the CB₁ receptor for[^3H]CP55,940; since the brains
were harvested 18 hours after the application of the ECS, these data suggest that a long-
lasting reduction in agonist affinity for the CB₁ receptor binding site is induced by a
single ECS exposure. However, the effect of single ECS treatment on the K_D for
[^3H]CP55,940 exhibits "tolerance" since the same parameter was not different from sham
in brains harvested 18 hours after the tenth session of ECS. While these data do not fully
investigate the temporal nature of ECS-induced alterations in endocannabinoid signaling,
they do suggest that ECS exerts long-term alterations in the balance between cortical and
subcortical endocannabinoid signaling.

Despite significant alterations in endocannabinoid signaling in the PFC and
amygdala, there was no robust alteration in the endocannabinoid system in the
hippocampus or the hypothalamus following ECS treatment. These data are surprising
given that the endocannabinoid system in these structures is sensitive to both stress exposure and antidepressant treatment (Chen and Bazan, 2005; Hill et al., 2005; Patel et al., 2004; data in Chapter 2 and 3). As such, the current data suggest that therapeutic changes elicited by ECS treatment do not involve altered endocannabinoid activity in these two regions; however, these data do not preclude the potential importance of endocannabinoid signaling in the hippocampus and hypothalamus in the pathology of depression.

An interesting finding of the current research was the ubiquitous reduction in binding affinity of $[^3H]CP55,940$ following a single ECS session. This reduction in binding affinity is likely mediated by a rapid biochemical event, possibly phosphorylation of the CB$_1$ receptor, which could shift the confirmation of the receptor into a low agonist affinity state; however, this hypothesis is speculative and would require validation. Alternately, an allosteric modulatory site has been identified on the CB$_1$ receptor, which when activated enhances the binding affinity of the CB$_1$ receptor (Price et al., 2005); thus, a single ECS exposure may have modified this site in a fashion such that the binding affinity of the CB$_1$ receptor became significantly reduced. Additionally, the presence of 5-HT has been documented to enhance the binding affinity of the CB$_1$ receptor (Devlin and Christopolous, 2002). As such, a rapid decline in 5-HT content could result in a reduction in binding affinity of the CB$_1$ receptor; however, ECS treatment results in an increase in 5-HT neurotransmission (Yoshida et al., 1998; Zis et al., 1992), arguing against this proposition. Irregardless of the mechanism by which a single ECS exposure modifies CB$_1$ receptor binding affinity, it is not clear that this change in affinity has
relevance to activation of CB₁ receptor signaling since a single ECS session did not affect CB₁-receptor mediated [³⁵S]GTPγS binding.

In the PFC, repeated ECS sessions resulted in a profound reduction in the density of binding sites for the CB₁ receptor. Given that binding of high affinity ligands to the CB₁ receptor is correlated with the ability of a ligand to induce a signaling response (Kearn et al., 1999) one would predict that the reduction in CB₁ receptor binding site density would be associated with diminished CB₁ receptor-mediated guanine nucleotide exchange; however, this was not the case. Repeated ECS treatments had no effect on CB₁ receptor-mediated [³⁵S]GTPγS binding in the PFC. While the possibility exists that the observed changes in CB₁ receptor binding are not associated with changes in signal capacity per se, there are alternative explanations. There is a possibility that ECS-induced changes in the G protein level may have influenced our data. For example, repeated ECS sessions have been shown to result in a reduction in basal GTPγS binding in the PFC exclusively, which could, in turn, reflect changes in either absolute content of G protein subunits or altered ability of these proteins to associate with a specific receptor (Nishida et al., 1990). Thus, the lack of change in CB₁ receptor-mediated GTPγS binding could be reflective of a change at the G protein level that compensates for a reduction in high affinity binding to the CB₁ receptor and, as a result, maintains functional levels of receptor signaling capacity. Further research is required to fully understand the nature of this dissociation and the functional consequence of the change in CB₁ receptor binding.

Regardless of the inconsistency between receptor binding and receptor signaling, the content of AEA in the PFC was significantly reduced after a single ECS session, and to a greater degree after repeated ECS sessions. Unlike the alterations in CB₁ receptor
binding affinity, maximal binding and reductions in AEA content in other regions after a
single ECS session, the reduction in prefrontal cortical AEA is the only change seen
following acute ECS that did not dissipate following chronic treatment, attesting to the
potential importance of this effect. As such, these data suggest that ECS treatment results
in a suppression of the endocannabinoid system in the PFC. Surprisingly, the content of
2-AG, the other major endocannabinoid, was unaffected by ECS treatment. This
reduction in AEA levels could be due to alterations in the activity of fatty acid amide
hydrolase (FAAH). However, our data demonstrate that both a single and repeated ECS
session reduced the maximal activity of FAAH while increasing the binding affinity of
AEA for FAAH. While an increase in the affinity of FAAH for AEA is consistent with
the reduced AEA levels in the PFC, the coincident reduction in total FAAH activity
would argue against this being the mechanism of action. In fact, the reduction in maximal
FAAH activity following ECS treatment could be a compensatory response to diminished
biosynthesis of AEA, which would support a hypothesis that the reduction in AEA
content is due to alterations in AEA synthesis rather than metabolism. In line with this
idea, ECS treatment has been shown to dramatically alter arachidonic acid liberation and
recycling (Peciconi et al., 1986; Reddy and Bazan, 1987). Specifically, there is a rapid
increase in free arachidonic acid following ECS treatment (Peciconi et al., 1986; Reddy
and Bazan, 1987), which in turn would result in a reduced pool of acylated arachidonic
acid for AEA biosynthesis. This mechanism is consistent with reductions in AEA after
both acute and chronic ECS treatments, and demonstrates that immediate changes in lipid
remodeling induced by ECS treatment could have functional consequences with regards
to biosynthesis and regulation of lipid derived neuroactive molecules such as the endocannabinoids.

Collectively, these data suggest that repeated ECS treatment is associated with a reduction in prefrontal cortical endocannabinoid activity. These findings are very intriguing in light of recent post-mortem studies which have demonstrated that suicidal depressed patients and suicidal, alcoholic depressives possess increased CB₁ receptor binding, expression and signaling as well as increased endocannabinoid levels in the PFC (Hungund et al., 2004; Vinod et al., 2005). Several reports have demonstrated that activation or blockade of the CB₁ receptor results in a decrease or increase in monoaminergic neurotransmission in the frontal cortex, respectively (Need et al., 2006; Sagredo et al., 2006; Tzavara et al., 2003). Furthermore, it has been suggested that reducing endocannabinoid transmission in the PFC could result in increased monoaminergic release, which is a mechanism common to conventional antidepressants (Witkin et al., 2005). Therefore, we hypothesize that repeated ECS treatment reduces endocannabinoid transmission in the PFC, resulting in disinhibition of monoamine release, which in turn contributes to the therapeutic actions of ECS.

While ECS treatment resulted in a significant reduction in prefrontal cortical endocannabinoid activity, a different response was seen in the amygdala. Specifically, a single ECS session resulted in an up-regulation of CB₁ receptor binding, which was not accompanied by significant changes in endocannabinoid content or CB₁ receptor mediated signal transduction. Following repeated ECS sessions, the increase in maximal binding of the CB₁ receptor seen after a single ECS session normalized, but a significant reduction in the EC50 of CB₁ receptor-mediated [³⁵S]GTPγS binding was found. These
findings suggest that successive ECS sessions result in a sensitization of CB₁ receptor-mediated signaling in the amygdala. Given the lack of concurrent change in CB₁ receptor binding following repeated ECS sessions, this increased sensitivity of the CB₁ receptor likely occurs at a downstream location. Accordingly, a comparable ECS regimen has been found to result in increased basal [³⁵S]GTP₇S binding in the amygdala, which is likely reflective of increased expression of specific G proteins (such as the G₀α) or enhancement of the ability of specific G proteins to interact with specific metabotropic receptors (Nishida et al., 1990), such as the CB₁ receptor. Despite the specific mechanism of this phenomenon, the current data indicate that repeated ECS sessions are associated with enhanced CB₁ receptor-mediated signal transduction in the amygdala.

Collectively, these data suggest that a shift in the equilibrium of cortical and subcortical endocannabinoid signaling is involved in the manifestation and/or maintenance of affective illness, and that normalization of this shift could be relevant for treatment of affective illness. This hypothesis is supported, to some degree, by the current data, as well as recent data demonstrating that chronic unpredictable stress increases cortical CB₁ mRNA expression and decreases subcortical CB₁ receptor binding (Bortolato et al., 2007; Hill et al., 2005) and the identification of increased CB₁ receptor activity in the PFC of depressed individuals who committed suicide (Hungund et al., 2004). Further, on a behavioral level, it has been shown that increased prefrontal cortical CB₁ receptor activity is associated with enhanced learning of emotionally aversive stimuli (Laviolette and Grace, 2006) and that reduced amygdalar endocannabinoid activity can result in impaired clearance of emotionally aversive memories (Marsicano et al., 2002), suggesting that a shift in the balance of cortical and subcortical
endocannabinoid signaling could foster the predominance of aversive memories, as seen in affective illness.

Additionally, this hypothesis also helps to reconcile the fact that both CB₁ receptor agonists and antagonists can elicit antidepressant effects (Gobbi et al., 2005; Grieben et al., 2005; Hill and Gorzalka 2005b; Shearman et al., 2003; Tzavara et al., 2003), as these agents may exhibit differential actions depending on whether cortical or subcortical CB₁ receptors are saturated. Examination of the current data supports this hypothesis as the binding affinity and signal transduction sensitivity of the CB₁ receptor is significantly higher in subcortical structures examined relative to the prefrontal cortex. As such, one would hypothesize that under conditions in which agonists to the CB₁ receptor are administered at low doses, CB₁ receptors in subcortical structures, such as the hippocampus or amygdala, would saturate prior to those in cortical structures, such as the prefrontal cortex. In line with this, the antidepressive effects of agents which increase CB₁ receptor activation occur following administration of low doses of these agents (Gobbi et al., 2005; Hill and Gorzalka, 2005b). Alternately, antidepressive responses to administration of CB₁ receptor antagonists occurs following administration of high doses of these drugs, which would saturate most of the available CB₁ receptor population and in turn is related to increases in monoamine neurotransmission in the frontal cortex as previously mentioned (Grieben et al., 2005; Need et al., 2006; Tzavara et al., 2003; Witkin et al., 2005). Future research employing discrete microinjections of cannabinoid CB₁ receptor agonists and antagonists is required to ultimately determine the feasibility of this hypothesis. These data add to the increasing body of evidence that the endocannabinoid system is involved in the pathophysiology and/or treatment of
depression (Hill and Gorzalka, 2005a; Mangieri and Piomelli, 2007; Vinod and Hungund, 2006; Witkin et al., 2005) and demonstrate the necessity to investigate the relative contributions of cortical and subcortical endocannabinoid signaling to the regulation of mood and stress reactivity to understand the exact role of this system in affective illness.
4.5 REFERENCES


CHAPTER 5
REGIONAL ALTERATIONS IN THE ENDOCANNABINOID SYSTEM IN AN ANIMAL MODEL OF DEPRESSION:
EFFECTS OF CONCURRENT ANTIDEPRESSANT TREATMENT

5.1 INTRODUCTION

Depressive illness is widespread and exhibits a lifetime prevalence rate of 16% (Kessler et al., 2003). Depressive illnesses are characterized by an array of disturbances in emotional behavior, memory, neurovegetative functions and hedonic processing. The neurobiological mechanisms subserving the development, manifestation and treatment of depression are complex, and there is ample evidence indicating that disturbances in monoaminergic signaling, glucocorticoid activity and neurotrophic/neuroplastic processes are involved (Duman and Monteggia, 2006; Holsboer, 2000; McEwen, 2005; Ressler and Nemeroff; 2000).

Our understanding of the biochemical processes in depression has been shaped by preclinical work employing animal models of depression. While an unequivocal animal model of depression is not available, several models have demonstrated a high degree of biological and behavioral concordance with major depression in humans (Nestler et al., 2002; O'Neil and Moore, 2003). In particular, the chronic unpredictable stress model (CUS; also referred to as chronic mild stress [CMS]) has been found to have reasonable

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face validity as a model of depression, largely because this model incorporates etiological factors that are similar to human depression (protracted exposure to mild, but unpredictable stressors) and elicits both biochemical (e.g. alterations in monoaminergic receptors and glucocorticoid signaling) and behavioral (e.g. anhedonia) responses that parallel those seen in human major depression (Lopez et al., 1998; Willner, 2005; however see Forbes et al., 1996; Reid et al., 1997 for a critique of the model). Accordingly, the CUS model represents a useful tool to examine potential systems involved in the pathophysiology of depression.

There is increasing evidence to support a role for the endocannabinoid system in the neurobiology of depression (Hill and Gorzalka, 2005a; Mangieri and Piomelli, 2007; Vinod and Hungund, 2006; Witkin et al., 2005). The endocannabinoid system is present in moderate to high levels in limbic brain regions, such as the prefrontal cortex, hippocampus and amygdala, where neuronal activity is known to be altered in depression (Bisogno et al., 1999; Egertova et al., 2003; Herkenham et al., 1991). Genetic deletion of the CB1 receptor in mice results in a phenotype that is reminiscent of the symptom profile of melancholic depression (reviewed in Hill and Gorzalka, 2005a), such as increased depressive-like and anxiety-like behaviors (Steiner et al., 2008; Uriguen et al., 2004), perseverence of emotionally aversive memories (Marsicano et al., 2002), enhanced activation and impaired inhibition of the hypothalamic-pituitary-adrenal (HPA) axis (Cota et al., 2007), diminished feeding behavior (Ravinet Trillou et al., 2004) and impaired responsiveness to rewarding stimuli (Sanchis-Segura et al., 2004). Consistent with the hypothesis that disruptions in endocannabinoid signaling may contribute to the development of depression, we have reported previously that CUS in rats induces a
robust reduction in both CB₁ receptor binding density and the tissue content of the endocannabinoid 2-AG in the hippocampus (Hill et al., 2005). In addition, pharmacological facilitation of endocannabinoid neurotransmission has been shown to produce an antidepressant response alone and to and enhance the effects of conventional antidepressants in several rodent models of antidepressant efficacy (Bortolato et al., 2007; Filip et al., 2006; Gobbi et al., 2005; Hill and Gorzalka, 2005b; Hill et al., 2007). Further, several modalities known to produce antidepressant effects in humans have been shown to increase endocannabinoid/CB₁ receptor signaling in the brain, including chronic treatment with the antidepressant desipramine (data in Chapter 2), voluntary exercise (data in Chapter 3), repeated electroconvulsive shock treatment (data in Chapter 4) and sleep deprivation (Chen and Bazan, 2005). Collectively, these studies support the hypothesis that hypofunctional endocannabinoid signaling contributes to the etiology or symptom spectrum of depressive illness and that increasing endocannabinoid signaling is associated with anti-depressant efficacy.

On the other hand, there is also evidence that hyperfunctional endocannabinoid signaling is associated with depression. There is a reliable association between long-term cannabis use and the development of suicidal ideations and depressive illness (Bovasso, 2001; Degenhardt et al., 2003; Lynskey et al., 2004), suggesting that overactive CB₁ receptor signaling promotes the development of depression. CB₁ receptor protein expression, binding site density and signal transduction are up-regulated in the prefrontal cortex of depressed individuals who have committed suicide (Hungund et al., 2004). Consistent with the clinical evidence, chronic mild stress exposure to rats (Bortolato et al., 2007) and CUS exposure to mice (C.J. Hillard and W.E. Cullinan, unpublished

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findings) increases CB$_1$ receptor mRNA in the prefrontal cortex. Moreover, 
electroconvulsive shock treatment down-regulates CB$_1$ receptor binding and tissue 
content of AEA within the prefrontal cortex (Chapter 4) and chronic treatment with the 
antidepressant fluoxetine decreases CB$_1$ receptor mRNA within the caudate putamen 
(Oliva et al., 2005). Both acute and chronic treatment with a CB$_1$ receptor antagonist has 
been found to elicit antidepressant responses in the forced swim test and chronic mild 
stress paradigm (Griebel et al., 2005; Shearman et al., 2003; Tzavara et al., 2003).

In an attempt to reconcile these apparently discrepant findings, we have 
previously suggested that the endocannabinoid system could differentially contribute to 
the symptomatology of depression in a region-specific manner (see Chapter 4). 
Specifically, the majority of evidence indicating that an increase in endocannabinoid 
activity is associated with depression has been localized to the prefrontal cortex, whereas 
the evidence that reductions in endocannabinoid signaling contribute to depression is 
largely based on findings in subcortical structures, such as the hippocampus, 
hypothalamus or amygdala. Thus, the aim of the current study is to attempt to examine 
this discrepancy from a neuroanatomical perspective by employing the CUS model of 
depression and performing a regional analysis of the endocannabinoid system and to 
explore the sensitivity of these changes to the antidepressant, imipramine. Given the 
equivocal nature of the CUS model for evoking "depression"-like symptoms (refer to 
Forbes et al., 1996; Reid et al., 1997; Willner, 2005) we have included herein a study of 
the effects of CUS on sexual motivation, a parameter which represents a measure of 
natural reward and motivational drive and is often impaired in depression (Williams and 
Reynolds, 2006). With this in mind, the current data confirm the hypothesis that
endocannabinoid signaling is regionally modulated in an animal model of depression, and that these changes are, to some degree, reversible following concurrent antidepressant treatment.

5.2 METHODS

5.2.1 Subjects

Male, Long-Evans rats (300 g; 70 days of age) housed in groups of three in triple mesh wire caging maintained at 21 °C, and on a 12 h light/dark cycle, with lights on at 0900 h. All rats were given ad libitum access to Purina Rat Chow and tap water, except during deprivation periods of the stressing protocol (outlined below). In addition to the experimental animals, female, Long-Evans rats were used as stimuli for sexual behavior testing. All females were bilaterally ovariectomized at 3 months of age using standard surgical procedures while anesthetized with 75 mg/kg ketamine hydrochloride and 7 mg/kg xylazine (intraperitoneal). All animal protocols were approved by the Canadian Council for Animal Care and the standards of the Animal Ethics Committee of the University of British Columbia.

5.2.2 Chronic Unpredictable Stress Protocol

For behavioral testing, one cohort of animals were either subjected to 21 days of CUS or acted as cage controls and were handled weekly. At the conclusion of this period, all animals were tested for copulatory behavior.

For biochemical assays, a different cohort of animals was employed with four treatment conditions generated to incorporate concurrent antidepressant treatment: 1) vehicle / no stress; 2) imipramine / no stress; 3) vehicle / CUS; 4) imipramine / CUS. Imipramine was dissolved in 0.9 % saline and was administered via intraperitoneal
injection at a dose of 10 mg/kg, and at a volume of 1 ml/kg. Injections were given daily between 1200h-1400h using 26 gauge 1/2" needles. The CUS paradigm employed has been repeatedly utilized by our laboratory for both behavioral and biochemical analysis (Brotto et al., 2001; Hill and Gorzalka, 2004; Hill et al., 2005), and is adapted from the original CMS paradigm (Willner et al., 1987). The CUS paradigm consisted of 2-3 stressors a day from the following list: 30 min tube restraint; 30 min exposure to social crowding with white noise/stroboscopic illumination; 5 min forced swim; 18 h food and/or water deprivation; 3 h cage rotation; and 18 h social isolation in damp bedding. All stressors were separated by a period of at least 2 h and were applied daily over the 21 day period. For the biochemical assays, all animals were rapidly decapitated in the morning after the 21st day of stress exposure following 18 h of overnight social isolation. Brains were removed and the prefrontal cortex (consisting of medial prefrontal cortex and anterior cingulate cortex), hippocampus, amygdala (consisting of central, basolateral and medial nuclei), hypothalamus, ventral striatum (consisting of the nucleus accumbens) and midbrain (including the cell bodies of monoaminergic nuclei in the raphe, ventral tegmental area and locus coeruleus) were sectioned out. Brains were frozen in liquid nitrogen within 5-7 min of decapitation and stored at -80 °C until analysis. Two independent cohorts of tissue were collected. One cohort of tissue (n = 7-8) was used for lipid extraction to determine endocannabinoid ligand content. The other cohort of tissue (n=4-5) was used as the source of membranes for CB1 receptor binding and FAAH activity assays. Trunk blood was collected upon decapitation for measurement of serum endocannabinoid content.
5.2.3 Sexual Behavior Testing

Females were induced into estrus using 10 µg estradiol benzoate 48 h prior to testing and 500 µg progesterone 4 h prior to testing. Both hormones were received from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in peanut oil and injected subcutaneously at a volume of 0.1 ml.

Prior to testing, males were exposed to an estrus-induced female on five occasions and then screened for sexual activity in two independent sessions. To achieve the criterion for sexual proficiency, male subjects had to ejaculate at least once during both of two 30 min screening sessions with a receptive female. Males that achieved this criterion were randomly assigned to be exposed to CUS or functioned as cage controls. All behavioral testing occurred during the middle third of the light cycle and was performed by trained observers who were blinded to the treatment groups. Testing occurred in cubical Plexiglas (30×30×30 cm) and cylindrical glass (30 cm diameter×45 cm height) chambers lined with contact bedding. Males were habituated to the chambers for 5 min prior to the beginning of testing. Test sessions began with the presentation of an estrus-induced female to a male in an individual testing chamber. The sexual behavior parameters scored were: frequency of mounts with pelvic thrusting prior to ejaculation, frequency of penile intromissions prior to ejaculation, frequency of ejaculations, latency to initiate mounting behavior, latency to initiate intromitting behavior, ejaculation latency (i.e., the period between the first intromission and the first ejaculation) and the postejaculatory interval (i.e., the period between ejaculation and the first intromission of the next copulatory bout). Stimulus females were rotated between males every 10 min to maintain sexual interest.
5.2.4 Biochemical Analysis

As stated, one cohort of tissue was converted to membrane fraction as described in Chapter 3. These membranes were subsequently used for CB₁ receptor binding (see Chapter 2) and FAAH activity assays (Chapter 3). A second cohort of tissue was generated for the extraction and analysis of endocannabinoid ligand content (see Chapter 2).

5.2.5 Statistics

Comparison of the effects of CUS on parameters of sexual behavior was performed using independent t-tests. Comparison of the effects of CUS exposure and/or imipramine administration on parameters of CB₁ receptor binding, FAAH activity and endocannabinoid ligand content in different brain regions were analyzed using a univariate analysis of variance (ANOVA), with stress exposure and drug treatment acting as fixed factors. When applicable, post-hoc analysis of each of these variables in each region was performed using a Tukey’s test. Significance was established against an alpha level of 0.05.

5.3 RESULTS

5.3.1 The Effects of CUS on Sexual Behavior

Exposure of male rats to CUS resulted in a significant increase in the latency of rats to initiate both mounting \[ t(12) = 3.41, p < 0.01; \text{Table 5.1}\] and intromitting \[ t(12) = 3.67, p < 0.01; \text{Table 5.1}\] behaviors with a receptive female rat. There was a near significant trend in the ability of CUS to increase the latency required for male rats to achieve ejaculation during copulation \[ t(12) = 1.87, p = 0.08; \text{Table 5.1}\] as well as to reduce the total number of ejaculations \[ t(12) = 1.70, p = 0.1; \text{Table 5.1}\]. There was no
effect of CUS exposure on the frequency of mounts [t (12) = 1.21, p > 0.05; Table 5.1];
intromissions [t (12) = 0.34, p > 0.05; Table 5.1]; or the latency required to re-initiate
sexual activity following the first ejaculation [t (12) = 0.01, p > 0.05; Table 5.1]. Thus,
exposure of male rats to CUS resulted in a significant diminution of motivation for sexual
activity, but did not significantly impair sexual performance once it was initiated.

Table 5.1: The effects of exposure to chronic, unpredictable stress (CUS) on
parameters of male sexual behavior.
Exposure of male rats to 21 days of CUS resulted in a significant impairment in sexual
motivation as demonstrated by the significant increase in the latency to engage in sexual
activity with a receptive female rat. The specificity of these effects are demonstrated by
the fact that sexual performance itself was not otherwise compromised by CUS exposure.
Significant differences from control animals (p < 0.05) denoted by *. Both treatment
conditions are n = 7. Data are presented as mean values +/- SEM.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CUS</th>
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</thead>
<tbody>
<tr>
<td>Mounts:</td>
<td>5.86 +/- 1.18</td>
<td>8.29 +/- 1.63</td>
</tr>
<tr>
<td>Intromissions:</td>
<td>11.57 +/- 0.95</td>
<td>12.29 +/- 1.26</td>
</tr>
<tr>
<td>Ejaculations:</td>
<td>2.00 +/- 0.31</td>
<td>1.29 +/- 0.29</td>
</tr>
<tr>
<td>Mount Latency (s):</td>
<td>19.43 +/- 6.26</td>
<td>68.57 +/- 12.97*</td>
</tr>
<tr>
<td>Intromission Latency (s):</td>
<td>26.57 +/- 7.39</td>
<td>80.57 +/- 12.72*</td>
</tr>
<tr>
<td>Ejaculation Latency (s):</td>
<td>303.43 +/- 18.52</td>
<td>358.00 +/- 22.51</td>
</tr>
<tr>
<td>Post Ejaculatory Interval (s):</td>
<td>311.71 +/- 12.29</td>
<td>311.43 +/- 16.27</td>
</tr>
</tbody>
</table>

5.3.2 Effects of CUS and/or Imipramine Treatment on CB₁ Receptor Binding

Parameters

In the prefrontal cortex (PFC), the agonist binding site density (B_max) of the CB₁
receptor was significantly increased in rats exposed to CUS compared to non-stressed
controls [F (1, 16) = 5.09, p < 0.04; Fig. 5.1a]; there was no effect of imipramine
treatment [F (1, 16) = 2.77, p > 0.05]; and there was no significant interaction between
CUS and imipramine treatment [F (1, 16) = 2.62, p > 0.05] on Bmax in the PFC. The
equilibrium dissociation constant (K_d) for [3H]CP55940 binding to the CB₁ receptor was
not affected by CUS exposure [F (1, 16) = 2.31, p > 0.05; Table 5.2] but was significantly reduced by imipramine compared to saline treated rats [F (1, 16) = 5.09, p < 0.04]. There was no significant interaction between CUS and imipramine treatment. [F (1, 16) = 0.09, p > 0.05].

Figure 5.1: The effects of exposure to 21 days of chronic unpredictable stress (CUS), imipramine (10 mg/kg; IMI) administration, or both regimens combined, on the maximal binding (B\text{max}) of [3H]CP55940 to the cannabinoid CB1 receptor in the a) prefrontal cortex; b) hippocampus; c) hypothalamus; d) ventral striatum; e) amygdala; f) midbrain. Values are denoted as means ± SEM. Significant differences from non-stressed (CON), vehicle (VEH) treated animals (p < 0.05) denoted by *. For all treatment conditions, n = 4-5.
In the hippocampus, CB1 receptor agonist binding site density was significantly decreased by CUS [F (1, 15) = 11.51, p < 0.005; Fig. 5.1b] and was unaffected by imipramine [F (1, 15) = 0.36, p > 0.05]. There was no significant interaction between the effects of CUS and imipramine treatment on the Bmax of the CB1 receptor [F (1, 15) = 0.40, p > 0.05]. There were no significant effects of CUS [F (1, 15) = 1.14, p > 0.05; Table 5.2] or imipramine [F (1, 15) = 0.46, p > 0.05], or a significant interaction between these two factors [F (1, 15) = 1.00, p > 0.05], on the Kd of [3H]CP55490 for binding to the CB1 receptor in the hippocampus.

Table 5.2: The effects of exposure to chronic, unpredictable stress (CUS) and/or concurrent treatment with the antidepressant imipramine (IMI; 10 mg/kg) on the dissociation constant (Kd) of [3H]-CP55940 from the cannabinoid CB1 receptor. Exposure of male rats to CUS for 21 days resulted in a significant reduction in the binding affinity (Kd) of the cannabinoid CB1 receptor in the hypothalamus, and this effect was prevented by concurrent IMI treatment. IMI treatment for 21 days reduced the Kd of the CB1 receptor in the prefrontal cortex and the ventral striatum. Significant differences from non-stressed (CON), vehicle (VEH) treated animals (p < 0.05) denoted by *. For all treatment conditions, n = 4-5. Data are presented as mean nM of [3H]-CP55940 +/- SEM.

<table>
<thead>
<tr>
<th></th>
<th>CON/VEH</th>
<th>CUS/VEH</th>
<th>CON/IMI</th>
<th>CUS/IMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prefrontal Cortex</td>
<td>0.61 +/- 0.14</td>
<td>0.83 +/- 0.10</td>
<td>0.38 +/- 0.15*</td>
<td>0.52 +/- 0.08</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.60 +/- 0.11</td>
<td>0.59 +/- 0.11</td>
<td>0.79 +/- 0.15</td>
<td>0.55 +/- 0.06</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>1.54 +/- 0.15</td>
<td>0.57 +/- 0.07*</td>
<td>1.02 +/- 0.06</td>
<td>1.22 +/- 0.45</td>
</tr>
<tr>
<td>Ventral Striatum</td>
<td>0.65 +/- 0.08</td>
<td>0.55 +/- 0.12</td>
<td>0.34 +/- 0.05*</td>
<td>0.37 +/- 0.02*</td>
</tr>
<tr>
<td>Amygdala</td>
<td>0.56 +/- 0.06</td>
<td>0.56 +/- 0.03</td>
<td>0.95 +/- 0.19</td>
<td>0.49 +/- 0.12</td>
</tr>
<tr>
<td>Midbrain</td>
<td>0.40 +/- 0.08</td>
<td>0.25 +/- 0.05</td>
<td>0.25 +/- 0.04</td>
<td>0.27 +/- 0.04</td>
</tr>
</tbody>
</table>

In the hypothalamus, analysis of variance revealed that there was a significant interaction between CUS and imipramine treatment on CB1 receptor binding site density
Post hoc analysis of these data indicate that the 
$B_{\text{max}}$ for $[^{3}\text{H}]$CP55940 binding to the CB$_1$ receptor binding site density was decreased both by CUS ($p < 0.01$) and imipramine treatment ($p < 0.05$); however, when CUS and imipramine were administered together, there was no significant difference in this group relative to control animals ($p > 0.05$). A similar pattern was seen in the effects of CUS and imipramine on the $K_d$ of $[^{3}\text{H}]$CP55940 for the CB$_1$ receptor in that there was a significant interaction between CUS and imipramine treatment [$F(1, 15) = 5.43, p < 0.05$; Table 5.2]. Post hoc analyses reveal that CUS produced a significant decrease in the $K_d$ of $[^{3}\text{H}]$CP55940 the CB$_1$ receptor ($p < 0.05$) while there was no effect of imipramine treatment alone ($p > 0.05$). When imipramine and CUS were administered concurrently, there was no difference in the $K_d$ of $[^{3}\text{H}]$CP55940 for binding to the CB$_1$ receptor relative to control animals ($p > 0.05$).

In the ventral striatum, there was a significant interaction between CUS and imipramine treatment on the $B_{\text{max}}$ of the CB$_1$ receptor [$F(1, 15) = 11.03, p < 0.01$; Fig. 5.1d]. Post hoc analyses revealed that both CUS ($p < 0.05$) and imipramine treatment ($p < 0.01$) induced significant reductions in $B_{\text{max}}$ that were not seen when both treatments were administered concurrently ($p > 0.05$). The $K_d$ for $[^{3}\text{H}]$CP55940 binding to the CB$_1$ receptor was not affected by CUS [$F(1, 15) = 0.20, p > 0.05$; Table 5.2] but was significantly reduced by imipramine [$F(1, 15) = 12.17, p < 0.01$]. There was no interaction between CUS and imipramine treatment [$F(1, 15) = 0.87, p > 0.05$].

In the amygdala, there was a significant interaction between CUS and imipramine treatments on the $B_{\text{max}}$ of the CB$_1$ receptor [$F(1, 14) = 5.10, p < 0.05$; Fig. 5.1e]; however, post hoc analysis demonstrated that this effect was due to an increase in $B_{\text{max}}$
following imipramine treatment (p < 0.05) that did not occur in rats exposed to CUS and imipramine treatment combined (p > 0.05). CUS alone did not affect the Bmax for the CB1 receptor (p > 0.05). The Kd of [3H]CP55940 binding in the amygdala was not affected by CUS [F (1, 14) = 3.76, p > 0.05; Table 5.2]; imipramine [F (1, 14) = 1.18, p > 0.05]; nor was there an interaction between the treatments [F (1, 14) = 3.76, p > 0.05].

Within the midbrain, there was no effect of CUS exposure on CB1 receptor binding site density [F (1, 16) = 2.04, p > 0.05], while imipramine produced a significant reduction in B_max [F (1, 16) = 4.54, p < 0.05]. There was no interaction between CUS exposure and imipramine treatment on the B_max of the CB1 receptor [F (1, 16) = 1.43, p > 0.05; Fig. 5.1f]. Neither CUS [F (1, 16) = 1.49, p > 0.05; Table 5.2] nor imipramine [F (1, 16) = 1.92, p > 0.05], affected the Kd of [3H]CP55940 for the CB1 receptor in the midbrain, nor was there a significant interaction between these two treatment regimens on this parameter [F (1, 16) = 2.75, p > 0.05].

5.3.3 Effects of CUS and/or Imipramine Treatment on Regional, Tissue Endocannabinoid Contents

CUS produced a significant reduction in AEA content in all brain regions examined (Fig. 5.2). Imipramine treatment alone had no effect on AEA content in any brain region and there were no significant interactions between CUS and imipramine. Therefore, CUS exerts a global and imipramine-insensitive reduction in AEA throughout the limbic system. ANOVA results for the effects of CUS, imipramine treatment, or both on the tissue content of AEA are as follows: Prefrontal cortex [CUS x IMI: F (1, 27) = 0.20, p > 0.05; CUS: F (1, 27) = 25.14, p < 0.001; IMI: F (1, 27) = 0.56, p > 0.05]; hippocampus [CUS x IMI: F (1, 26) = 0.08, p > 0.05; CUS: F (1, 26) = 20.86, p < 0.001;
IMI: F (1, 26) = 0.13, p > 0.05; hypothalamus [CUS x IMI: F (1, 25) = 0.89, p > 0.05; CUS: F (1, 25) = 8.41, p < 0.01; IMI: F (1, 25) = 0.62, p > 0.05]; ventral striatum [CUS x IMI: F (1, 27) = 2.06, p > 0.05; CUS: F (1, 27) = 25.28, p < 0.001; F (1, 27) = 0.02, p > 0.05]; amygdale [CUS x IMI: F (1, 25) = 0.24, p > 0.05; CUS: F (1, 25) = 12.14, p < 0.005; IMI: F (1, 25) = 0.12, p > 0.05]; and midbrain [CUS x IMI: F (1, 27) = 0.24, p > 0.05; CUS: F (1, 27) = 16.18, p < 0.001; IMI: F (1, 27) = 2.02, p > 0.05].

**Figure 5.2:** The effects of exposure to 21 days of chronic unpredictable stress (CUS), imipramine (10 mg/kg; IMI) administration, or both regimens combined, on the tissue content of the endocannabinoid ligand anandamide (AEA) in the a) prefrontal cortex; b) hippocampus; c) hypothalamus; d) ventral striatum; e) amygdale; f) midbrain. Values are denoted as means ± SEM. Significant differences between groups (p < 0.05) denoted by *. For all treatment conditions are, n = 7-8.
In contrast, tissue 2-AG contents were not affected in a global manner but were selectively increased by CUS in the hypothalamus and the midbrain. Similar to the changes in AEA content, these regional increases were unaffected by imipramine treatment (see Table 5.3 for both data and ANOVA results).

**Table 5.3: The effects of exposure to chronic, unpredictable stress (CUS) and/or concurrent treatment with the antidepressant imipramine (IMI; 10 mg/kg) on the tissue content (nmol/g tissue) of the endocannabinoid 2-arachidonylglycerol (2-AG).**

Exposure of male rats to 21 days of CUS resulted in a significant increase in 2-AG content within the hypothalamus and the midbrain, neither of which was reversed by concurrent IMI treatment. Significant differences from non-stressed (CON), vehicle (VEH) treated animals (p < 0.05) denoted by *. For all treatment conditions, n = 7-8.

Data are presented as mean nmol/g tissue +/- SEM.

<table>
<thead>
<tr>
<th>Region</th>
<th>CON/VEH</th>
<th>CUS/VEH</th>
<th>CON/IMI</th>
<th>CUS/IMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prefrontal Cortex</td>
<td>4.35 +/- 0.29</td>
<td>5.16 +/- 0.14</td>
<td>5.60 +/- 0.45</td>
<td>5.63 +/- 0.52</td>
</tr>
<tr>
<td>CUS x IMI: F (1, 27) = 0.82, p &gt; 0.05; CUS: F (1, 27) = 0.93, p &gt; 0.05; IMI: F (1, 27) = 3.93, p &gt; 0.05</td>
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<tr>
<td>Hippocampus</td>
<td>6.37 +/- 0.64</td>
<td>6.11 +/- 0.45</td>
<td>6.82 +/- 0.69</td>
<td>7.04 +/- 0.59</td>
</tr>
<tr>
<td>CUS x IMI: F (1, 26) = 0.08, p &gt; 0.05; CUS: F (1, 26) = 0.00, p &gt; 0.05; IMI: F (1, 26) = 1.28, p &gt; 0.05</td>
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<tr>
<td>Hypothalamus</td>
<td>8.25 +/- 0.96</td>
<td>12.92 +/- 0.60*</td>
<td>8.38 +/- 0.76</td>
<td>13.56 +/- 0.98*</td>
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<tr>
<td>CUS x IMI: F (1, 25) = 0.10, p &gt; 0.05; CUS: F (1, 25) = 35.76, p &lt; 0.001; IMI: F (1, 25) = 0.22, p &gt; 0.05</td>
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<tr>
<td>Ventral Striatum</td>
<td>5.81 +/- 0.77</td>
<td>6.26 +/- 0.45</td>
<td>4.71 +/- 0.27</td>
<td>5.68 +/- 0.26</td>
</tr>
<tr>
<td>CUS x IMI: F (1, 27) = 0.32, p &gt; 0.05; CUS: F (1, 27) = 2.41, p &gt; 0.05; IMI: F (1, 27) = 3.33, p &gt; 0.05</td>
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<tr>
<td>Amygdala</td>
<td>9.57 +/- 0.68</td>
<td>8.84 +/- 1.04</td>
<td>7.91 +/- 0.94</td>
<td>10.60 +/- 1.08</td>
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<tr>
<td>CUS x IMI: F (1, 23) = 3.00, p &gt; 0.05; CUS: F (1, 23) = 0.98, p &gt; 0.05; IMI: F (1, 23) = 0.00, p &gt; 0.05</td>
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<tr>
<td>Midbrain</td>
<td>4.00 +/- 0.31</td>
<td>5.71 +/- 0.44*</td>
<td>5.02 +/- 0.40</td>
<td>5.73 +/- 0.19*</td>
</tr>
<tr>
<td>CUS x IMI: F (1, 27) = 2.03, p &gt; 0.05; CUS: F (1, 27) = 11.89, p &lt; 0.005; IMI: F (1, 27) = 2.20, p &gt; 0.05</td>
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**5.3.4 Effects of CUS and/or Imipramine Treatment on FAAH Activity**

The Vmax (see Fig. 5.3) values for the hydrolysis of AEA by membranes were determined in a region-specific manner. There were no effects of either CUS or imipramine alone on the maximal hydrolytic activity of FAAH in any brain region.
examined; however, within the ventral striatum and midbrain there was a significant interaction between CUS and imipramine treatment such that the combination of these treatments increased the $V_{\text{max}}$ of FAAH in these structures. ANOVA results for the effects of CUS, imipramine treatment, or both on the maximal hydrolytic activity ($V_{\text{max}}$) of FAAH are as follows: prefrontal cortex [CUS x IMI: $F(1, 15) = 0.48, p > 0.05$; CUS: $F(1, 15) = 0.66, p > 0.05$; IMI: $F(1, 15) = 0.00, p > 0.05$]; hippocampus [CUS x IMI: $F(1, 16) = 0.52, p > 0.05$; CUS: $F(1, 16) = 0.41, p > 0.05$; IMI: $F(1, 16) = 0.08, p > 0.05$]; hypothalamus [CUS x IMI: $F(1, 14) = 2.13, p > 0.05$; CUS: $F(1, 14) = 0.59, p > 0.05$; IMI: $F(1, 14) = 0.09, p > 0.05$]; amygdala [CUS x IMI: $F(1, 12) = 0.22, p > 0.05$; CUS: $F(1, 12) = 1.00, p > 0.05$; IMI: $F(1, 12) = 0.06, p > 0.05$]; ventral striatum [CUS x IMI: $F(1, 14) = 9.57, p < 0.01$; post hoc analysis revealed that CUS/IMI is significantly different ($p < 0.05$) relative to no stress/vehicle, CUS/vehicle and no stress/IMI]; midbrain [CUS x IMI: $F(1, 16) = 4.76, p < 0.05$; post hoc analysis revealed that CUS/IMI is significantly different ($p < 0.05$) relative to no stress/vehicle, CUS/vehicle and no stress/IMI). The binding affinity of AEA for FAAH ($K_m$) was also determined in a region specific manner. The $K_m$ of FAAH was not effected by CUS alone in any brain region. Similarly, the $K_m$ of FAAH was not effected by imipramine alone, except in the midbrain. Also, the combination of CUS and imipramine increased the $K_m$ of FAAH in the ventral striatum exclusively (data and ANOVA results in Table 5.4).
Figure 5.3: The effects of exposure to 21 days of chronic unpredictable stress (CUS), imipramine (10 mg/kg; IMI) administration, or both regimens combined, on the maximal hydrolysis of anandamide by fatty acid amide hydrolase ($V_{\text{max}}$) in the a) prefrontal cortex; b) hippocampus; c) hypothalamus; d) ventral striatum; e) amygdala; f) midbrain. Values are denoted as means ± SEM. Significant differences from non-stressed (CON), vehicle (VEH) treated animals ($p < 0.05$) denoted by *.

For all treatment conditions, n = 4-5.
Table 5.4: The effects of exposure to chronic, unpredictable stress (CUS) and/or concurrent treatment with the antidepressant imipramine (IMI; 10 mg/kg) on the binding affinity (Kₘ) of fatty acid amide hydrolase for anandamide.

Exposure of male rats to 21 days of CUS did not modify the binding affinity of fatty acid amide hydrolase (FAAH) for anandamide. Administration of IMI alone increased the Kₘ of FAAH, exclusively in the midbrain. However, the joint administration of CUS and IMI resulted in an increase in the Kₘ of FAAH in the ventral striatum and the midbrain.

Significant differences from non-stressed (CON), vehicle (VEH) treated animals (p < 0.05) denoted by *. For all treatment conditions, n = 4-5. Data are presented as mean nM of 3H-arachidonylethanolamide +/- SEM.

<table>
<thead>
<tr>
<th></th>
<th>CON/VEH</th>
<th>CUS/VEH</th>
<th>CON/IMI</th>
<th>CUS/IMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prefrontal Cortex</td>
<td>0.61 +/- 0.08</td>
<td>0.65 +/- 0.09</td>
<td>0.65 +/- 0.10</td>
<td>0.46 +/- 0.05</td>
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<td></td>
<td>CUS x IMI: F (1, 15) = 1.92, p &gt; 0.05; CUS: F (1, 15) = 0.68, p &gt; 0.05; IMI: F (1, 15) = 0.80, p &gt; 0.05</td>
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<tr>
<td>Hippocampus</td>
<td>0.64 +/- 0.12</td>
<td>0.69 +/- 0.08</td>
<td>0.80 +/- 0.28</td>
<td>0.71 +/- 0.10</td>
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<td>CUS x IMI: F (1, 16) = 0.17, p &gt; 0.05; CUS: F (1, 16) = 0.02, p &gt; 0.05; IMI: F (1, 16) = 0.28, p &gt; 0.05</td>
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<tr>
<td>Hypothalamus</td>
<td>0.62 +/- 0.10</td>
<td>0.42 +/- 0.05</td>
<td>0.44 +/- 0.03</td>
<td>0.55 +/- 0.12</td>
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<td>CUS x IMI: F (1, 14) = 2.81, p &gt; 0.05; CUS: F (1, 14) = 0.20, p &gt; 0.05; IMI: F (1, 14) = 0.07, p &gt; 0.05</td>
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<tr>
<td>Ventral Striatum</td>
<td>0.37 +/- 0.05</td>
<td>0.37 +/- 0.06</td>
<td>0.37 +/- 0.04</td>
<td>0.69 +/- 0.08*</td>
</tr>
<tr>
<td></td>
<td>CUS x IMI: F (1, 14) = 7.32, p &lt; 0.02; CUS: F (1, 14) = 2.11, p &gt; 0.05; IMI: F (1, 14) = 1.90</td>
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<tr>
<td>Amygdala</td>
<td>0.81 +/- 0.14</td>
<td>0.83 +/- 0.17</td>
<td>0.95 +/- 0.14</td>
<td>0.53 +/- 0.10</td>
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<td>CUS x IMI: F (1, 12) = 2.42, p &gt; 0.05; CUS: F (1, 12) = 2.11, p &gt; 0.05; IMI: F (1, 12) = 0.24, p &gt; 0.05</td>
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<tr>
<td>Midbrain</td>
<td>0.30 +/- 0.03</td>
<td>0.45 +/- 0.05</td>
<td>0.60 +/- 0.07*</td>
<td>0.61 +/- 0.11*</td>
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<td></td>
<td>CUS x IMI: F (1, 16) = 1.11, p &gt; 0.05; CUS: F (1, 16) = 1.36, p &gt; 0.05; IMI: F (1, 16) = 10.35, p &lt; 0.01</td>
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5.4 DISCUSSION

There has been considerable debate over the validity of the CUS/CMS model to induce behavioral responses akin to the anhedonia seen in human depression (Forbes et al., 1996; Reid et al., 1997). In animal models, anhedonia is typically defined and measured as a deficit in motivational drive for a normally rewarding behavior (Willner et al., 1992). One of the prominent symptoms of depression is a lack of motivation for rewarding activities, which is frequently manifested as a loss of sex drive (Williams and Reynolds, 2006). In the present study, we found that exposure of male rats to three
weeks of CUS resulted in a significant impairment in motivation for sexual activity without affecting performance after sexual activity was initiated. These data are consistent with the hypothesis that CUS results in a deficit in motivation for a natural reward and is a useful model for the studies of the neurochemical changes that accompany anhedonia.

CUS produced changes in both CB₁ receptor agonist binding site density and in the tissue content of the endocannabinoid ligands. Exposure to CUS produced a reduction in the tissue content of AEA in all of the brain regions examined (PFC, hippocampus, hypothalamus, ventral striatum, amygdala and midbrain). The reduction in AEA content was not due to accelerated metabolism, as membranes from CUS exposed rats did not exhibit changes in the hydrolysis of AEA. The widespread nature of this reduction is surprising in light of the generally accepted view that endocannabinoid synthesis/release is driven by local changes in neuronal activity. These data support an alternative hypothesis that AEA content in the brain is regulated more broadly and suggest that one of the functions of AEA is to provide a common set point for CB₁ receptor activation throughout the brain. Interestingly, this effect of CUS was unaltered by concurrent treatment with the tricyclic antidepressant, imipramine. These data argue against the hypothesis that the global change in AEA is secondary to the effects of CUS on monoaminergic signaling. This inability of antidepressant treatment to reverse the effects of CUS on AEA content could represent a mechanism limiting the efficacy of these drugs, as it has previously been shown that augmentation of AEA/CB₁ receptor signaling during CMS is sufficient to reverse the “depressive”-like effects of this paradigm (Bortolato et al. 2007). These data support the argument that inhibition of
FAAH could represent a valid pharmacological target for the development of novel monotherapies or adjunctive treatments for depression (Bortolato et al., 2007; Hill et al., 2007; Mangieri and Piomelli, 2007).

Rats exposed to CUS exhibited a significant increase in CB₁ receptor binding site density in the PFC, a finding that is consistent with other reports that CUS/CMS results in a significant increase in CB₁ receptor mRNA in the PFC (Bortolato et al., 2007; C.J. Hillard and W.E. Cullinan, unpublished findings). Therefore, it is likely that the increase in PFC CB₁ receptor agonist binding site density reflects an up-regulation of CB₁ receptor expression within specific neuronal populations in the PFC. However, the identity of the neurons which exhibit increased CB₁ receptor expression has yet to be determined and will greatly influence the interpretation of the current data.

The functional relevance of changes in prefrontal cortical CB₁ receptors following CUS has yet to be determined but is consistent with clinical studies demonstrating that CB₁ receptor protein expression, binding site density and signal transduction are also increased in the PFC of depressed humans who died by suicide (Hungund et al., 2004; Pazos et al., 2006; Vinod et al., 2005). Recent work has demonstrated that increased endocannabinoid/CB₁ receptor signaling in the PFC can increase voluntary alcohol intake (Hansson et al., 2007), suggesting that increased prefrontal cortical CB₁ receptor binding in depression could be related to the high prevalence of alcoholism associated with this disease, as has previously been suggested (Vinod et al., 2005). Moreover, increases in CB₁ receptor signaling within the PFC enhances the association of aversive cues (Laviolette and Grace, 2005), which suggests that increased CB₁ receptor binding in the PFC in depression could contribute to the enhancement in aversive emotional memory.
that often characterizes this disease. On the other hand, CB₁ receptors in the
ventromedial prefrontal cortex mediate the antidepressant actions of CB₁ receptor
agonists through trans-synaptic activation of dorsal raphe serotonergic neurons (Bambico
et al., 2007), suggesting that increased CB₁ receptor density in the PFC could be a
compensatory response that dampens the effects of CUS and/or depression.

In contrast to the PFC, CUS exposure resulted in a significant reduction in CB₁
receptor agonist binding site density in the hippocampus. This finding is in agreement
with other reports that CUS reduces hippocampal CB₁ receptor mRNA and protein
expression, binding site density and activation of GDP/GTP exchange (Hill et al., 2005;
Perez-Rial et al., 2004; Reich et al., 2007; C.J. Hillard and W.E. Cullinan, unpublished
findings). It is likely that this effect of CUS is mediated by increased glucocorticoid
secretion since CUS elicits glucocorticoid hypersecretion (Herman et al., 1995; Hill and
Gorzalka, 2004; Hill et al., 2005) and 21 days of corticosterone administration, in the
absence of stress, elicits a comparable reduction in CB₁ receptor binding and protein
expression in the hippocampus (Hill et al., 2008). It is surprising that this effect was not
modulated by antidepressant treatment as we have recently found that chronic treatment
with a similar tricyclic antidepressant, desipramine, results in an up-regulation of CB₁
receptor binding in the hippocampus (Chapter 2), and that local infusions of a CB₁
receptor agonist into the dorsal hippocampus elicit an antidepressant-like response in the
forced swim test (McLaughlin et al., 2007). While this result does not support a role for
hippocampal CB₁ receptor density effects in the mechanism of action of imipramine, it
does not rule out a role for these changes in the spectrum of CUS-induced behavioral and
neurochemical effects.
In an earlier study, we found that CUS reduced 2-AG content in the hippocampus of male rats (Hill et al., 2005); however, we did not observe the same effect in this study. Moreover, in our previous study CUS did not affect AEA content in the hippocampus (Hill et al., 2005), while in the current study, CUS induced a significant reduction in AEA. The most plausible explanation of this discrepancy is a diurnal effect; specifically, both AEA and 2-AG exhibit a diurnal rhythm in that AEA levels are higher in the dark phase and 2-AG levels are higher in the light phase (Valenti et al., 2004). In our previous study, animals were sacrificed at the end of the light phase, while in the current study, animals were sacrificed at the end of the dark cycle. Thus, perhaps CUS causes a reduction in both AEA and 2-AG content in the hippocampus, but this reduction only occurs during the peak of the daily rhythm, and not during the trough. This would result in a net reduction of endocannabinoid/CB1 receptor signaling during both phases of the daily rhythm.

Similar to what was seen in the hippocampus, CUS reduced the CB1 receptor agonist binding site density in the hypothalamus. Several studies have established a critical role of the hypothalamic endocannabinoid system in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis; in particular, endocannabinoid/CB1 receptor signaling inhibits activation of the HPA axis while loss of CB1 receptors and CB1 receptor antagonists enhance HPA axis reactivity (Cota et al., 2003, 2007; Patel et al., 2004, 2005). Disturbances in the HPA axis, such as overactivity and/or resistance to feedback inhibition, are common in melancholic depression (Wong et al., 2000) and are a consequence of CUS (Herman et al., 1995; Hill and Gorzalka, 2004; Hill et al., 2005). Therefore, the reduction in the CB1 receptor density in the hypothalamus is consistent
with the increased activation of the HPA axis seen following CUS. Normalization of HPA axis dysfunction is correlated with remission of depression (Greden et al., 1983; Ribeiro et al., 1993) and antidepressant therapies can diminish HPA axis responsivity in depressed humans and rats exposed to CUS (Deuschle et al., 2003; Duncan et al., 1996; Lopez et al., 1998; Chapter 2). We reported previously that chronic treatment with the antidepressant, desipramine, increases hypothalamic CB1 receptor agonist binding site density in unstressed rats, which contributes desipramine’s ability to dampen HPA axis activation (Chapter 2). Similarly, the present data demonstrate that concurrent imipramine attenuates the effects of CUS to decrease CB1 receptor binding site density in the hypothalamus. Taken together, these and earlier findings are consistent with the hypotheses that reduced CB1 receptor expression contributes to HPA axis hyper-reactivity following CUS and that anti-depressant-induced reversal of this change contributes to the mechanism by which this class of drugs normalizes HPA function in stressed rodents and, possibly, depressed humans.

In addition to these changes in the hypothalamic endocannabinoid system, the tissue content of 2-AG in the hypothalamus was increased following CUS. We have shown previously that acute stress reduces hypothalamic 2-AG content in mice which correlates with increased HPA axis activation (Patel et al., 2004). Furthermore, we found that repeated exposure to a homotypic stressor resulted in both a significant increase in hypothalamic 2-AG and habituation of HPA axis activation response to the stressor. These data support the hypothesis that elevated 2-AG content in the hypothalamus dampens HPA axis activation; if this is correct, then increased hypothalamic 2-AG following CUS would function to oppose HPA hyperactivity. As discussed above,
however, CUS also reduced hypothalamic CB₁ receptor density which would produce the opposite effect on HPA activity. It is possible that the change in 2-AG compensates for the loss of CB₁ receptors; or, alternatively, the increase in 2-AG results in CB₁ receptor down-regulation.

The effects of CUS and imipramine on the CB₁ receptor in the ventral striatum were similar to those in the hypothalamus; CB₁ receptor binding densities were reduced by CUS and partially reversed by concurrent imipramine treatment. Activation of CB₁ receptors in the nucleus accumbens within the ventral striatum increases dopamine release and enhances the rewarding properties of both natural and artificial rewards (Caille et al., 2007; Mahler et al., 2007), suggesting, in turn, that decreased CB₁ receptor signaling in this structure could impair hedonic processing. Anhedonia is a core symptom of major depression, and a behavioral phenomenon typically seen in rodents following CUS/CMS (Willner, 2005; Willner et al., 1992). It has recently been shown that the administration of a FAAH inhibitor during CMS (Bortolato et al., 2007) and to mice exposed to repeated restraint (Rademacher and Hillard, 2007) attenuates the development of anhedonia. The current data present the first evidence that CUS-induced anhedonia could be due to local impairments in CB₁ receptor activity within the nucleus accumbens, and that the reversal of this effect by antidepressants (or FAAH inhibition) may, in part, be related to normalization of CB₁ receptor signaling in this region; however, this remains to be experimentally demonstrated.

Surprisingly, an interaction between CUS and imipramine occurred in the ventral striatum and midbrain, such that co-treatment of imipramine and CUS resulted in a robust activation of FAAH activity although neither alone had an effect on activity. These data
are interesting and suggest that FAAH activity is sensitive to imipramine only in the context of CUS; and, alternatively, that FAAH activity is sensitive to CUS only in the context of imipramine treatment. If these data are interpreted in relationship to the model above that a decrease in ventral striatal AEA content is associated with anhedonia, then they suggest that this effect of imipramine to increase FAAH activity in the presence of CUS is an undesirable side effect of the antidepressant that is countering the clinical efficacy of these drugs. In support of this suggestion, preclinical data demonstrate that administration of a FAAH inhibitor can augment the behavioral effects of imipramine treatment (Filip et al., 2006).

Accumulating evidence implicates the endocannabinoid system in the pathophysiology of depression (Gorzalka et al., 2008; Hill and Gorzalka, 2005a; Mangieri and Piomelli, 2007; Vinod and Hungund, 2006; Witkin et al., 2005). The results reported herein support that role and demonstrate significant and region-specific changes in the CB₁ receptor and/or endocannabinoids. CB₁ receptor binding site density in the hippocampus, hypothalamus and ventral striatum is significantly reduced while density is increased in the PFC, in the CUS model of depression, suggesting an imbalance between cortical and subcortical anandamide/CB₁ receptor signaling reminiscent of the changes in dopamine signaling seen in schizophrenia (Deutch, 1992; as proposed in Chapter 4). The increase in cortical CB₁ receptor binding site densities following CUS could represent either a compensatory response to maintain serotonergic firing activity (Bambico et al., 2007) or a driving force in the development of depression (Hungund et al., 2004). Alternately, the reduction of AEA/CB₁ receptor signaling in subcortical structures could contribute to the changes in HPA axis drive, hedonic valuation and
emotional behavior seen in depression. The fact that AEA is so profoundly reduced, that this effect is insensitive to antidepressant treatment and that antidepressants even appear to activate FAAH in the face of concurrent stress, supports the contention that inhibition of FAAH should be investigated as a pharmacological target for the development of novel antidepressants or adjuncts to conventional antidepressants (Bortolato et al., 2007; Gobbi et al., 2005; Hill et al., 2007).
5.5 REFERENCES


CHAPTER 6

CIRCULATING ENDOCANNABINOID CONTENT IS ALTERED IN DEPRESSIVE ILLNESS\textsuperscript{5}

6.1 INTRODUCTION

The traditional theory, that major depression is a consequence of deficient monoamine activity has been revised by the addition of novel theories suggesting that disturbances in other systems are important for the pathophysiology of depression (Hindmarch, 2002). In particular, there is evidence that glucocorticoids, cytokines and neurotrophins are involved in the manifestation and treatment of this disease (Holsboer, 2000; Leonard and Song, 1996; Schiepers et al., 2005; Vaidya and Duman, 2001).

Mounting preclinical evidence implicates the endocannabinoid system in the pathophysiology of depression. For example, chronic unpredictable stress, which often elicits biochemical changes reminiscent of those seen in depressed populations (Lopez et al., 1998), has been shown to both reduce the tissue content of the endocannabinoid ligands 2-arachidonylglycerol (2-AG) and anandamide (AEA), and down-regulate the central cannabinoid receptor (CB\textsubscript{1}) in the subcortical structures, such as the hippocampus and hypothalamus, of rats (Hill et al., 2005; Reich et al., 2007; Chapter 5). These data suggest that endocannabinoid activity could be compromised in depression, a suggestion that is supported by findings that pharmacological activation of the

endocannabinoid system elicits an antidepressant-like response in the forced swim test in rodents (Bortolato et al., 2007; Gobbi et al., 2005; Hill and Gorzalka, 2005a; Hill et al., 2007; Rutkowska and Jachimczuk, 2004) and that regimens which evoke antidepressant responses increase endocannabinoid activity in several subcortical structures (Chapter 2, 3 and 4). Furthermore, mice that are deficient in the CB1 receptor exhibit a constellation of behavioral changes that are reminiscent of symptoms of depression such as anhedonia, anxiety, inability to extinguish aversive memories, heightened stress responsiveness and disturbances in neurovegetative functions such as feeding behavior and weight regulation (Barna et al., 2004; Cota et al., 2007; Marsicano et al., 2002; Martin et al., 2002; Sanchis-Segura et al., 2004; Uriguen et al., 2004; reviewed in Hill and Gorzalka, 2005b). Together, these data imply that deficient endocannabinoid activity could be associated with the manifestation and progression of depression.

However, there is an equally abundant collection of data suggesting that up-regulation of the endocannabinoid system contributes to the symptoms of depression. Specifically, antagonism of the endocannabinoid system also produces antidepressant-like effects in preclinical animal models of antidepressant efficacy (Griebel et al., 2005; Shearman et al., 2003; Tzavara et al., 2003). Furthermore, preclinical studies have demonstrated an increase in prefrontal cortical CB1 receptor mRNA and binding site density in animal models of depression (Bortolato et al., 2007; Chapter 5), and a reduction in prefrontal cortical endocannabinoid activity following electroconvulsive shock treatment (Chapter 4). Post-mortem studies have revealed that the CB1 receptor is up-regulated in the prefrontal cortex of depressed, suicide victims (Hungund et al., 2004). While this would appear to suggest that the endocannabinoid system is up-regulated in
depression, it should be noted that no endocannabinoid ligand measurements were done on this population, so the functional consequences of this change are not clear. The only published study to date of endocannabinoids in a population with affective disorders examined cerebrospinal fluid (CSF) content of N-arachidonylethanolamine (anandamide; AEA) in patients with affective disorders that were being used as a psychiatric control group; notably, these subjects were not a homogeneous population of depressed patients but included bipolar patients currently in a manic phase (Giuffrida et al., 2004). This study failed to find differences in the CSF AEA between control subjects and those with affective disorders, which could have been due, in part, to the diagnostic heterogeneity of the participants. This study also did not examine the content of the other primary endocannabinoid, 2-AG. The current state of knowledge would suggest that the endocannabinoid system could be involved in depression; however, due to a paucity of data from clinical populations, hypotheses concerning the nature of this involvement are premature.

Serum endocannabinoids present an interesting variable to examine in depressed populations for two reasons. First, changes in serum endocannabinoids could be representative of changes in CNS endocannabinoid content. It is known that endocannabinoids cross the blood brain barrier (Glaser et al., 2006; Mechoulam et al., 1998; Willoughby et al., 1997), and AEA content has been found to be increased in both the CSF and serum of schizophrenic patients (De Marchi et al., 2003; Giuffrida et al., 2004; Leweke et al., 1999); however, it should be noted that no direct correlation has been found between serum AEA and CSF AEA (Giuffrida et al., 2004). Furthermore, recent clinical work has demonstrated that increases in serum endocannabinoid content
following osteopathic treatment are correlated to specific subjective and behavioral measures suggestive of a cannabimimetic response, indicating that serum endocannabinoids are likely reflective of changes in central endocannabinoid activity (McPartland et al., 2005). Second, peripheral actions of endocannabinoids could also be relevant in the context of depression. Endocannabinoids are known to potently modulate immunomodulatory processes and cardiovascular function (Klein et al., 2003; Kunos et al., 2000), both of which have been associated with morbidity and illness associated with depression. Thus, the peripheral actions of endocannabinoids themselves should not be discounted in this scenario.

The goal of the present study was to examine serum endocannabinoid content in clinically depressed individuals. We have quantified the endocannabinoids AEA and 2-AG in the serum of ambulatory, medication free, female subjects suffering from minor or major depression and compared them to psychiatrically healthy controls matched for demographic characteristics.

6.2 METHODS

6.2.1 Participants

A total of 56 adult women from Saint Louis, MO USA participated in the study. Half of them (n= 28) met DSM-IV diagnostic criteria for clinical depression (American Psychiatric Association, 1994); the other half (n=28) had no lifetime history of psychiatric illness. The depressed and control subjects were matched on a case-by-case basis with respect to age and ethnicity. All subjects were in good health, defined as having (a) no history of chronic medical illness, (b) no indications of acute infectious disease at study entry, as evidenced by self-report of symptoms and a normal complete
blood count, and (c) no prescribed medication regimen, other than oral contraceptives, in the past six months including anti-depressants. Candidates were excluded if they were older than 55; had been pregnant in the past year; were menopausal, postmenopausal, or had irregular menses; were undernourished as evidenced by serum albumin ≤ 3.3 g/dL; or reported abusing illicit substances including cannabis, cocaine, and heroin.

Depressed patients were recruited through advertisements in local newspapers seeking individuals “feeling down and depressed, losing interest in enjoyable activities, or having trouble with eating, sleeping, or concentration.” To qualify for the study, depressed patients had to meet criteria for a current Major Depressive Episode (N=16) or Minor Depressive Episode (N=12) according to DSM-IV (American Psychiatric Association, 1994). Diagnoses were made by trained interviewers utilizing the Depression Interview and Structured Hamilton (Freedland et al., 2002). This instrument combines the probes needed to diagnose clinical depression according to DSM-IV with those needed to judge symptom severity on the 17-item Hamilton Rating Scale for Depression (Williams, 1988). Patients with comorbid psychotic, eating, alcohol, substance (other than nicotine dependence), or anxiety disorders (other than generalized anxiety disorder) were excluded using modules from the Diagnostic Interview Schedule (Robins et al., 1981) and the Primary Care Evaluation of Mental Disorders (Spitzer et al., 1994). Control subjects were also recruited through newspaper advertisements; these postings sought “medically healthy adults for a study of mood and health.” To qualify for the study, control subjects had to match a depressed subject in terms of age and ethnicity, and have a lifetime history free of psychiatric illness, as documented in structured interviews using the Depression Interview and Structured Hamilton, and modules from
the Diagnostic Interview Schedule and the Primary Care Evaluation of Mental Disorders. They also needed to score < 5 on the 10-item Center for Epidemiologic Studies Depression Scale (Radloff, 1977).

6.2.2 Procedures

During an initial session at the laboratory, research assistants explained study procedures, and subjects provided written informed consent. A battery of structured psychiatric interviews was then administered to determine eligibility, namely the Depression Interview and Structured Hamilton, and modules from the Diagnostic Interview Schedule and the Primary Care Evaluation of Mental Disorders. Eligible subjects were interviewed regarding their medical history, completed a battery of questionnaires about their health practices, and underwent a series of anthropometric and cardiovascular assessments (data not shown). Next, subjects were seated in a comfortable chair and had 35-ml of blood drawn through antecubital venipuncture in serum separating tubes. The blood was subsequently centrifuged for 15 min at 1000 x g, and the serum was aspirated, divided into aliquots, and frozen at -70° C until the end of the study. All serum samples were frozen by 120 minutes following venipuncture. Thawed serum was later used to assess contents of 2-AG and AEA. All blood draws were performed between 0900h and 1200h to control for diurnal variation. Upon completion of the study, participants were compensated $150. These procedures were approved by the Institutional Review Board of Washington University, USA.

6.2.3 Serum Endocannabinoids Extraction and Measurement

All extractions were performed using Bond Elut C18 solid-phase extraction columns (1 ml; Varian Inc, Lake Forest, CA). Serum samples (0.5 ml each) were thawed
and made up to 15% ethanol, to which the internal standards [\(^2\text{H}_8\)]-AEA (16.9 pmol) and [\(^2\text{H}_8\)]-2-AG (46.5 pmol) (Cayman Chemicals, Ann Arbor, MI) were added. Samples were then vortexed and centrifuged at 1000 \(\times\) g for 4 min. The supernatant was loaded on C18 columns, which have been conditioned with 1 ml redistilled ethanol and 3 ml of double distilled water (ddH\(_2\)O). The remaining pellet was washed with 100 \(\mu\)l of 15% ethanol and centrifuged again for 3 min. The resulting supernatant was also loaded onto the C18 column. Columns were washed with 5 ml ddH\(_2\)O and eluted with 1 ml of ethyl acetate. The ethyl acetate layer in the resulting elute was removed and dried under N\(_2\). Lipids in the residual ddH\(_2\)O phase were extracted by mixing with an additional 1 ml of ethyl acetate, which was added to the original ethyl acetate solution. Once dried, samples were resuspended in 20 \(\mu\)l of methanol and stored at –80\(^\circ\)C. AEA and 2-AG were quantified using liquid chromatography/mass spectrometry as described in Chapter 2.

### 6.2.4 Statistics

Comparisons between individuals with depression and matched controls on serum endocannabinoid contents were performed using independent samples t-tests. To verify that observed associations were not inflated by potential confounders, a series of univariate analyses of variance were then performed. These analyses involved comparing endocannabinoid contents across depressed and control groups, while covarying for factors that differed across diagnostic group, which were body mass index (BMI) and percentage of daily smokers. Bivariate correlations were also performed to examine the relationships between serum endocannabinoids and the clinical profiles of patients, including the duration of their current episode, its severity, and the intensity of particular symptom clusters. It should be noted that the sample from one control subject (control to
an individual with minor depression) was compromised during the extraction procedure and thus was not included in any analysis.

6.3 RESULTS

The demographic profile of the population that was used in this analysis can be seen in Table 6.1. For the sample of individuals with major depression and their matched controls, there were no differences in age (p = 0.93), race (p = 1.00) or years of education (p = .15). Individuals with major depression exhibited a significantly higher body mass index (BMI; p = 0.001) compared with their matched controls, and they were also more likely to be daily smokers (p = 0.02). The number of alcoholic drinks consumed in a week was higher, but not significantly, in individuals with major depression (p = 0.07).

For the sample of individuals with minor depression and their matched controls, there were no differences between the two groups on age (p = 0.80), race (p = 0.84), percentage of daily smokers (p = 0.17) or alcoholic drinks consumed per week (p = 0.62), however years of total education was non-significantly lower in individuals with minor depression (p = 0.06).

Table 6.1: Demographic characteristics of sample population (mean variable +/- SD)

<table>
<thead>
<tr>
<th></th>
<th>Major Depression (n=16)</th>
<th>Control (n=16)</th>
<th>Minor Depression (n=12)</th>
<th>Control (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>27.6 +/- 9.7</td>
<td>27.9 +/- 9.2</td>
<td>31.0 +/- 8.0</td>
<td>30.2 +/- 6.9</td>
</tr>
<tr>
<td>BMI</td>
<td>31.2 +/- 8.1*</td>
<td>23.8 +/- 2.4</td>
<td>31.8 +/- 10.9</td>
<td>27.6 +/- 6.7</td>
</tr>
<tr>
<td>Education (years)</td>
<td>14.4 +/- 1.8</td>
<td>15.4 +/- 1.8</td>
<td>14.6 +/- 2.0</td>
<td>16.2 +/- 1.9</td>
</tr>
<tr>
<td>% daily smokers</td>
<td>31.3 +/- 47.9*</td>
<td>0 +/- 0</td>
<td>16.7 +/- 38.9</td>
<td>0 +/- 0</td>
</tr>
<tr>
<td>Alcoholic drinks / week</td>
<td>3.4 +/- 5.7</td>
<td>0.6 +/- 1.3</td>
<td>1.5 +/- 3.1</td>
<td>2.4 +/- 5.6</td>
</tr>
<tr>
<td>% recurrent depression</td>
<td>75%</td>
<td>N/A</td>
<td>75%</td>
<td>N/A</td>
</tr>
<tr>
<td>Total HAM-D Score</td>
<td>20 +/- 4.0</td>
<td>N/A</td>
<td>15.3 +/- 4.7</td>
<td>N/A</td>
</tr>
<tr>
<td>Length of Current Episode (weeks)</td>
<td>N/A</td>
<td>N/A</td>
<td>41.3 +/- 51.2</td>
<td>N/A</td>
</tr>
<tr>
<td>% currently in therapy</td>
<td>12.5 +/- 34.2</td>
<td>N/A</td>
<td>0% +/- 0</td>
<td>N/A</td>
</tr>
<tr>
<td>Race‡</td>
<td>6C; 8AA; 1H; 1A</td>
<td>6C; 8AA; 1H; 1A</td>
<td>6C; 6AA</td>
<td>6C; 5AA</td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td>‡ C=Caucasian; AA=African American; H=Hispanic; A=Asian</td>
<td></td>
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<tr>
<td>* = significantly different from their matched control group, p &lt; 0.05.</td>
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</tbody>
</table>
In women with major depression, serum 2-AG was significantly reduced [$t(30) = 2.098, p = 0.04$; Fig. 6.1a] (serum 2-AG for major depression: $12.5 +/- 5.6$ pmol/ml serum vs. matched controls: $19.6 +/- 12.5$ pmol/ml serum), with an effect size of 0.57 standard deviations. This effect was marginally reduced by controlling for tobacco use ($p = 0.08$), and marginally enhanced by controlling for BMI ($p = 0.03$).

**Figure 6.1:** Serum content of the endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) in women with: a) major depression and their matched controls ($n=16$/group); or b) women with minor depression and their matched controls ($n=12$ for minor depression; $n=11$ for controls). Values are denoted in pmol/ml of serum AEA or 2-AG content. * Significantly different from control ($p < .05$).

To examine if this reduction in serum 2-AG was related to severity, chronicity or symptom profile of major depression, we examined the correlation of serum 2-AG to
of these variables in the major depression cohort. Serum 2-AG content was found to exhibit a significant negative correlation with duration of current depressive episode ($r = -.492$, $p = 0.05$; Fig. 6.2). Within the sample of subjects with major depression, there was no difference in serum 2-AG between those with recurrent major depression and those in their first episode [$t (14) = 0.133$, $p = 0.90$]. Serum 2-AG did not significantly correlate with total Hamilton score ($r = -.09$, $p = 0.74$), suggesting that this reduction of 2-AG was not directly related to depression severity.

**Figure 6.2:** Serum content of the endocannabinoid 2-arachidonoylglycerol (2-AG) exhibited a significant negative correlation ($r = -.492$) with the duration of the current depressive episode (in weeks).

Serum AEA content did not differ between subjects with major depression and their matched controls [$t (30) = 0.205$, $p = 0.84$; Fig. 6.1a] (serum AEA for major depression: 0.74 +/- 0.32 pmol/ml serum vs. matched controls: 0.72 +/- 0.29 pmol/ml serum). While serum AEA was not correlated with total Hamilton score ($r = -.24$, $p = 0.38$), serum AEA exhibited a highly significant, negative correlation with scores on both the Hamilton variable for cognitive anxiety ($r = -.647$, $p < 0.01$; Fig. 6.3a) and somatic
anxiety ($r = -0.674$, $p < 0.01$; Fig. 6.3b), such that individuals who demonstrated higher measures of anxiety, exhibited lower serum AEA content. Serum AEA content did not correlate with any other Hamilton rating. Serum AEA did not correlate with duration of current depressive episode ($r = 0.30$, $p = 0.25$), nor did serum AEA content differ between subjects who experienced recurrent depression as opposed to those who were experiencing their first episode [$t (14) = 1.12$, $p = 0.28$].

**Figure 6.3:** (a) Serum content of the endocannabinoid $N$-arachidonyl ethanolamine (anandamide; AEA) exhibited a significant negative correlation ($r = -0.647$) with Hamilton ratings for cognitive anxiety; (b) serum content of the endocannabinoid AEA exhibited a significant negative correlation ($r = -0.674$) with Hamilton ratings for somatic anxiety.
Serum AEA content was significantly increased in patients with minor depression compared to their matched controls \([t(21) = 2.48, p = 0.02; \text{Fig. 6.1b}]\) (serum AEA for minor depression: 0.95 +/- 0.44 pmol/ml serum vs. matched controls: 0.60 +/- 0.18 pmol/ml serum), with a robust effect size of 1.96 standard deviations. Serum 2-AG was higher in patients with minor depression; however, this was not significant \([t(21) = 1.26, p = 0.22; \text{Fig. 6.1b}]\) (2-AG in minor depression: 26.4 +/- 18.32 pmol/ml serum vs matched controls: 18.18 +/- 12.37 pmol/ml serum, effect size = 0.67 standard deviations). The length of the current depressive episode appeared to correlate with both serum AEA \((r = .51, p = .09)\) and serum 2-AG \((r = .50, p = .10)\), such that serum contents of both these molecules were higher the longer the duration of the episode. However, perhaps due to the small number of patients in these analyses, these effects were not statistically significant. There were no significant differences in serum AEA \([t(10) = -.169, p = .12]\) or serum 2-AG \([t(10) = 1.76, p = .11]\) between those experiencing a first episode of depression versus recurrent depression. There was no significant correlation between total Hamilton score and serum AEA \((r = -.19, p = .55)\). The correlation between total Hamilton scores and serum 2-AG content was positive \((r = .51, p = .10)\), but nonsignificant because of the sample size of 12 subjects. It should be noted that one sample from the control group to the minor depression patients was compromised during the extraction procedure and thus data from this individual is not included in the data set.

### 6.4 DISCUSSION

The major finding of this study was that in a population of ambulatory, medication free females diagnosed with major depression, serum content of the endocannabinoid 2-AG was significantly decreased. The magnitude of this decrease was
significantly related to the duration of the current depressive episode, such that as an episode progressed, 2-AG content decreased more substantially. These data provide the first demonstration that major depression could be associated with a hypoactive endocannabinoid system.

While serum AEA content was not significantly altered in major depression, amounts of this molecule were strongly and negatively correlated with somatic and cognitive anxiety. That is, patients with high anxiety also exhibited low serum AEA. This finding suggests that while AEA may not be related to depression per se, it could be inversely related to the extent of anxiety present. Consistent with this, animal research has demonstrated that selective inhibition of AEA metabolism elicits robust anxiolytic effects (Kathuria et al., 2003; Patel and Hillard, 2006). Together, these data support the idea that low AEA may be associated with the manifestation anxiety and that inhibition of AEA metabolism is a logical target for the development of anxiolytic drugs (Gaetani et al., 2003; Kathuria et al., 2003; Patel et al., 2004).

The relationship between serum endocannabinoid content and central endocannabinoid content is not known. Since endocannabinoids possess the ability to cross the blood brain barrier (Glaser et al., 2006; Mechoulam et al., 1998; Willoughby et al., 1997), it is certainly plausible that significant changes in central endocannabinoid content would result in spillover into the serum. Alternatively, it is also possible that endocannabinoid synthesis in the periphery may regulate to some degree endocannabinoid levels in the central nervous system. The finding that significant correlations were found in this study between serum endocannabinoids and specific clinical profiles on the Hamilton scale is circumstantial evidence that serum
endocannabinoids reflect central endocannabinoid content to some degree. This hypothesis is substantiated by recent work demonstrating that cannabimimetic-like responses to osteopathic manipulations are associated with increases in serum endocannabinoid content (McPartland et al., 2005).

An unexpected finding in this study was that the changes in serum endocannabinoids observed in minor depression were the opposite of those seen in major depression. Specifically, AEA was significantly higher whereas 2-AG was appreciably, but not significantly, elevated among women with minor depression compared to their matched controls. Given that minor depression is a less severe variant of major depression it is surprising that these opposite effects were found. One possible interpretation of this finding is that endocannabinoids may act as a protective buffer against the progression of affective disease. Animals that are deficient in the CB1 receptor are more susceptible to the depressive-like effects of chronic stress (Martin et al., 2002), suggesting that the endocannabinoid system acts as a buffer against the shift of stress into depression; however, the current data require replication and extension before this hypothesis can be further substantiated.

There are several limitations to this research that require attention. For example, the effect that has been documented here is exclusively in a population of women, thus a determination of whether this effect is present in both genders or is limited to women is critical to the advancement of theories concerning the role of endocannabinoids in depression. In line with this limitation, menstrual cycle was not controlled for in this study; however, fluctuations in serum endocannabinoid content throughout the menstrual cycle would likely introduce increased variability that would occlude detection of a
significant finding. Furthermore, analysis of CSF endocannabinoids, especially 2-AG, in depressive disorders is essential to determine if a deficiency is consistently observed in the central nervous system. Another limitation with these findings is that these data are cross-sectional and thus do not give a direct understanding of the direction of causality, or insights into how these changes in endocannabinoid serum content progress throughout remission and relapse within a given individual. Finally, the possibility does exist that an undetermined third variable could mediate the effects documented here.

Analysis of covariance determined that the deficit in 2-AG seen in major depression was somewhat reduced when tobacco use was covaried; however, this covariance accounted for only a small proportion of the documented effect. We view this as an unlikely explanation for our findings, however, as it cannot parsimoniously account for why the direction of change of serum endocannabinoids was opposite for minor and major depression when both groups possessed more smokers than their matched controls.

Additionally, while exclusion criteria for this study included cannabis dependence, it is unknown whether patients occasionally consumed cannabis in the weeks leading up to assessment. Some depressed patients do use cannabis as self medication (Gruber et al., 1996), and it is possible that such use could disrupt endogenous production of 2-AG or AEA. However, animal research has suggested that repeated administration of exogenous cannabinoid ligands does not reliably reduce brain endocannabinoid content (Gonzalez et al., 2004), and, as with smoking, this effect could not explain the bidirectional alterations seen in the two depressed populations.

These data are the first demonstration of an alteration in the endocannabinoid system in clinical depression. It is our hypothesis, based upon converging human and
animal evidence, that the endocannabinoid system acts as a protective buffer system and that the enhanced endocannabinoid signaling seen in minor depression is a compensatory response that retards its progression into major depression. However, the association of a deficit in 2-AG signaling with major depression suggests that at some point in the progression of the disease, not only does the endocannabinoid compensation disappear, but a significant down-regulation of endocannabinoid signaling occurs, which could result in worsening of the depression. Collectively, these data suggest that the development of agents which non-selectively inhibit uptake or metabolism of AEA and 2-AG could be an effective form of treatment for comorbid depression-anxiety disorders.
6.5 REFERENCES


CHAPTER 7

GENERAL DISCUSSION

Only recently has the endocannabinoid system been considered to contribute to emotional behavior, and thus possibly play a role in the etiology and/or treatment of affective illness (Hill and Gorzalka, 2005b; Mangieri and Piomelli, 2007; Serra and Fratta, 2007). The research described in the current dissertation extended this knowledge by investigating whether the endocannabinoid system is regulated by antidepressant regimens, and determining the extent to which the endocannabinoid system is dysregulated in both an animal model of depression and in women diagnosed with major depression. The main findings of this dissertation are: 1) that treatment with the tricyclic antidepressant desipramine for three weeks resulted in an increase in the binding site density of the CB₁ receptor in the hippocampus and the hypothalamus, which in turn contributed to the ability of desipramine treatment to attenuate stress responsivity (Chapter 2); 2) that voluntary exercise increased CB₁ receptor binding and intracellular signaling, and the tissue content of the endocannabinoid AEA, within the hippocampus, which subsequently contributed to the ability of exercise to augment proliferation of progenitor cells within the dentate gyrus of the hippocampus (Chapter 3); 3) that electroconvulsive shock treatment robustly reduced CB₁ receptor binding and AEA content within the prefrontal cortex while enhancing CB₁ receptor signaling within the amgdala (Chapter 4); 4) that CB₁ receptor binding was reduced in the hippocampus, hypothalamus and ventral striatum in the CUS model of depression, while CB₁ receptor binding was increased in the prefrontal cortex; all of these effects, except for those in the hippocampus, were reversed to some degree by concurrent antidepressant treatment.
Moreover, CUS reduced AEA content in the prefrontal cortex, hippocampus, hypothalamus, amygdala, ventral striatum and midbrain; this reduction in AEA was not mediated by an increase in the hydrolytic activity of FAAH nor was the reduction of AEA reversed by concurrent antidepressant treatment (Chapter 5); and 5) that women diagnosed with major depression exhibit a significant reduction in the circulating levels of the endocannabinoid 2-AG, while women diagnosed with minor depression exhibit a significant increase in the circulating content of the endocannabinoid AEA (Chapter 6). Collectively, these data suggest that the endocannabinoid system may be involved in both the pathophysiology and a mechanism of action for the treatment of depression. The relevance of the current data for both of these aspects of depression will be discussed independently.

7.1 TOWARDS AN ENDOCANNABINOID THEORY OF DEPRESSION

Prior to the research performed in this thesis, it was hypothesized that depressive illness, may in part be due to deficits in endocannabinoid signaling (Hill and Gorzalka, 2005b). This hypothesis was primarily based on three observations: 1) deletion of the CB1 receptor produced a neurobehavioral phenotype in mice that is strikingly reminiscent to the symptom profile of depressive illness, particularly melancholic depression (Aso et al., 2008; Hill and Gorzalka, 2005b; Martin et al., 2002; Steiner et al., 2008b); 2) phenomena which can instigate depressive episodes, such as protracted stress, can downregulate endocannabinoid signaling in the hippocampus (Hill et al., 2005); 3) activation of the CB1 receptor, via either exogenous or endogenous ligands, elicits an antidepressant-like response in an array of preclinical paradigms, such as the forced swim test, tail suspension test and chronic mild stress model (Bambico et al., 2007; Bortolato et
Furthering our understanding of the role of the endocannabinoid system in depression, the current dissertation provides ample empirical support for the hypothesis that endocannabinoid signaling is impaired in depression, and that this impairment in endocannabinoid activity may be functionally relevant to the manifestation of particular symptoms of this disease.

Within the current dissertation, it was discovered that central endocannabinoid signaling was profoundly suppressed in an animal model of depression (with the exception of the prefrontal cortex, which will be discussed below), and that peripheral endocannabinoid signaling was reduced in major depression. Given that impairments in CB₁ receptor activity can produce depressive-like behaviors, and that endocannabinoid signaling is dampened in depression (or the animal corollary), it is tempting to speculate that a deficit in endocannabinoid activity could increase the susceptibility to or promote the development of a depressive episode. Interpretation of these data in light of the functional neuroanatomy of endocannabinoid signaling provides a template of how a deficit in this system could produce this response.

Within the hippocampus, there is evidence that a deficit in endocannabinoid signaling could impair neurotrophic and neuroplastic processes, and in turn interfere with adaptive behavior designed to cope with stress. Specifically, genetic deletion of the CB₁ receptor reduces proliferation of progenitor cells, neurogenesis and BDNF expression in the hippocampus (Aguado et al., 2005; Aso et al., 2008; Jin et al., 2004; Kim et al., 2006; Steiner et al., 2008b). While a demonstration of impaired neurogenesis in clinical depression has yet to be documented, reduced levels of hippocampal BDNF have been
found in major depression (Karege et al., 2005); similarly, many animal models of
depression exhibit reductions in cell proliferation, neurogenesis and BDNF (Gronli et al.,
2006; Tsankova et al., 2006). The relevance of these changes is highlighted by the fact
that the depressive phenotype of mice lacking the CB₁ receptor is reversed by
administration of BDNF into the hippocampus (Aso et al., 2008), as well as the finding
that hippocampal BDNF is essential for antidepressant pharmacological agents to elicit
their behavioral effects (Adachi et al., 2008). Thus, a deficit in endocannabinoid activity
within the hippocampus (as was seen in the CUS model of depression; Chapter 5), could
impair hippocampal neurotrophic activity, which in turn would increase depressive-like
behaviors.

Endocannabinoid signaling within the hypothalamus is known to be important for
both neuroendocrine and neurovegetative functioning. CB₁ receptors in the PVN gate
excitatory input to CRH neurosecretory cells, and thus function as a negative regulator of
HPA axis activity (Di et al., 2003; Gorzalka, et al., 2008a). Deficits in hypothalamic
endocannabinoid signaling result in increased CRH expression, hyperactivity of the HPA
axis and impaired glucocorticoid negative feedback (Cota et al., 2003, 2007). There is
ample evidence for a breakdown in glucocorticoid feedback, and subsequent
hypersecretion of glucocorticoids, in depressive illness, as well as animal models of
depression (Herman et al., 1995; Holsboer, 2000; Parker et al., 2003; Young et al., 1990).
In support of a coupling of these two phenomena, we have recently found that
pharmacological inhibition of FAAH is capable of preventing the basal hypersecretion of
glucocorticoids that occurs following chronic stress (M.N. Hill, V. Viau and B.B.
Gorzalka, unpublished findings). Thus, it is reasonable to predict that the reduction in
hypothalamic AEA/CB₁ signaling following CUS would be a driving force in the facilitation of adrenocortical activity. Putatively, a similar mechanism could be involved in the dysregulation of the HPA axis in major depression.

In addition to neuroendocrine regulation, hypothalamic endocannabinoid activity has also been implicated in neurovegetative functions, such as feeding and sleep/wakefulness cycles (Matias et al., 2006; Murillo-Rodriguez et al., 2006), which are disturbed in depression (DSM-IV, 2000). A reduction in hypothalamic endocannabinoid signaling is compatible with the reductions in appetite, weight gain and enhanced arousal seen in depression, particularly the melancholic subtype (Gold and Chrousos, 2002), and may be contributing to the reduced weight gain and altered sleep architecture associated with the CUS model of depression (Bortolato et al., 2007; Cheeta et al., 1997).

Endocannabinoid signaling in the amygdala has been found to be integral to the extinction of aversive memories (Marsicano et al., 2002). Genetic or pharmacological blockade of the CB₁ receptor impairs the clearance of emotionally aversive memories (Cannich et al., 2004; Chhawal et al., 2005; Kamprath et al., 2006; Marsicano et al., 2002). Depression is known to be characterized by ruminative coping patterns in which individuals continuously perseverate on emotionally negative thoughts (Ciesla and Roberts, 2007; Donaldson et al., 2007). Rumination could represent a clinical corollary of deficient aversive memory extinction, or at least deficient emotional cognitive flexibility, and accordingly, may be related to reduced amygdalar endocannabinoid activity. This hypothesis is highlighted by the growing consensus that amygdalar endocannabinoid signaling is specific for adaptation to aversive, but not positive, stimuli (Holter et al., 2005; Kamprath et al., 2006; Niyuhire et al., 2007).
The classical reward circuit is composed of the mesolimbic dopamine system, where dopaminergic neurons originating in the ventral tegmental area (VTA) project to the nucleus accumbens (Grace et al., 2007). Dopamine release within the nucleus accumbens is associated with rewarding stimuli and is believed to be involved with motivational salience (Berridge, 2007; Schultz, 2002). Integration of endocannabinoid signaling into this pre-existing reward circuitry has occurred over the past few years (Gardener, 2005). Accordingly, endocannabinoid actions within the VTA can promote dopamine neuron firing through a reduction in local GABAergic inhibition (Matyas et al., 2008; Pan et al., 2008; Szabo et al., 2002), and endocannabinoid activity within the nucleus accumbens promotes rewarding responses to an array of stimuli, including palatable food and recreational drugs (Caille et al., 2007; Mahler et al., 2007; Soria-Gomez et al., 2007). Similarly, impairments in endocannabinoid signaling, presumably within the reward circuitry, result in impaired motivational behavior and reductions in dopaminergic transmission in response to rewarding stimuli (Cheer et al., 2007; Melis et al., 2007; Sanchis-Segura et al., 2004). Given the inherent association of depressive illness and deficient reward salience, it is interesting to note that within the CUS model of depression, AEA content was significantly reduced in the midbrain (which contains the VTA) and AEA content and CB1 receptor binding density were both significantly reduced in the ventral striatum (primarily composed of the nucleus accumbens; Chapter 5). Furthermore, inhibition of FAAH has been found to prevent the development of anhedonia following chronic stress (Bortolato et al., 2007; Rademacher and Hillard, 2007). This effect that may be mediated by reinstating AEA/CB1 receptor signaling within the reward circuit. Thus, a deficiency in endocannabinoid signaling within the
mesolimbic dopamine system may foster an anhedonic state, similar to what is seen in depression.

At the peripheral level, circulating endocannabinoids are potent modulators of immune and cardiovascular processes (Hillard, 2000; Ullrich et al., 2007). The primary physiological actions of endocannabinoids in these systems are to constrain cardiovascular reactivity (via regulation of sympathetic activation and vascular smooth muscle excitability; Ashton and Smith, 2007; Gauthier et al., 2005; Pakdeechote et al., 2007) and inflammatory processes (Batkai et al., 2007; Chang et al., 2001; Karsak et al., 2007). Accordingly, reductions in circulating endocannabinoid ligands could theoretically result in an increase in blood pressure, sympathetic activity and production of inflammatory mediators. It is probably not a coincidence that all of these phenomena are seen in major depression (Carney et al., 2007; Miller et al., 2003; Raison et al., 2006). As has been suggested with other systems, the deficit in peripheral endocannabinoid signaling seen in major depression (Chapter 6) may be a contributing factor in the increased prevalence of cardiovascular and inflammatory diseases comorbid with affective illness (Carney et al., 2002).

This collection of data, inferring that a deficit in endocannabinoid signaling is functionally related to the development of depression is further supported by two other findings. First, while the clinical data presented in Chapter 6 are preliminary, it should be noted that we have recently replicated this finding in an independent population (Hill et al., under review). Specifically, we have found significant reductions in circulating levels of both AEA and 2-AG in a separate group of women diagnosed with major depression (Hill et al., under review). At this point, there is no obvious explanation as to why only
reductions in serum 2-AG in major depression were documented in the current study, whereas reductions in both AEA and 2-AG were documented in a separate population. Regardless of this discrepancy, these data collectively provide the first clinical demonstrations of reduced levels of endocannabinoids in major depression and support the hypothesis that depressive illness is coupled to a deficiency in endocannabinoid activity.

Second, clinical trials employing the CB₁ receptor antagonist Rimonabant for the treatment of obesity found that a significant proportion of individuals developed notable indices of anxiety and depression following consumption of Rimonabant (Christensen et al., 2007; van Gaal et al., 2005). These data indicate that, similar to what has been demonstrated in rodents (Gorzalka et al., 2008a; Hill and Gorzalka, 2005b; Martin et al., 2002; Steiner et al., 2008b; Viveros et al., 2005), endocannabinoid signaling is critical for the regulation of human emotional behavior. Furthermore, by experimentally demonstrating (through placebo controlled trials) that pharmacological inhibition of endocannabinoid activity in humans can directly promote the development of anxiety and depression, these data also support the major hypothesis developed in this dissertation, that impairments in endocannabinoid signaling may promote affective illness (Christensen et al., 2007; van Gaal et al., 2005).

Based on the evidence, the following hypothesis can be proposed. Either due to genetic factors (i.e., variants in the CB₁ receptor that influence signal capacity) or environmental conditions (i.e., chronic stress which decreases endocannabinoid/CB₁ receptor activity), an impairment in endocannabinoid signaling may increase the susceptibility to or promote the development of depressive illness. Within subcortical
structures, an endocannabinoid deficit may contribute to the impairments in neurotrophic expression and neuroplasticity, basal hyperactivity of the HPA axis, increases in anxiety, emotionality and anhedonia and reductions in neurovegetative functions seen in major depression. Within the periphery, a deficit in endocannabinoid activity may contribute to the increases in inflammatory processes and cardiovascular problems associated with depressive illness. Thus, a deficiency in endocannabinoid signaling is sufficient to produce most, if not all, of the behavioral and physiological alterations seen in depression at both the central and peripheral level. While it is very improbable that the endocannabinoid system is the primary mediator of affective illness, the current data attest to its potential importance in playing a major role in depression.

One particular point should be addressed here regarding the putative role of the endocannabinoid system in depression. Clinically, depression is typically manifested at a 2:1 ratio in women to men (Grigoriadis and Robinson, 2007); thus, any potential theory of depression should be able to account for this dramatic gender difference in disease prevalence. With regards to gender differences in endocannabinoid function, there is little known. At the hormonal level, there is evidence that female sex steroids, such as estradiol, can modulate endocannabinoid function. There is evidence that the FAAH gene may be under negative genomic regulation by estrogen (Waleh et al., 2002), which is consistent with the finding that estrogen downregulates FAAH expression within the uterus (Maccarrone et al., 2000). This would suggest that estrogen may enhance AEA signaling, a hypothesis which is substantiated by the findings that estradiol treatment increases hypothalamic AEA content (Scorticati et al., 2004), as well as the fact that estradiol administration modulates emotional behavior in a CB1 receptor dependent
fashion, that is also mimicked by pharmacological inhibition of FAAH (Hill et al., 2007). At the gender level, this effect of estrogen may be relevant as females have been found to exhibit higher levels of AEA within the pituitary than males, while 2-AG did not differ between genders (Gonzalez et al., 2000a). However, on the other side of the coin, estrogens also downregulate CB1 receptor transcription (Gonzalez et al., 2000a,b) and male rodents have been found to exhibit higher levels of CB1 mRNA than females within the pituitary gland (Gonzalez et al., 2000a) and throughout the brain, the binding affinity of the CB1 receptor is significantly weaker in females as opposed to males (Rodriguez de Fonseca et al., 1994). In line with this, a recent in-vivo positron emission tomography study employing a radioligand for the CB1 receptor has found that in humans, men exhibit a significantly higher density of the CB1 receptor within frontocortical subregions and limbic structures than women (van Laere et al., 2008). Taken together, these data present a somewhat paradoxical gender difference in endocannabinoid signaling, with females appearing to exhibit higher levels of AEA, but males possessing higher densities of CB1 receptor binding. With regards to how this may relate to an endocannabinoid theory of depression, it is possible that lower densities/affinity of corticolimbic CB1 receptors may predispose women to be more susceptible to depressive illness. This is in accordance with the aforementioned hypothesis that steady state reductions in endocannabinoid signaling may subsequently impair individuals coping and emotional responses to life stress. Furthermore, as AEA is a rapidly synthesized ligand, the regulation of AEA is much more dynamic than that of CB1 receptor expression. Thus, despite the fact that females may possess higher levels of AEA than males, fluctuations in menstrual cycle or stress (both which are known to effect AEA concentrations, centrally
and peripherally; Gonzalez et al., 2000a; Habayeb et al., 2004; Hill et al., under review; Lazzarin et al., 2004; Patel et al., 2005) may decrease AEA and result in periods of dampened CB₁ receptor signaling (relative to men), that could be a prognosticating factor in a depressive episode. However, this hypothesis is highly speculative, and further research investigating gender differences in endocannabinoid function and regulation of emotion would be required to fully understand if this theory can account for the robust gender difference that is seen in the prevalence of depressive illness.

### 7.1.1 The Case of the Prefrontal Cortex

The consensus of the data generated in this thesis was primarily that a deficit in endocannabinoid signaling is involved in the development of depression; however, the prefrontal cortex seems to be the one neuroanatomical site which does not exhibit a reduction in endocannabinoid signaling in depression. Individuals diagnosed with major depression, who died by suicide (as well as alcoholic individuals who have died by suicide), have been found to exhibit increased protein expression, maximal binding site density and intracellular signal transduction of the CB₁ receptor in the dorsolateral prefrontal cortex (Hungund et al., 2004; Pazos et al., 2006; Vinod et al., 2005). Recent reports have revealed that exposure of rats or mice to the chronic unpredictable (or mild) stress models of depression results in an increase in the expression of CB₁ receptor mRNA in the prefrontal cortex (Bortolato et al., 2007; C.J. Hillard and W.E. Cullinan, unpublished data). Data from the current thesis replicated and extended these findings, as exposure of rodents to the CUS model of depression resulted in a significant increase in CB₁ receptor binding site density in the prefrontal cortex (Chapter 5).
The majority of neurons within the prefrontal cortex are either local interneurons (of a GABAergic phenotype; Gabbott and Bacon, 1996; Hendry et al., 1987) or pyramidal neurons (of a glutamatergic phenotype) which extend their axons to sites throughout the brain such as the amygdala and ventral tegmental area (Brinley-Reed et al., 1995; Carr and Seasack, 2000; Likhtik et al., 2005). Accordingly, changes in CB\textsubscript{1} receptor mRNA in this structure may not necessarily equate to changes in CB\textsubscript{1} receptor binding, as the CB\textsubscript{1} receptor is typically transported to axonal terminals following transcription and assembly (Leterrier et al., 2006). The current data argue that the increase in CB\textsubscript{1} receptor binding seen in animal models of depression (and possibly in major depression) is likely occurring on local interneurons given the concordance between gene expression and functional receptor expression. This in turn will have significance for the interpretation of these changes. For example, previous data have indicated that infusion of a CB\textsubscript{1} receptor agonist into the ventromedial prefrontal cortex increases activation of prefrontal cortical projections to the dorsal raphe, in turn promoting serotonergic neurotransmission (Bambico et al., 2007). This phenomenon would likely be mediated by activation of CB\textsubscript{1} receptors on local GABAergic interneurons which tonically suppress pyramidal neurons projecting to the dorsal raphe. Thus, an increase in CB\textsubscript{1} receptor expression on GABAergic interneurons in the ventromedial prefrontal cortex following CUS exposure (and possibly in major depression) could be a compensatory response elicited to increase serotonergic neurotransmission and counter the development or worsening of depression-like behaviors. This hypothesis is supported by the fact that local administration of a CB\textsubscript{1} receptor agonist into the ventromedial prefrontal cortex evokes an antidepressant
response that is mediated by a serotonergic mechanism (Bambico et al., 2007; R.J. McLaughlin, M.N. Hill & B.B. Gorzalka, unpublished findings). Thus, while the CB₁ receptor is found to be increased in the prefrontal cortex of individuals with major depression and animals exposed to the CUS model of depression, this response is likely not involved in the development of depression, but more so, is a response aimed at curbing it.

7.2 ENHANCEMENT OF ENDOCANNBINOID SIGNALING IS A NOVEL THERAPEUTIC TARGET FOR THE PHARMACOTHERAPY OF DEPRESSION

As it is hypothesized that a deficiency in endocannabinoid signaling is contributing to the etiology of depression, it is logical likewise to predict that pharmacological augmentation of this system would provide benefit to the treatment of this disease. One manner in which to examine the therapeutic potential of the endocannabinoid system would be to determine if this system is a target for a variety of antidepressant treatment regimens which elicit clinical benefit. Within the current dissertation it was demonstrated that chronic tricyclic antidepressant treatment, repeated electroconvulsive shock and engagement in physical activity (each of which is clinically effective in treating depression) all increased some aspect of subcortical endocannabinoid signaling. Specifically, endocannabinoid activity in the hippocampus was increased by chronic antidepressant treatment (Chapter 2) and voluntary exercise (Chapter 3), while amygdalar endocannabinoid activity was increased by electroconvulsive shock treatment (Chapter 4) and hypothalamic endocannabinoid activity was increased by antidepressant treatment (Chapter 2). Furthermore, the relevance of these changes in endocannabinoid signaling was illustrated by the fact that stress-attenuating effects of antidepressant
treatment and the neuroplastic effects of voluntary exercise were abrogated by antagonism of the CB1 receptor (Chapter 2 and 3, respectively). Thus, congruent with a theoretical deficit in endocannabinoid signaling in depression, several distinct antidepressant treatments increase endocannabinoid activity, supporting the argument that this system is a valid target for the development of novel antidepressants.

There is an abundance of preclinical evidence to support the contention that enhancement of this system elicits antidepressant-like responses. Inhibition of endocannabinoid uptake and/or FAAH activity elicits antidepressant effects in the forced swim test (Filip et al., 2006; Gobbi et al., 2005; Hill and Gorzalka, 2005a; Hill et al., 2007), the tail suspension test (Gobbi et al., 2005) and the chronic mild stress model (Bortolato et al., 2007), and facilitates the actions of conventional antidepressants (Filip et al., 2006). Thus, at the behavioral level, an enhancement in endocannabinoid activity elicits effects reminiscent of a typical antidepressant agent.

In addition to behavioral effects, there are several “biochemical signatures” that are induced by protracted administration of an antidepressant regimen. For example, treatment with every class of antidepressant agent and somatic treatments for depression (such as electroconvulsive shock treatment or voluntary exercise) have been found to increase hippocampal BDNF, cell proliferation and neurogenesis (Castren et al., 2007; Hashimoto et al., 2004; Malberg and Schechter, 2005). An enhancement in endocannabinoid neurotransmission would produce similar effects to these as stimulation of the CB1 receptor increases BDNF expression and cell proliferation in the hippocampus (Aguado et al., 2005; Derkinderen et al., 2003), and prolonged administration of a CB1 receptor agonist has been found to increase hippocampal neurogenesis (Jiang et al.,
In line with this, the current data demonstrate that the ability of voluntary exercise to increase cell proliferation in the hippocampus is via an up-regulation of endogenous cannabinoid signaling (Chapter 3), indicating that the endocannabinoid system is not only able to modulate hippocampal plasticity, but it can mediate the effects of other relevant treatments.

Similarly, chronic administration of antidepressant agents in humans and other species dampens both basal and stress-induced HPA axis activity (Butterweck et al., 2001; de Bellis et al., 1993; Michelson et al., 1997; Reul et al., 1993). These effects are believed to be relevant to the clinical efficacy of these agents, as individuals who do not exhibit normalization of HPA axis function in response to pharmacotherapy exhibit a higher likelihood of symptomatic relapse (Ribeiro et al., 1993; Zobel et al., 2001). The current data reveal that the ability of chronic antidepressant treatment to dampen HPA axis activity is via recruitment of CB1 receptor signaling (Chapter 2), indicating a functional role of this system in HPA axis regulation by antidepressants. Agents which facilitate endocannabinoid neurotransmission have been found to attenuate stress-induced activation of the HPA axis (Patel et al., 2004), and we have recently determined that concurrent inhibition of FAAH during chronic stress exposure reverses the basal hypersecretion of glucocorticoids produced by this regimen (M.N. Hill, V. Viau and B.B. Gorzalka, unpublished findings). Thus, an increase in endocannabinoid signaling would dampen HPA axis activity in a similar fashion to protracted treatment with a conventional antidepressant.

In addition to mimicking the responses evoked by long-term antidepressant treatment, the endocannabinoid system also exhibits a high degree of interaction with
monoaminergic circuits that become relevant under the umbrella of affective illness. CB₁ receptors are found throughout serotonergic, noradrenergic and dopaminergic cell bodies in the midbrain (Haring et al., 2007; Matyas et al., 2008; Oropeza et al., 2001), and stimulation of CB₁ receptors increases the firing rate of these neurons and as well as terminal release of monoamines in regions such as the frontal cortex, hippocampus and nucleus accumbens (Bambico et al., 2007; Gobbi et al., 2005; Pillolla et al., 2007; Oropeza et al., 2005; Solinas et al., 2006). At the synaptic level, there is some evidence that cannabinoids inhibit monoamine reuptake, similar to the actions of conventional antidepressants (Banerjee et al., 1975; Steffens and Feuerstein, 2004). Similarly, AEA has been found to inhibit 5-HT reuptake in human platelets (Velenovska and Fisar, 2007); however, these effects of AEA on 5-HT reuptake in platelets and synaptosomes occurs independent of CB₁ receptor activation (Steffens and Feuerstein, 2004; Velenovska and Fisar, 2007). With respect to specific receptors, there is extensive preclinical and clinical evidence that antagonism of 5-HT₂A receptors enhances the efficacy of conventional antidepressants (Marek et al., 2003). At the behavioral level, activation of the CB₁ receptor (by both exogenous and endogenous ligands) has been found to attenuate stereotyped behaviors evoked by 5-HT₂A receptor stimulation (Cheer et al., 1999; Darmani 2001; Egashira et al., 2004; Gorzalka et al., 2005; Rutkowska and Jachimczuk, 2004). More so, both of the endocannabinoid ligands, AEA and 2-AG, have been found to inhibit 5-HT₂A receptor mediated signal transduction (Boger et al., 1998) and, at high concentrations, AEA can inhibit 5-HT₂A receptor binding (Kimura et al., 1998).

Collectively, it appears that pharmacological enhancement of endocannabinoid signaling can produce all of the behavioral and biochemical effects of conventional
antidepressants. Furthermore, while potentiation of endocannabinoid activity can reverse stress-induced anhedonia (Bortolato et al., 2007; Rademacher and Hillard, 2007), an effect also produced by conventional antidepressants (see Willner, 2005), this pharmacological strategy has no apparent addictive or rewarding properties of its own (Gobbi et al., 2005; Scherma et al., 2008). Furthermore, pharmacological agents targeting endocannabinoid signaling appear to exhibit minimal effects on sexual activity (Gorzalka et al., 2008b), suggesting that drugs of this class would not exhibit sexual side effects, as have been reported with SSRI antidepressants (Meston and Gorzalka, 1992; Montgomery et al., 2002). As such, agents which augment endocannabinoid neurotransmission produce all of the desired cellular, neuroendocrine and behavioral responses of a standard antidepressant drug, but with less potential addictive and adverse side effects, and accordingly, represent a putative class of agents which should be clinically examined for the pharmacotherapy of depression.

7.2.1 The Case of the Antidepressant Effects of CB1 Receptor Antagonists

Despite substantial evidence arguing that enhancement of endocannabinoid transmission exerts antidepressant effects, several researchers have demonstrated that administration of CB1 receptor antagonists to rodents elicits antidepressant-like effects in a variety of preclinical paradigms (Griebel et al., 2005; Shearman et al., 2003; Steiner et al., 2008a; Tzavara et al., 2003). These data are congruent with the finding that electroconvulsive shock, which is a very effective treatment for depression, decreased both CB1 receptor binding and AEA tissue content in the prefrontal cortex (Chapter 4). Together, these data suggest the possibility that a reduction in prefrontal cortical endocannabinoid signaling may be antidepressant, and that the antidepressant effect of
CB₁ receptor antagonists may be mediated by blockade of CB₁ receptors within the prefrontal cortex. While these data appear to be incompatible with the finding that activation of CB₁ receptors in the ventromedial prefrontal cortex is antidepressant (Bambico et al., 2007; R.J. McLaughlin, M.N. Hill & B.B. Gorzalka, unpublished findings), it must be considered that within the electroconvulsive shock treatment studies, the chunk of prefrontal cortex tissue used for analysis was composed of both the ventromedial prefrontal cortex and anterior cingulate cortex. Accordingly, administration of a CB₁ receptor antagonist into the anterior cingulate cortex has recently been found to evoke a robust antidepressant response (R.J. McLaughlin, M.N. Hill & B.B. Gorzalka, unpublished findings). This effect had some degree of specificity as administration of a CB₁ receptor antagonist into the ventromedial prefrontal cortex has no effect on depression-like behaviors in the forced swim test (Bambico et al., 2007; R.J. McLaughlin, M.N. Hill & B.B. Gorzalka, unpublished findings). Therefore, it is possible that systemically administered CB₁ receptor antagonists exert their antidepressant effects in preclinical paradigms through a blockade of CB₁ receptors within the anterior cingulate cortex. Furthermore, electroconvulsive shock may preferentially suppress endocannabinoid signaling within the anterior cingulate cortex, which in turn may contribute to its antidepressant effects. However, CB₁ receptor antagonists would be inappropriate as antidepressants, given that in some individuals such agents can actually increase the expression of anxiety and depression (Christensen et al., 2007; van Gaal et al., 2005), likely as a consequence of disrupting limbic endocannabinoid signaling. In conclusion, these clinical data indicate that the antidepressant effect elicited by CB₁ receptor antagonists in animal paradigms (Griebel et al., 2005; Shearman et al., 2003;
Steiner et al., 2008a; Tzavara et al., 2003), is likely a false positive of preclinical research, is not relevant to the treatment of human affective pathologies and thus, should not detract from the antidepressant potential of agents which enhance endocannabinoid neurotransmission.

7.2.2 Limitations

While the theory presented in this dissertation has substantial support, there are some limitations that have arisen in these data that require discussion. A major limitation of these data is the lack of consistency among differing classes of antidepressant treatments. In particular, the differences between the effects of chronic treatment with desipramine and imipramine were quite surprising. Desipramine increased CB₁ receptor binding in the hippocampus and hypothalamus, while imipramine treatment (in the absence of stress) increased CB₁ receptor binding in the amygdala, and reduced CB₁ receptor binding in the hypothalamus. The increased amygdalar CB₁ receptor binding following imipramine is similar to what was seen following electroconvulsive shock and is not inconsistent with the greater theory; however, the reduction of hypothalamic CB₁ receptor binding observed following imipramine treatment is quite incongruent with the changes seen in this structure following desipramine treatment. This difference is surprising given that both of these compounds evoke comparable behavioral effects at the clinical and preclinical level (Araki et al., 1985; Einarson et al., 1999; Wieland and Lucki, 1990); however, the monoamine systems which these agents target do differ, which may account for this discrepancy. In particular, desipramine is a norepinephrine reuptake inhibitor while imipramine predominately inhibits 5-HT reuptake (Frazer, 1997). A recent study has revealed that chronic treatment with the antidepressant
citalopram (which is a 5-HT reuptake inhibitor) results in a significant reduction in CB₁ receptor mediated GTPγS within distinct hypothalamic nuclei (Hesketh et al., 2008), which is concordant with the reduced hypothalamic CB₁ receptor binding seen following imipramine and supports a potential dissociation of serotonergic and noradrenergic effects. The relevance of this dissociation is intriguing as several reports have documented that 5-HT based agents (such as SSRI’s) do not consistently suppress HPA axis function in a manner analogous to tricyclic antidepressants which target noradrenergic systems (Connor et al., 2000; Duncan et al., 1996; Young et al., 2004). The differential regulation of hypothalamic endocannabinoid signaling by noradrenergic and serotonergic agents may account for this discrepancy. This putative differentiation of serotonergic and noradrenergic regulation of the CB₁ receptor (at least within the hypothalamus), does not necessarily preclude the role of the endocannabinoid system in the antidepressant response. More so, these data suggest that the endocannabinoid system is one mechanism that can be recruited by antidepressants, but is not necessarily a target for all classes of antidepressants. The extent to which hypothalamic endocannabinoid signaling is recruited by an antidepressant, however, may in turn be related to the ability of that agent to modify HPA axis function.

A similar limitation with these data is the extent to which these effects are generalizable to a broader context. For example, while it was found that hippocampal endocannabinoid signaling is engaged by voluntary exercise to contribute to the increased cell proliferation elicited by this treatment (Chapter 3), this does not necessarily mean that the endocannabinoid system is a universal mediator of the effects of antidepressants on hippocampal neuroplasticity. Given that desipramine treatment did increase
hippocampal CB₁ receptor density, it is plausible that endocannabinoids may contribute to the effects of this drug treatment on hippocampal plasticity (Gur et al., 2007). However, both electroconvulsive shock treatment and imipramine treatment did not increase hippocampal endocannabinoid signaling, and imipramine was unable to reverse the reductions in hippocampal endocannabinoid signaling following CUS. Electroconvulsive shock treatment is the most robust antidepressant regimen to increase cell proliferation, neurogenesis and BDNF expression (Malberg et al., 2000; Nibuya et al., 1995), despite having no effect on hippocampal endocannabinoid activity. Furthermore, imipramine is able to stimulate neurogenesis and reverse the suppression of neurogenesis seen in animal models of depression (Keilhoff et al., 2006; Sairanen et al., 2005; Xu et al., 2007), again despite its inability to modulate endocannabinoid signaling in the hippocampus. As such, it would appear that the hippocampal endocannabinoid system is not necessarily relevant for all treatment modalities of depression. While some regimens (such as exercise) may recruit endocannabinoids to modulate plasticity, other regimens (such as electroconvulsive shock treatment) may recruit independent pathways that produce the same end result. The most parsimonious reconciliation of this is that activation of the hippocampal endocannabinoid system is sufficient to promote neuroplastic and antidepressant-like effects, but is not necessary for all forms of antidepressant treatment to be effective.

7.3 CONCLUSION

The primary aim of the research in this dissertation was to further our understanding of the putative role of the endocannabinoid system in the pathophysiology and treatment of affective illness, and major depression in particular. The experimental
evidence presented here reveals that the endocannabinoid system is impaired in both clinical major depression and an animal model of depression, and that endocannabinoid activity is functionally enhanced by a variety of antidepressant treatment regimens. Fluctuations in endocannabinoid signaling within distinct neuroanatomical circuits can sufficiently account for the neurobehavioral and neuroendocrine alterations seen both in major depression and following antidepressant treatment. Thus, it is plausible to hypothesize that endocannabinoid signaling is dysfunctional in depressive illness, and that this impairment is a driving force in the development of this disease in some individuals. Accordingly, pharmacological facilitation of endocannabinoid signaling should be seriously examined at the clinical level to determine potential efficacy in the treatment of major depression. Based on the current understanding of the pharmacological and neurobehavioral properties of endocannabinoid signaling, some of which were the result of research in this dissertation, it can reasonably be hypothesized that this pharmacotherapeutic strategy would be as effective as conventional antidepressants, with a less adverse side effect profile.
7.4 REFERENCES


Hill MN, Miller GE, Carrier EJ, Gorzalka BB, Hillard CJ under review Circulating endocannabinoid and N-acyl ethanolamines are differentially regulated by stress. J Clin Endocrinol Metab


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ANIMAL CARE CERTIFICATE

Application Number: A06-0243

Investigator or Course Director: Boris Gorzalka

Department: Psychology, Department of

Animals: rats Sprague-Dawley 350

Start Date: May 1, 2006  Approval Date: June 26, 2008

Funding Sources:

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Regulation of the endocannabinoid system by stress and antidepressants

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

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