PREFRONTAL ENDOCANNABINOID SIGNALING MEDIATES NEUROENDOCRINE AND BEHAVIORAL COPING RESPONSES TO STRESS

by

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Abstract

Major depression is a heterogeneous disease often precipitated by dysfunction within the neuroendocrine stress circuitry, leading to profound deficits in prefrontocortical function. The endocannabinoid system has recently emerged as a vital component of the stress response; however, the mechanisms by which endocannabinoid signaling in the prefrontal cortex (PFC) modulates neuroendocrine and behavioral responses to stress has yet to be elucidated. In Chapter 2, genetic deletion of the CB_1 receptor prolonged corticosterone secretion following cessation of stress, which was recapitulated by CB_1 receptor antagonism within the medial PFC. Acute stress produced a delayed elevation in 2-arachidonoylglycerol (2-AG) content in the medial PFC that was reversed by glucocorticoid receptor antagonism. Immunohistochemical and electrophysiological data demonstrated the presence of CB_1 receptors in inhibitory-type terminals impinging upon principal neurons within layer V of the medial PFC. Furthermore, application of corticosterone to prefrontocortical slices suppressed γ-aminobutyric acid release onto layer V principal neurons, which was prevented by CB_1 receptor antagonism. Hence, the ability of glucocorticoids to terminate HPA axis activity is mediated by local recruitment of 2-AG in the medial PFC. In Chapter 3, forced swim stress rapidly suppressed anandamide (AEA) content in the medial PFC. Local inhibition of AEA hydrolysis decreased passive coping and increased active coping strategies in the forced swim test (FST) in a CB_1 receptor-dependent and serotonin-mediated manner. Furthermore, local inhibition of AEA hydrolysis increased the firing rate of serotonin neurons, suggesting that prefrontocortical AEA signaling modulates stress coping behaviors via regulation of serotonergic neurotransmission. In Chapter 4, rats exposed to chronic unpredictable

stress (CUS) displayed increased CB_1 receptor binding specifically within the ventromedial PFC. CUS exposure increased passive coping and decreased active coping strategies in the FST, which was further augmented by ventromedial PFC CB_1 receptor blockade. Thus, the increase in CB_1 receptor binding observed in the ventromedial PFC of CUS-exposed rodents serves a compensatory role that maintains proactive coping strategies under chronically stressful conditions. Collectively, this body of research indicates that prefrontocortical endocannabinoid signaling is a critical mediator of neuroendocrine and behavioral stress responses and may represent an appealing target for future therapeutic strategies aimed at combating stress-related disorders.

Preface

A version of Chapter 2 has been published in: Hill MN*, McLaughlin RJ*, Pan B, Fitzgerald ML, Roberts CJ, Lee TT, Karatsoreos IN, Mackie K, Viau V, Pickel VM, McEwen BS, Liu QS, Gorzalka BB, Hillard CJ. (2011). Recruitment of prefrontal cortical endocannabinoid signaling by glucocorticoids contributes to termination of the stress response. J Neurosci, 31(29), 10506-15. I conducted all of the experiments except those in Figure 2.1C (Figure 1C in the published manuscript), Figures 2.3A and 2.3C (Figures 3A and 3C in the published manuscript), Figure 2.4 (Figure 5 in the published manuscript), and Figure 2.5 (Figure 6 in the published manuscript). CJ Roberts performed the CB_1 receptor knockout mice experiment in Figure 2.1C. IN Karatsoreos and MN Hill conducted the immunohistochemical analyses in mice in Figures 2.3A and 2.3C. B Pan conducted the in *vitro* electrophysiology experiments in Figures 2.5 and 2.6. I conducted all of the microinfusion studies, lipid extractions, performed all central surgeries, histology, immunohistochemical receptor staining in rats, shared behavioral testing duties, and co-wrote the manuscript with MN Hill (* denotes co-first author status).

A version of Chapter 3 has been accepted for publication in *European Neuropsychopharmacology*. McLaughlin RJ, Hill MN, Bambico FR, Stuhr KL, Gobbi G, Hillard CJ, Gorzalka BB. Prefrontal cortical anandamide signaling coordinates coping responses to stress through a serotonergic pathway. I conducted all of the experiments except those in Figure 3.4, which were performed by FR Bambico. I performed all of the cannula implantations, conducted all of the microinfusions and behavioral testing, histology, lipid extractions, analyzed the data, and wrote the manuscript.

A version of Chapter 4 is currently under review for publication. McLaughlin RJ, Hill MN, Dang S, Hillard CJ, Gorzalka BB. Up-regulation of cannabinoid CB_1 receptor binding in the ventromedial prefrontal cortex is adaptive following chronic unpredictable stress exposure. S Dang assisted in running the chronic unpredictable stress and forced swim test. I performed all of the cannula implantations, microinfusions, brain removals, tissue extractions, and neurochemical receptor binding assays, assisted in behavioral testing, and wrote the manuscript.

Check the first pages of these chapters to see footnotes with similar information.

All rats were housed and cared for, and tissue was harvested, according to the guidelines of the University of British Columbia and the Canadian Council for Animal Care, except rats used for Chapter 3.2.4 which were housed and cared for according to guidelines set by the Canadian Institutes of Health Research for animal care and scientific use. All research in this dissertation using rats was approved by the University of British Columbia Animal Care Committee (certificate numbers A07-0243 and A09-0220), except for research conducted in Chapter 3.2.4, which was approved by the Animal Care Committee of McGill University (protocol number 4805). All mice were housed and cared for according to the Guide for the Care and Use of Laboratory Animals and research using mice was approved by the Institutional Animal Use and Care Committee of the Medical College of Wisconsin (AUA certificate number 141).

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List of Abbreviations

2-AG	2-arachidonoylglycerol
5-HT	5-hydroxytryptamine (serotonin)
AC	Adenylyl cyclase
ACSF	Artificial cerebrospinal fluid
ACTH	Adrenocorticotropic hormone
AEA	N-arachidonylethanolamide (anandamide)
ANOVA	Analysis of variance
AP	Anterior-posterior
BDNF	Brain-derived neurotophic factor
BLA	Basolateral amygdala
B_{max}	Maximal binding site density
BNST	Bed nucleus of the stria terminalis
BSA	Bovine serum albumin
C	Celsius
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CB_1R-/-	CB_1 receptor knockout
cm	Centimeter
CNR1	CB_1 receptor gene
CON	Control
CRH	Corticotropin-releasing hormone
CUS	Chronic unpredictable stress
D1	Day 1
D2	Day 2
DA	Dopamine
DAG	Diacylglycerol
DGL	Diacylglycerol lipase
dmPFC	Dorsomedial PFC
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

DSE	Depolarization-induced suppression of excitation
DSI	Depolarization-induced suppression of inhibition
DV	Dorsal-ventral
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
FAAH	Fatty acid amide hydrolase
FST	Forced swim test
g	Gram
GABA	γ-aminobutyric acid
GPCR	G-protein-coupled receptor
GTP	Guanosine triphosphate
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPA	Hypothalamic-pituitary-adrenocortical
hr	Hour
Hz	Hertz
IP	Intraperitoneal
IPSC	Inhibitory post-synaptic current
K^+	Potassium
K_D	Dissociation constant
kg	Kilogram
KOH	Potassium hydroxide
LTDi	Long-term depression at inhibitory synapses
M	Molar units
MΩ	Mega ohm
MGL	Monoacylglycerol Lipase
MGluR5	Metabotropic glutamate receptor 5
min	Minute
mIPSC	Miniature inhibitory post-synaptic current
mRNA	Messenger ribonucleic acid
MT	Membrane transporter

μA	Microamp
μg	Microgram
μl	Microliter
μm	Micrometer
μM	Micromolar
mg	Milligram
MgATP	Magnesium adenosine triphosphate
$MgCl_2$	Magnesium chloride
mM	Millimolar
ml	Milliliter
ML	Medial-lateral
mm	Millimeter
ms	Millisecond
mV	Millivolt
N_2	Nitrogen
Na^+	Sodium
NA	Noradrenaline
NAPE	N-acyl phosphatidylethanolamine
ng	Nanogram
nl	Nanoliter
nM	Nanomolar
OBX	Olfactory bulbectomy
OEA	Oleoylethanolamide
PBS	Phosphate-buffered saline
pCPA	p-chlorophenylalanine
PEA	Palmitoylethanolamide
PFC	Prefrontal cortex
PKA	Protein kinase A
pmol	Picomoles
PPR	Paired-pulse ratio
PVN	Paraventricular nucleus of the hypothalamus

s	Second
SEM	Standard error of the mean
SSRI	Selective serotonin reuptake inhibitor
THC	Δ^9-tetrahydrocannabinol
TME	Tris-HCl- $MgCl_2$-Ethylenediaminetetraacetic acid
TTX	Tetrodotoxin
VEH	Vehicle
vmPFC	Ventromedial PFC
VTA	Ventral Tegmental Area
WT	Wild-type

Acknowledgements

Well, it's been a long journey, one that I couldn't have possibly endured without the vast support and encouragement from mentors, family, and friends to whom I owe tremendous gratitude. First and foremost, I must thank my supervisor, Dr. Boris Gorzalka, for allowing me to become an independent, self-sufficient researcher but still providing guidance and advice when I needed it. You have been the ideal supervisor over these past 5 years and I am forever grateful that you accepted me into your laboratory. I must also express my appreciation to my PhD supervisory committee, Dr. Joanne Weinberg and Dr. Todd Handy, for offering their guidance, feedback, and support throughout the writing of this dissertation. Many thanks also goes out to my various collaborators, Drs. Cecilia Hillard, Victor Viau, Liisa Galea, Gabriella Gobbi, and Bruce McEwen, who have greatly influenced my research and kindly offered their laboratory, resources, and expertise to conduct this research. I also owe utmost gratitude to Dr. Matthew Hill for being my colleague, mentor, and friend throughout my graduate studies and beyond. You have taught me so much in these past 5 years, reinvigorating my passion for neuroscience and serving as an exemplary scientist to which I will continually strive to emulate. My appreciation for everything you have done for me, both inside and outside the laboratory, truly knows no bounds. I must also thank my fellow lab members, Tiffany Lee, Caitlin Riebe, Anna Morrish, and Silvain Dang who have always been available when I needed their assistance, any time, day or night. Your help over these past years certainly does not go unnoticed and I am extremely appreciative for everything you have done. Outside the laboratory, I owe considerable thanks to my parents, Debbie and Gary McLaughlin. Without your words of encouragement and

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To my wife and son…

1. General Introduction

1.1. Major Depression

Major depression is a devastating mental illness that produces profound emotional, motivational, cognitive, and neurovegetative disturbances that cause significant distress or impairment in personal, social, and/or occupational aspects of daily life functioning. Emotionally, individuals suffering from major depression are characterized by depressed mood, accompanied by increased anxiety, feelings of worthlessness, excessive or inappropriate guilt, or even suicidal ideations (American Psychiatric Association, 2000). A loss of pleasure or interest in daily life activities (i.e., anhedonia) is typically regarded as a hallmark symptom of major depression (American Psychiatric Association, 2000). Cognitive functioning is also often impaired, resulting in a diminished ability to think, concentrate, or make executive decisions (American Psychiatric Association, 2000). Moreover, most major homeostatic systems, including feeding, sleeping, stress, and reproductive drive, exhibit some form of disturbance, however, the manifestation of these disturbances can be highly variable (American Psychiatric Association, 2000). For instance, individuals suffering from major depression can be characterized by insomnia or hypersomnia, excessive weight loss or weight gain, hyperarousal or hypoarousal, and psychomotor agitation or retardation (American Psychiatric Association, 2000). Thus, despite a common diagnostic description, major depression is now viewed as a complex and heterogeneous disease that can manifest as a variety of diverse phenotypes.

Indeed, major depression is unequivocally viewed as a heterogeneous disease that can be partitioned into distinct subtypes (Parker, 2000). It is estimated that approximately

25-30% of depressed individuals are classified as the melancholic subtype (Gold and Chrousos, 2002). Melancholic depression is the most treatment-resistant subtype and exhibits disturbances such as anhedonia, increased anxiety, reduced appetite and weight loss, insomnia, hyperarousal, impaired behavioral flexibility, and a predominance of aversive memories (Rush and Weissenburger, 1994; Gold and Chrousos, 2002; Hill and Gorzalka, 2005a). In direct contrast, approximately 15-30% of depressed individuals are diagnosed with atypical depression, presenting with a symptom cluster of hypersomnia, hyperphagia, weight gain, hypoarousal, and immunological anergy (Gold and Chrousos, 2002; Gold et al., 2002). Thus, the classification of atypical depression is fundamentally the opposite of the melancholic subtype, suggesting that the neurobiological underpinnings for these two classes are inherently different (Gold and Chrousos, 2002).

The lifetime prevalence rates of major depression are currently at an all-time high, with an estimated 16.5% of the population expected to experience at least one depressive episode during their lives (Kessler et al., 2005). In 75% of cases of major depression, the disease course is recurrent and is manifested as multiple cycles of remission and exacerbation (Frank and Thase, 1999). The economic burden of major depression is estimated to be as high as $44 billion per year in lost work production (Stewart et al., 2003), while the World Heath Organization has predicted that it will become the second most prevalent cause of illness-induced disability by the year 2020 (Murray and Lopez, 1997). For these reasons and numerous others, the impetus for elucidating the underlying neuropathology of major depression and the need for more effective therapeutic treatment strategies has never been greater.

1.2. The Neurobiology of Stress

Stress is fundamentally defined as a state of strain resulting from a challenge to homeostasis following exposure to a real or perceived threat to an organism's well-being. Hans Selye, a pioneer of research examining the biological effects of exposure to stressful stimuli, first provided the empirical foundation for this field of work by describing what he termed the General Adaptation Syndrome, which consisted of an enlargement of the adrenal gland, atrophy of the thymus, spleen, and other lymphyoid tissue, and gastric ulcerations (Selye, 1936). Dr. Selye suggested that this syndrome develops in three stages. The first stage was referred to as the "general alarm reaction", where the organism is suddenly confronted with a threat or critical situation. This stage is followed by a period of prolonged resistance, whereby the organism preferentially increases production of thyrotopic and adrenotropic factors while ceasing production of growth and gonadotropic hormones, which are less urgently needed in such emergencies. Finally, if the organism is under a perpetual state of distress where tolerance does not develop, resistance is lost and the organism succumbs to the final stage of exhaustion, and in some cases, death (Selye, 1936). It is remarkable how this description of the stress response is still largely applicable today, given the many advances in this field since these early discoveries.

Although Dr. Selye had originally postulated that all threats to homeostasis are nonspecific, it is now known that physiological stressors such as cold, hypoxia, hypoglycemia, and hemorrhage are processed differently than psychological stressors, which may be perceived as stressful even though they do not directly disrupt physiological homeostasis. As a result, physiological stressors are commonly referred to

as “reactive” stressors since they present a direct challenge to homeostatic function, while psychological stressors are referred to as “anticipatory” stressors since the organism only anticipates a threat to homeostasis based on prior experiences or innate species-specific preconceptions (Herman et al., 2003; Riebe and Wotjak, 2011). This distinction between reactive and anticipatory responses is also experience-dependent; the environment in which a reactive stressor is experienced can also be conditioned, resulting in an anticipatory response upon subsequent exposure to that environment (Herman et al., 2003).

The underlying neural circuitries that coordinate reactive vs. anticipatory responses to stressors are fundamentally distinct. Physical stressors are initially perceived by sensory fibers in the periphery, which is transmitted through the spinal cord to regions of the hindbrain including the nucleus of the solitary tract and the ventrolateral medulla (Swanson et al., 1983). These hindbrain projections are predominantly excitatory, largely mediated by catecholamines including noradrenaline (NA), and are directly responsible for engaging the neuroendocrine stress response (Cunningham and Sawchenko, 1988; Cunningham et al., 1990). In contrast, psychological stressors depend on indirect forebrain inputs from corticolimbic structures such as the amygdala, hippocampus, and prefrontal cortex (PFC), which are responsible for discriminating between threatening and non-threatening stimuli (Herman et al., 2003).

Regardless of the pathway by which the biological stress response is initiated, it subsequently elicits a generalized activation of an autonomic reflex arc, characterized by activation of the hypothalamic-sympathetic-adrenomedullary axis and triggered by sympathetic neural efferents that stimulate the release of adrenomedullary

catecholamines, and a neuroendocrine reflex arc, characterized by activation of the hypothalamic-pituitary-adrenocortical (HPA) axis and triggered by the secretion of hypothalamic-releasing hormones (Tasker and Herman, 2011).

The HPA axis represents the major neuroendocrine system responsible for the maintenance of homeostatic balance in response to stressful stimuli (Herman et al., 2003). Following exposure to a stressor, corticotropin-releasing hormone (CRH) neurons in the parvocellular region of the paraventricular nucleus of the hypothalamus (PVN) become activated, releasing CRH into the median eminence where it is then transported to the anterior pituitary, stimulating the release of adrenocorticotropic hormone (ACTH) into the bloodstream. In turn, ACTH induces the release of glucocorticoids (cortisol in humans, corticosterone in rodents) from the adrenal cortex into general circulation. Upon release from the adrenal cortex, glucocorticoid hormones are able to cross the blood-brain barrier and bind to glucocorticoid receptors located in the PVN, as well as extrahypothalamic limbic structures including the amygdala, hippocampus, and PFC to exert both delayed (genomic) and rapid (non-genomic) feedback modulation of the HPA axis (Dallman, 2005; Ulrich-Lai and Herman, 2009). The classical delayed transcriptional regulatory effects of glucocorticoids are mediated by intracellular glucocorticoid receptors (McEwen, 1973), while the rapid non-genomic glucocorticoid actions are thought to be mediated by activation of putative membrane-associated glucocorticoid receptors (Orchinik et al., 1991). Mineralocorticoids are also released in response to stress, although at much lower levels (<1000-fold), and as a consequence are not considered to be important in the acute systemic stress response (Tasker and Herman, 2011).

Both the autonomic and neuroendocrine reflex arcs converge at the adrenal glands but on different time scales, with the autonomic response resulting in rapid catecholamine secretion and the neuroendocrine response resulting in delayed glucocorticoid secretion (Tasker and Herman, 2011). Because of the rapid nature of the autonomic response, it is thought to directly contribute to the fight-or-flight response, thereby promoting survival. In contrast, the neuroendocrine glucocorticoid response is thought to provide support for the stress response by mobilizing glucose stores from the muscle and liver, enhancing cardiovascular function, and inhibiting growth, reproductive, and inflammatory responses, thereby diverting energy supplies and allowing for maximum availability to attend to the threat at hand (Tasker and Herman, 2011). The rapid actions of glucocorticoids in peripheral tissue are incredibly valuable, supporting the survival of the organism in the minutes following a stressful challenge.

Glucocorticoids also serve an equally important purpose in the brain, effectively terminating the neuroendocrine stress response (i.e., promoting feedback inhibition of the HPA axis) and restoring homeostasis. This feedback inhibition is important, as it prevents depletion of hypothalamic and pituitary stress hormones, thereby allowing the organism to mount successive stress responses (Sapolsky et al., 2000). It also protects the organism against the excitotoxic effects of excessive CRH and glucocorticoid secretion, as it is well established that chronically elevated levels of glucocorticoids can produce a multitude of detrimental health effects (McEwen, 2008). Moreover, deficient feedback inhibition and glucocorticoid hypersecretion can also lead to insulin resistance, visceral fat deposition, osteoporosis, inhibition of T-helper-1-directed cellular immunity, excessive fear, and chronic suppression of the mesolimbic dopamine (DA) reward

system, thereby promoting susceptibility to an array of pathological disease states ranging from mood disorders to type II diabetes (Gold and Chrousos, 2002; Hill and McEwen, 2010).

1.3. Stress and Major Depression

The precise neurobiological mechanisms responsible for the development and maintenance of depressive symptoms are multi-factorial and considerably complex. However, a vast amount of research has demonstrated that repeated or prolonged exposure to social, psychological, and/or environmental stress is one of the greatest instigating factors in the development of major depression (Paykel, 2001; Hammen, 2005). Individuals suffering from major depression report experiencing more stressful life events as well as a higher level of uncontrollable or unpredictable stress when compared to non-depressed individuals (Ravindran et al., 2002; Ilgen and Hutchison, 2005). Cumulative life stressors strongly predict the lifetime prevalence of affective disorders (Caspi et al., 2003; McEwen, 2004). Moreover, psychological stress frequently precedes the onset of affective episodes (Brown et al., 1987; Lewinsohn et al., 1988; Kendler et al., 1993), predicts depression severity (Hammen et al., 1992) and relapse (Paykel and Tanner, 1976), and is related to an inferior antidepressant response (Lloyd et al., 1981). Accordingly, dysfunction within the neural network that mediates the neuroendocrine response to stress is a key precipitating factor that can augment susceptibility to depressive episodes later in life.

Indeed, a large proportion of individuals with major depression, particularly those afflicted with the melancholic subtype, display abnormalities at various levels of the neuroendocrine stress response. For instance, these individuals display increased

concentrations of CRH in the cerebrospinal fluid, increased CRH messenger ribonucleic acid (mRNA) and protein levels in the PVN, and a blunted ACTH response to a CRH challenge (likely reflecting downregulation of CRH receptors) (Nemeroff et al., 1984; Gold et al., 1988a, b; Holsboer, 2000). Moreover, depressed patients often do not exhibit appropriate negative feedback responses to administration of the synthetic glucocorticoid dexamethasone, suggesting that inhibitory feedback of the HPA axis may be impaired in depression, and thus exists in a chronic feed-forward state (Gold et al., 1988a, b; Murphy, 1991; Holsboer, 2000). Although stress-induced alterations at the level of the hypothalamus indeed contribute to HPA axis disturbances and glucocorticoid hypersecretion, it is now thought that dysfunction within upstream inhibitory glucocorticoid feedback structures such as the hippocampus and PFC may underlie the neuroendocrine, emotional, and cognitive deficits present in stress-related illnesses (Furay et al., 2008). This literature review largely focuses on the role of the PFC, a brain region that exerts complex hierarchical control over the HPA axis.

1.4. The Prefrontal Cortex

1.4.1. Organization and Homology

The PFC represents the center for executive functioning, responsible for mediating a range of cognitive, behavioral, and neuroendocrine processes that are necessary to plan, control, and direct behavior according to shifting environmental demands. The PFC is a structurally and functionally heterogeneous brain region, and subregions of the PFC have been classically defined based on the presence or absence of a granular zone and its strong reciprocal connections with the dorsomedial nucleus of the thalamus (Rose and Woolsey, 1948; Uylings and van Eden, 1990). Current classifications

also account for the functional (i.e. electrophysiological and behavioral) properties of the subregion, the presence and distribution of different neurochemicals and neurotransmitter systems, and its embryological development when comparing homologies between cortical areas in different species (Uylings et al., 2003).

The primate PFC is roughly divided into three anatomically and functionally distinct subregions; a medial region, an orbital region, and a dorsolateral region (Barbas, 1992; Carmichael and Price, 1994). The medial region is the most evolutionarily conserved of the three subdivisions and provides the major cortical output to visceromotor structures in the hypothalamus and brainstem (Ongur and Price, 2000). The orbital subregion has been implicated in social learning and coding of affective stimuli, and receives inputs from several sensory modalities, including olfaction, taste, vision, visceral afferents, and somatic sensation (Ongur and Price, 2000). The dorsolateral PFC by contrast, has evolved into the most highly specialized cortical region in primates, vital for executive functioning tasks such as working memory, behavioral flexibility, attentional control, decision-making, and temporal organization of behavior (Brown and Bowman, 2002).

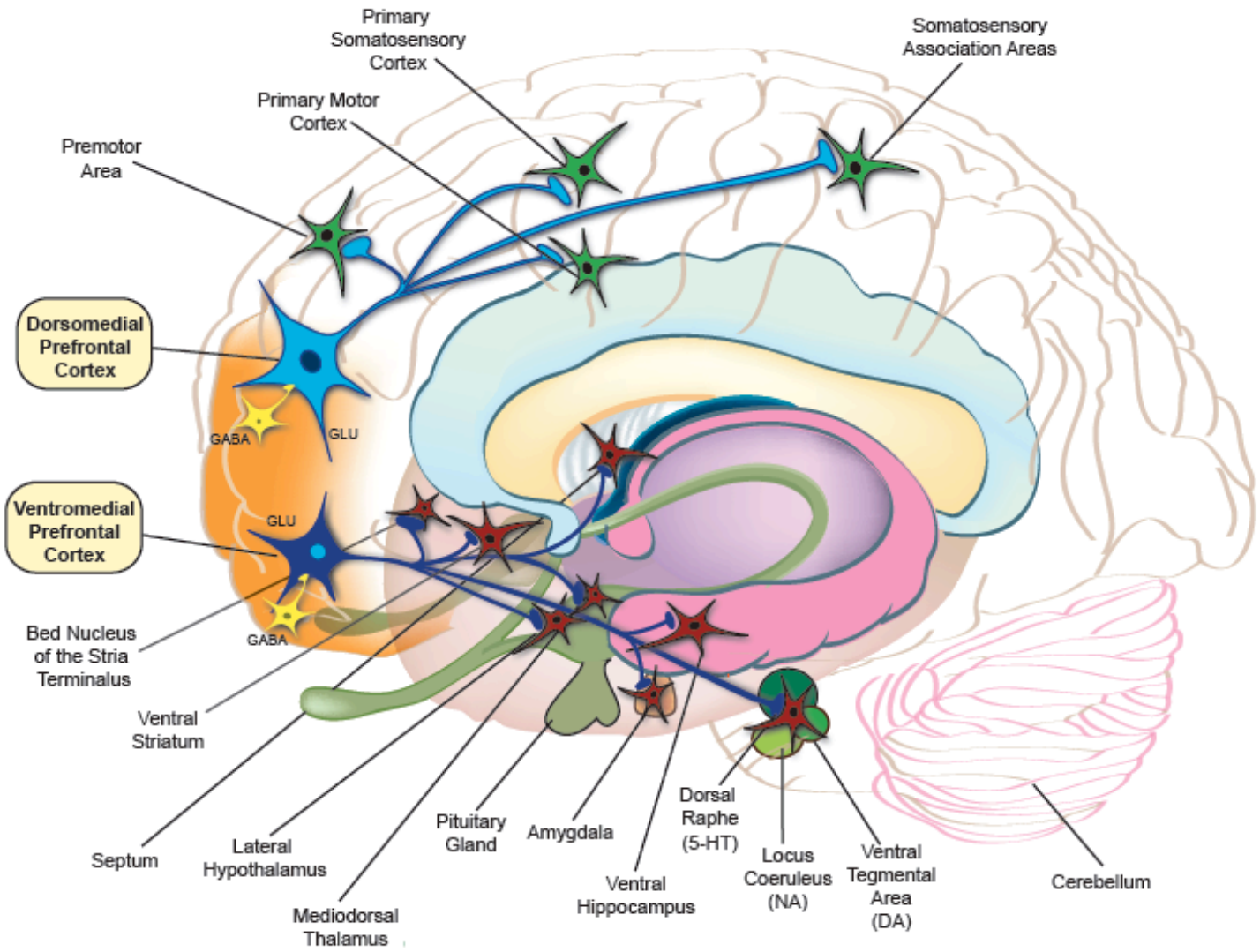
Similar to the primate PFC, the rodent PFC is also divided into three topographically distinct territories (Kolb, 1984; Brown and Bowman, 2002; Heidbreder and Groenewegen, 2003; Uylings et al., 2003; Dalley et al., 2004). First is the medial PFC, which can be further sub-divided into a dorsomedial region that includes precentral, aganular and anterior cingulate cortices, and a ventromedial region that encompasses the prelimbic, infralimbic, and medial orbital cortices (Heidbreder and Groenewegen, 2003). Second is a ventrally located region termed the orbital PFC that encompasses the ventral

and ventrolateral orbital cortices. Third is a lateral region of the PFC that includes the dorsal and ventral agranular insular cortices. The medial and orbital regions of the rodent PFC are structurally and functionally quite similar to corresponding regions in the primate PFC, but debate has surrounded whether rodents have a region homologous to the evolutionarily advanced primate dorsolateral PFC, due to significant cross-species variation in neural connectivity and cytoarchitectonic characteristics (Preuss, 1995). For instance, neurons in the medial dorsal nucleus of the thalamus lack projections to dorsolateral regions of the rodent PFC, which is a defining feature of prefrontal classification (Uylings et al., 2003). However, imaging, lesion, and electrophysiology studies have demonstrated that executive functioning and emotional learning tasks similar to those mediated by the primate dorsolateral PFC are carried out by distinct subregions of the medial PFC, and to a lesser extent, the orbital PFC in rodents (Uylings et al., 2003; Dalley et al., 2004; Seamans et al., 2008).

Each division of the medial PFC receives a unique set of afferent projections. There is a dorsoventral shift along the medial PFC, such that connections with the dorsomedial PFC (specifically the agranular and anterior cingulate cortices) are predominantly with sensorimotor areas, while inputs to and from the ventromedial PFC (specifically the prelimbic and infralimbic cortices) are primarily limbic in nature (Hoover and Vertes, 2007). The dorsomedial PFC receives widespread afferent projections from areas of the cortex and associated thalamic nuclei representing all sensory modalities. This information is presumably integrated at, and utilized by, the dorsomedial PFC in goal-directed actions (Hoover and Vertes, 2007). In contrast, the ventromedial PFC shares strong reciprocal connections with subcortical limbic brain

structures including the amygdala, ventral hippocampus, lateral hypothalamus, septum, thalamus, striatum, and the bed nucleus of the stria terminalis (BNST) (Heidbreder and Groenewegen, 2003; Ishikawa and Nakamura, 2003; Vertes, 2006; Drevets et al., 2008a). Moreover, the ventromedial PFC also provides forebrain modulation over visceral control centers in the brainstem, including cholinergic neurons originating from the basal forebrain (Gaykema et al., 1991), NA neurons from the locus coeruleus (Jodo and Aston-Jones, 1997; Jodo et al., 1998), DA neurons emanating from the ventral tegmental area (VTA) and sustantia nigra (Loughlin and Fallon, 1984; Carr and Sesack, 2000a, b), and 5-hydroxytryptamine (serotonin; 5-HT) neurons projecting from the dorsal and median raphe nucleus (Hajos et al., 1998). Hence, the ventromedial PFC (encompassing the prelimbic and infralimbic cortices) is ideally situated to modulate the output of limbic and monoaminergic neuronal networks that have long been implicated in the etiology and treatment of mood disorders, and as such, will be the primary focus of this review (see Figure 1.1 for a simplified diagram illustrating the primary downstream projection sites from the dorsomedial and ventromedial PFC).

Figure 1.1. Notable downstream projections from the dorsomedial and ventromedial prefrontal cortex.



The medial prefrontal cortex (PFC) shares reciprocal connections with various cortical and subcortical structures, however, the specific targets of these projections vary widely according to the anatomical site of origin. In general, the dorsomedial PFC shares connections with other cortical sensorimotor areas such as the primary motor and somatosensory cortices (depicted as green neurons). This information is integrated within the dorsomedial PFC and coordinates goal-directed actions. In contrast, the ventromedial PFC shares reciprocal projections with subcortical limbic structures known to be involved in the regulation of stress and emotionality. These include the amygdala, ventral hippocampus, lateral hypothalamus, septum, thalamus, striatum, bed nucleus of the stria terminalis, and midbrain monoaminergic cell bodies such as the serotonin-producing dorsal raphe, the dopamine-producing ventral tegmental area, and the noradrenaline-producing locus coeruleus (depicted as red neurons). Hence, the ventromedial PFC (encompassing the prelimbic and infralimbic cortices) is ideally situated to modulate the output of the limbic system and monoaminergic neuronal networks that have long been implicated in the etiology and treatment of mood disorders.

1.4.2. Medial Prefrontal Cortex and Acute Stress

The medial PFC is a vital part of a distributed extrahypothalamic network that modulates activation and feedback inhibition of the HPA axis. Convergent evidence from both human and rodent studies demonstrates that subregions of the medial PFC differentially modulate the behavioral and systemic response to psychological stress (Holmes and Wellman, 2009). Acute exposure to stressful stimuli induces robust activation of the immediate early gene c-*fos* (a marker of neuronal activation) and enhanced glucose mobilization in all subdivisions of the medial PFC (Duncan et al., 1993; Cullinan et al., 1995); however, lesion studies have revealed markedly different roles for the dorsomedial and ventromedial subregions in regulating HPA axis activation. For instance, bilateral lesions to the dorsomedial PFC centered in the anterior cingulate and prelimbic cortices have been shown to enhance ACTH and corticosterone secretion as well as c-*fos* activation and CRH mRNA expression in the PVN following restraint stress (Diorio et al., 1993; Figueiredo et al., 2003; Radley et al., 2006a). In contrast, lesions to the ventromedial PFC, centered in the infralimbic cortex, produce an opposite effect, suppressing the activation of CRH-secreting PVN neurons and improving HPA axis recovery (Radley et al., 2006a). Thus, the dorsally located prelimbic region of the medial PFC serves to suppress the HPA axis response to acute psychological stress, while the ventrally located infralimbic cortex serves to activate autonomic PVN outputs and promote stress-induced activation of the HPA axis.

The prelimbic and infralimbic cortex do not innervate the PVN directly, but instead relay through various subcortical intermediaries to modulate HPA responsivity. The infralimbic cortex sends direct projections to the lateral septum, anteroventral region

of the BNST, the medial, basomedial, and central amygdala, as well as the nucleus of the solitary tract, all regions that have been implicated in activation of the HPA axis (Hurley et al., 1991; Herman et al., 2003; Vertes, 2004). Conversely, the prelimbic cortex projects sparingly to these regions. Instead, this subregion heavily innervates several inhibitory stress-integrative structures, including the GABAergic peri-PVN zone that surrounds the PVN, the paraventricular thalamus, anterior and dorsomedial regions of the BNST, ventral subiculum, and basolateral amygdala (BLA) (Hurley et al., 1991; Vertes, 2004; Jankord and Herman, 2008; Radley et al., 2009).

Anatomical tracing experiments have further revealed that projection neurons in the prelimbic cortex send excitatory input to γ-aminobutyric acid (GABA) cell groups in the anterior BNST that exert an inhibitory influence over the PVN (Radley et al., 2009). Moreover, these researchers have recently extended this finding to show that extrinsic projections from the hippocampal ventral subiculum converge with these prelimbic projections onto a common relay in the anterior BNST, and synergize to potently inhibit the HPA axis in an additive fashion (Radley and Sawchenko, 2011). Chronic stress-induced dysfunction and glucocorticoid-mediated excitotoxicity within the medial PFC and/or the hippocampus is known to contribute to HPA axis hyperactivity and can increase vulnerability to stress-related illnesses later in life (McEwen, 2006). Therefore, characterizing alterations in the neural mechanisms that mediate PFC- and hippocampal-mediated inhibitory feedback processes is of paramount importance to understanding the underlying pathology of stress-related illnesses.

1.4.3. Medial Prefrontal Cortex, Stress, and Major Depression

Dysfunction within the PFC and the interrelated circuitry that connects the medial PFC to other cortical and limbic structures can account for the disturbances in emotional behavior, cognitive performance, neurotransmission, autonomic regulation, and neuroendocrine responses that are associated with stress-related affective disorders (Drevets et al., 2008a). Indeed, a number of structural and functional abnormalities arise in the PFC following chronic exposure to stress and/or glucocorticoids that may contribute to the development of depressive-like symptoms. For instance, in rodents, chronic exposure to stress or repeated corticosterone administration causes dramatic, albeit reversible, retraction of superficial apical dendrites in prefrontal pyramidal neurons that is accompanied by decreased spine density in the medial PFC (Wellman, 2001; Cook and Wellman, 2004; Radley et al., 2005; Radley et al., 2006b; Michelsen et al., 2007; Radley et al., 2008). The susceptibility of apical dendrites to changes in the corticosteroid environment is worth noting, as reciprocal projections from limbic regions including the ventral hippocampus, lateral hypothalamus, striatum, and amygdaloid complex terminate in these superficial layers of the medial PFC, where they preferentially contact apical dendrites (Room et al., 1985; Takagishi and Chiba, 1991; Chiba, 2000; Gabbott et al., 2005). Furthermore, chronic stress or corticosterone administration induces significant atrophy in the PFC that is correlated with impairments in working memory, behavioral flexibility, and reappraisal, processes that are largely dependent on the integrity of this structure (Cerqueira et al., 2007b; Dias-Ferreira et al., 2009). Researchers have postulated that this deficit may arise from alterations in synaptic plasticity, as chronic stress also reduces the induction of long-term potentiation in prefrontal inputs from the

hippocampus (Cerqueira et al., 2007a). Chronic stress also significantly reduces prefrontal 5-HT and DA content (Mizoguchi et al., 2000; Mizoguchi et al., 2002), which persists for up to 3 months and is coupled with negative feedback resistance in the dexamethasone suppression test and a behaviorally depressed state (Mizoguchi et al., 2008). Accordingly, many conventional antidepressants restore extracellular concentrations of DA and 5-HT in the PFC (Tanda et al., 1994; Matsumoto et al., 1999), suggesting that normalization of monoaminergic signaling within this PFC circuit may be related to the stable remission of depressive symptomology.

Observations from clinical, neuropsychological, and neuroimaging studies support the notion that prefrontal dysfunction contributes to the development and maintenance of the depressive phenotype (Drevets et al., 1998a; Pizzagalli et al., 2004; Drevets et al., 2008b; Myers-Schulz and Koenigs, 2011). However, the ramifications of PFC abnormalities largely depend on the precise subregion implicated. For instance, individuals with bilateral dorsal PFC lesions have been shown to be especially susceptible to major depression, whereas those expressing bilateral ventromedial PFC lesions actually appear to exhibit enhanced resistance to depressive symptoms (Koenigs et al., 2008). This suggests that the dorsal and ventromedial PFC are both causally implicated in depression, but appear to differentially mediate resilience and vulnerability. Neuroimaging studies have further revealed that the ventrally located subgenual region of the PFC undergoes a substantial loss in gray matter volume in unipolar and bipolar depressed patients, coupled to a pronounced loss of glial cells and reduced glucose metabolism in this region (Drevets et al., 1997; Drevets et al., 1998a; Drevets et al., 1998b; Ongur et al., 1998; Botteron et al., 2002; Drevets et al., 2008b). These findings

have been extended to show that during a tryptophan or catecholamine depletion-induced depressive episode, metabolic activity is elevated in this region compared to levels observed during remission (Neumeister et al., 2004; Hasler et al., 2008). Notably, various clinical interventions including pharmacological antidepressants (Mayberg et al., 2000; Drevets et al., 2002; Holthoff et al., 2004), electroconvulsive shock treatment (Nobler et al., 2001), and deep brain stimulation (Mayberg et al., 2005) have all been associated with altered subgenual cortical activity that coincides with symptom improvement.

The subgenual region of the PFC in humans appears to be functionally and structurally homologous to the ventromedial region of the PFC in rodents (Takagishi and Chiba, 1991; Ongur et al., 2003) and interestingly, Hamani and colleagues have demonstrated that deep brain stimulation of the ventromedial PFC in rats also promotes anxiolysis as well as a robust antidepressant-like response that is dependent on the integrity of the 5-HT system, thus providing a parallel to the clinical findings (Hamani et al., 2010a; Hamani et al., 2010b). Similarly, this group has recently shown that deep brain stimulation is also capable of reversing chronic stress-induced deficits in sucrose preference and hippocampal BDNF, but only in rats not receiving 5-HT-depleting lesions within the dorsal raphe (Hamani et al., 2012). Moreover, electrical stimulation of the medial (but not lateral) PFC in rats produces substantial increases in limbic 5-HT output that may contribute to the rapidly induced antidepressant effects of electroconvulsive therapy and deep brain stimulation (Juckel et al., 1999). Thus, the ventromedial PFC is an especially appealing target for novel antidepressant treatment strategies and represents a vital site of convergence for preclinical research on stress and emotionality and clinical research on major depression in patient populations.

1.5. The Endocannabinoid System

For centuries, extracts of the *Cannabis sativa* plant have been used for their therapeutic and mood-enhancing properties. The discovery and characterization of Δ^9-tetrahydrocannabinol (THC), the primary psychoactive constituent of marijuana in 1964, along with the synthesis of biologically active analogs (collectively termed 'cannabinoids') has served as the foundation for a relatively new field of research focused on understanding the pharmacological and biochemical properties of these compounds and how they produce their physiological and behavioral effects. Significant progress was made in the early 1990's with the identification and cloning of the cannabinoid CB_1 receptor in rodent brain tissue (Howlett et al., 1990; Matsuda et al., 1990), and the later characterization of the CB_2 receptor in spleen macrophages (Munro et al., 1993).

Both cannabinoid receptors are inhibitory G-protein coupled receptors (GPCRs) with the CB_1 receptor coupling to intracellular $G_{i/o}$ proteins, while CB_2 receptors couple exclusively to G_i proteins (Howlett, 2002). CB_1 receptors represent the most abundant class of GPCRs in the brain, as neuroanatomical studies have confirmed widespread expression throughout the forebrain, basal ganglia, and limbic system, suggestive of a ubiquitous neuromodulatory role for this receptor subtype in both humans (Glass et al., 1997; Mato and Pazos, 2004) and rodents (Herkenham et al., 1990; Herkenham et al., 1991). In rodents, intense CB_1 receptor staining has been observed in the neocortex, hippocampus, striatum, substantia nigra, and the cerebellum, while moderate CB_1 receptor immunoreactivity has been detected in the cingulate, entorhinal and piriform cortical areas, olfactory bulbs, amygdala, and nucleus accumbens (Herkenham et al.,

1990). CB_1 receptor density is much lower in the thalamus, hypothalamus, area postrema, and the midbrain, and essentially null in the medulla (Herkenham et al., 1990; Tsou et al., 1998).

Activation of CB_1 receptors inhibits adenylyl cyclase (AC) activity leading to a subsequent reduction in the cyclic adenosine monophosphate (cAMP) cascade, augmentation of inward-rectifying potassium channels, and inhibition of subsequent calcium influx via voltage-gated calcium channels (Howlett, 1995). Furthermore, CB_1 receptors are located on presynaptic axon terminals of glutamatergic principal neurons as well as on a subpopulation of non-calbindin and cholecystokinin-positive GABAergic basket cells (Hajos and Freund, 2002; Freund et al., 2003; Katona et al., 2006). Thus, CB_1 receptors are ideally positioned to modulate the balance of excitation and inhibition within a given neural circuit.

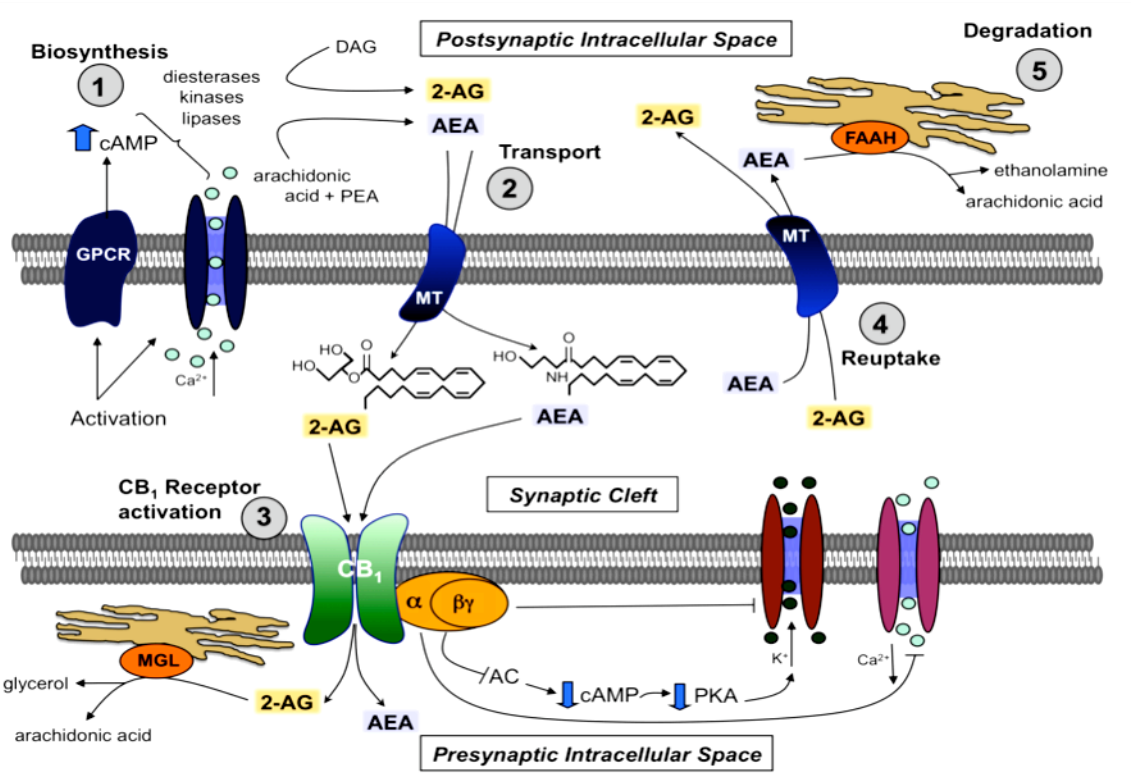
Further investigation into cannabinoid receptor pharmacology paved the way for the discovery and characterization of naturally occurring endogenous ligands. These are the arachidonate-derived lipophilic molecules N-arachidonylethanolamide (anandamide; AEA) and 2-arachidonylglycerol (2-AG) (Devane et al., 1992; Sugiura et al., 1995). Several other putative endocannabinoids have been isolated; however, greater attention has been given to AEA and 2-AG because of their potent agonistic activity at the CB_1 receptor. Both AEA and 2-AG are formed postsynaptically by activity-dependent cleavage of phospholipid head groups via activation of specific enzymes. The biosynthesis of 2-AG is mediated by the conversion of phosphatidylinositol by phospholipase C into diacylglycerol, which is subsequently converted to 2-AG via diacylglycerol lipase (DGL) (Hillard, 2000; Sugiura et al., 2002). The pathways

mediating AEA synthesis are not quite as clear. To date, three distinct and independent mechanisms have been shown to synthesize AEA, but the original described pathway involves a two-step process that begins with the conversion of phospholipid precursors to N-acyl phosphatidylethanolamine (NAPE) via a calcium-dependent transacylase. Phospholipase D then hydrolyzes NAPE to produce N-acyl ethanolamines, including AEA (Ahn et al., 2008; Bisogno, 2008).

Endocannabinoids are unlike traditional neurotransmitters in that they are not stored in vesicles but are instead synthesized on demand in postsynaptic cells following postsynaptic membrane depolarization. They are then released into the synapse where they travel in a retrograde manner to activate CB_1 receptors located on the presynaptic membrane, hyperpolarizing it and thereby reducing postsynaptic currents and depressing subsequent neurotransmitter release (Di Marzo et al., 1999). Termination of AEA and 2-AG signaling begins with transport across the plasma membrane followed by enzymatic hydrolysis into arachidonic acid and ethanolamine or glycerol, respectively (Ahn et al., 2008). This is accomplished via their respective hydrolytic enzymes; fatty acid amide hydrolase (FAAH) is the catabolic enzyme for AEA, while 2-AG is primarily metabolized by monoacylglycerol lipase (MGL) (Bisogno, 2008). The life cycle of these endocannabinoids is illustrated in Figure 1.2.

Independent studies have revealed that brain tissue concentrations of 2-AG are greater than AEA, although it should be noted that the magnitude of this difference varies considerably in the literature depending on the method of extraction. For instance, whole brain concentrations of 2-AG have been estimated to be as much as 1000-fold greater than concentrations of AEA in mass spectrometry analyses (Stella et al., 1997; Kathuria

Figure 1.2. A simplified schematic diagram illustrating the life cycle of the endocannabinoids anandamide and 2-arachidonylglycerol.



(1) Anandamide (AEA) and 2-arachidonylglycerol (2-AG) are synthesized on demand through the action of multiple diesterases, kinases and lipases. Stimulation of postsynaptic G protein-coupled receptors (GPCR) and Ca^{2+} channels increases adenylyl cyclase (AC) activity and cyclic adenosine monophosphate (cAMP) production, which facilitates the conversion of endocannabinoid precursors N-arachidonoylphosphatidylethanolamine (from arachidonic acid and phosphatidylethanolamine) and diacylglycerol (DAG) into AEA and 2-AG, respectively. Other putative biosynthetic pathways may also be involved (not shown). ***(2)*** AEA and 2-AG are transported into the synaptic cleft through non-vesicular trafficking that involves a yet uncharacterized membrane transporter (MT). ***(3)*** AEA and 2-AG bind to presynaptically located metabotropic CB_1 receptors (CB_1). CB_1 receptor activation hyperpolarizes the presynaptic membrane by activating inward rectifying K^+ channels, reducing protein kinase A (PKA) phosphorylation, and inhibiting L, N and P/Q-type Ca^{2+} channels (through the $G_{i/o}$ protein α subunit). ***(4)*** AEA and 2-AG are transported back into the cell via a yet uncharacterized MT. ***(5)*** In the cell, AEA and 2-AG are metabolized through different catabolic pathways. AEA is degraded in the post-synapse by fatty acid amide hydrolase (FAAH; located predominantly on the membrane surface of cytoplasmic organelles) into arachidonic acid and ethanolamine. 2-AG is degraded in the presynapse by monoacylglycerol lipase (MGL) into arachidonic acid and glycerol, although other post-synaptic 2-AG hydrolases may also participate in this process (not shown). ↑: stimulation, ⊤ or ↓: inhibition.

et al., 2003). However, this notion has recently been challenged by studies employing in *vivo* microdialysis, revealing baseline interstitial levels of both 2-AG and AEA to be similarly within the low nanomolar (nM) range (Buczynski and Parsons, 2010). This suggests that synaptic availability of 2-AG and AEA is evidently similar despite the considerable variation present in bulk tissue measurements. It is possible that intracellular accumulation of 2-AG may account for the discrepancy in endocannabinoid tissue content (Buczynski and Parsons, 2010). Alternatively, post-mortem accumulation of 2-AG is known to occur much more rapidly than AEA, with a five-fold increase in 2-AG being observed as early as 30 s post-decapitation (Sugiura et al., 2001; Patel et al., 2005a). This may help to explain why the 2-AG/AEA ratio measured in bulk tissue is much higher than in *vivo* microdialysates (Buczynski and Parsons, 2010). Nevertheless, additional research is necessary before quantitative measures of endocannabinoid tissue content can be accepted as accurate estimates of the 2-AG/AEA synaptic signaling pools.

It is not entirely clear why two endogenous ligands exist for the same receptor, but there are slight pharmacokinetic differences that may produce differential signaling patterns. For instance, AEA exhibits a high affinity for the CB_1 receptor (approximately 50-100 nM), but its efficacy at inducing intracellular signal transduction is somewhat poor, with only partial agonist properties (Hillard, 2000). In contrast, 2-AG has a lower affinity for the CB_1 receptor (approximately 1-10 μM) but induces a robust intracellular response (Hillard, 2000). Thus, 2-AG is thought to induce a rapid and robust CB_1 receptor response that is required for modulation of activity-induced synaptic plasticity, while AEA evokes more tonic, mild CB_1 receptor stimulation that may have greater implications for proper behavioral functions (Gorzalka et al., 2008).

1.6. Cannabinoids and Emotionality: Insights from Clinical Studies

Anecdotal reports from healthy cannabis smokers characterize its acute effects as consisting of relaxation, reduced anxiety, stress relief, euphoria, increased well-being and sociability, heightened sensory experience and imagination, distortion of time perception, and feelings of depersonalization (Hollister and Overall, 1975; Bambico and Gobbi, 2008). In recent years, there has been a growing interest in the therapeutic potential of cannabinoids for mood disorders. This interest has been inspired by several reports documenting their capacity to improve mood in healthy individuals and mitigate symptoms of major depression in patients being treated for other illnesses such as multiple sclerosis and human immunodeficiency virus (Consroe et al., 1997; Page et al., 2003; Ellis et al., 2009). Those who have reported self-medicating with marijuana or other synthetic cannabinoids have generally reported antidepressant and anxiolytic effects. For instance, over 95% of patients surveyed in the United Kingdom believed to have obtained various medicinal benefits from cannabis (Ware et al., 2005), and European surveys have revealed that depression is among the most frequently cited reasons for its use (Schnelle et al., 1999). Furthermore, a recent controlled trial examining the effects of cannabis consumption on chronic neuropathic pain revealed significant improvements in measures of anxiety and depression, in addition to reduced pain and better quality of sleep (Ware et al., 2010). Among multiple sclerosis patients, mood dysfunction, depression, anxiety, appetite loss, pain, stress, and sleep disturbance were the symptoms most often reported to be relieved by cannabis consumption (Consroe et al., 1997; Page et al., 2003; Amtmann et al., 2004; Clark et al., 2004). These effects are attributed to activation of the CB_1 receptor, as co-administration of CB_1 receptor

antagonists has been shown to abrogate acute subjective and physiological components of cannabinoid-induced mood alterations (Huestis et al., 2001; Huestis et al., 2007).

Despite these encouraging findings, no large-scale double-blind study to date has directly tested the anxiolytic and antidepressant effects of cannabis or cannabis-derived drugs on patients suffering from mood disorders. This may be due to the diversity of experiences associated with cannabis intoxication, which are dependent on many factors including baseline emotional states, genetic background, personality and expectations of the user, environmental setting, and the dose of the drug ingested (Bambico and Gobbi, 2008). For example, in contrast to low dose cannabinoid administration, high dose cannabinoid exposure has been shown to elicit anxiety, panic, and psychotomimetic effects (Curran et al., 2002). Therefore, the variable and complex effects of direct cannabinoid agonists on emotional states may present an unstable therapeutic window that is not particularly conducive to the treatment of mood disorders.

Another major obstacle in the implementation of cannabis-derived drugs for mood disorders is that long-term heavy cannabis use, particularly during adolescence, is associated with increased risk of contracting depressive-like symptoms that persist into adulthood (Bovasso, 2001; Patton et al., 2002). A constellation of maladaptive behaviors including diminished drive and ambition, increased apathy, dysphoria, decreased ability to carry out long-term plans, and a difficulty dealing with frustration – collectively referred to as amotivational syndrome – is thought to be induced by long-term cannabis abuse (Campbell, 1976). Notably, these component symptoms overlap with those of major depression, suggesting that chronic adolescent cannabis use could have a detrimental impact on the development of mood, motivation, and reward processing

pathways in the brain (Campbell, 1976; Musty and Kaback, 1995). Recent preclinical research supports this notion, as chronic CB_1 receptor agonist administration in adolescent rats promotes depressogenic responding along with dysregulated monoaminergic neural firing that persists into adulthood (Bambico et al., 2010b). Given the detrimental effects of chronic cannabinoid exposure during this developmental phase, it is conceivable to speculate that the pathogenesis of major depression could partially be due to alterations in the endocannabinoid system.

1.7. Effects of Endocannabinoid Manipulations on Emotional Behavior

1.7.1. CB_1 Receptor Disruption

The generation of transgenic mice lacking the CB_1 receptor has offered tremendous insight into the role of this signaling system in the regulation of emotional states. CB_1 receptor knockout mice exhibit increased depressive-like passive coping responses (i.e., immobility) in the forced swim test (FST) and tail suspension test (Aso et al., 2008; Steiner et al., 2008c), two commonly implemented paradigms used to assess the antidepressant potential of novel pharmacotherapeutic compounds. Similarly, these mice are particularly susceptible to the anhedonic effects of chronic stress (Martin et al., 2002), and exhibit reduced responsiveness to rewarding stimuli such as sucrose and ethanol (Poncelet et al., 2003; Sanchis-Segura et al., 2004), in addition to reductions in food intake and weight gain (Cota et al., 2003). CB_1 receptor-deficient mice also display an increase in anxiogenic traits in tests such as the light-dark box (Martin et al., 2002), elevated plus maze (Haller et al., 2002; Haller et al., 2004a; Haller et al., 2004b), and social interaction test (Martin et al., 2002), and exhibit strongly impaired short-term and long-term extinction in auditory fear-conditioning tests (Marsicano et al., 2002).

CB_1 receptor-deficient mice also exhibit physiological disturbances that are commonly associated with emotional dysfunction. For instance, 5-HT negative feedback is severely impaired in these mice. CB_1 receptor-deficient mice also display increased basal 5-HT extracellular levels and attenuated fluoxetine-induced promotion of 5-HT extracellular levels in the PFC (Aso et al., 2009). These mice also exhibit a reduction in the binding site density of the 5-HT transporter in the PFC and hippocampus, functional desensitization of 5-HT_{1A} autoreceptors in the dorsal raphe, and downregulation of 5-HT_{2C} receptor expression in the dorsal raphe, nucleus accumbens, and hypothalamic PVN (Aso et al., 2009). Increases in both basal and stress-induced activity of the HPA axis have also been documented in the form of pronounced CRH, ACTH, and corticosterone release (Cota et al., 2003; Barna et al., 2004; Haller et al., 2004a). Lastly, these mice exhibit impaired hippocampal neurogenesis (Jin et al., 2004) and reduced release of brain-derived neurotrophic factor (BDNF) in response to neurotoxic insults (Khaspekov et al., 2004).

Genetic deletion of the CB_1 receptor also reduces the dendritic complexity of medial PFC pyramidal neurons and promotes expansion of dendrites in amygdala pyramidal neurons, coinciding with increased anxiety-like behavior (Hill et al., 2011a). These morphological alterations parallel volumetric changes that have been documented in clinical populations (Price and Drevets, 2010) and are similar to the changes produced by exposure to chronic stress in rodents (McLaughlin et al., 2009). Given the hierarchical control the medial PFC exerts over the amygdala, compromised CB_1 receptor signaling may be a consequence of alterations in structural morphology within these regions, resulting in an amygdalocentric form of information processing which favors an

increased salience to aversive environmental cues and the subsequent development of pathological mood states (Hill et al., 2011a).

Collectively, these data demonstrate that CB_1 receptor-deficient mice exhibit increased anxiety, behavioral despair, anhedonia, disrupted sleep/feeding cycles, perseveration of aversive memories, deficient 5-HT feedback, HPA axis hyperactivation, reduced hippocampal neurogenesis, and altered dendritic branching in the medial PFC and amygdala. Thus, CB_1 receptor deletion promotes a phenotype that is strikingly reminiscent of the symptomatic profile of melancholic depression (Hill and Gorzalka, 2005a).

Although the similarities between CB_1 receptor-deficient mice and individuals suffering from major depression have been repeatedly documented, the effects of global pharmacological CB_1 receptor blockade in preclinical models of emotionality have been somewhat equivocal. For instance, chronic treatment with the CB_1 receptor antagonist rimonabant has been shown to mimic the effects of chronic stress, increasing immobility time in the FST and reducing consumption of sucrose-sweetened water, which is indicative of depressive-like responding and anhedonia, respectively (Beyer et al., 2010). Moreover, chronic rimonabant administration decreases 5-HT levels in the frontal cortex, reduces hippocampal cell proliferation, survival, and BDNF levels, and increases concentrations of pro-inflammatory cytokines (Beyer et al., 2010). However, CB_1 receptor antagonism has also been shown to exert antidepressant (Shearman et al., 2003; Griebel et al., 2005; Steiner et al., 2008a), anxiolytic (Griebel et al., 2005), anxiogenic (Navarro et al., 1997), or null effects (Adamczyk et al., 2008). These equivocal results

may be due to a number of factors, including differences in species, strain, dosing, testing conditions, or off-target effects of the agents employed (Hill and Gorzalka, 2009b).

1.7.2. Facilitation of Endocannabinoid Signaling

Transgenic mice lacking the AEA-degrading enzyme FAAH have proven to be an invaluable tool in which to ascertain the functional impact of enhanced endogenous cannabinoid signaling on emotional behavior. FAAH knockout mice are severely impaired in their ability to degrade AEA, exhibiting 10-15-fold higher concentrations of this endocannabinoid compared to wild-type mice (Cravatt et al., 2001), while retaining normal CB_1 receptor densities (Basavarajappa et al., 2006). In direct contrast to the behavioral phenotype of CB_1 receptor knockout mice, FAAH knockout mice display robust antidepressant-like responses in the FST and tail suspension test (Bambico et al., 2010a), anxiolytic responses in the open field test and light-dark box (Naidu et al., 2007; Moreira et al., 2008; Bambico et al., 2010a; Cassano et al., 2011), and enhanced aversive memory extinction (Varvel et al., 2007), all of which are abolished by pretreatment with a CB_1 receptor antagonist. Genetic deletion of FAAH has also been shown to promote social interaction (Cassano et al., 2011) and prevent the anxiogenic phenotype that develops following exposure to social defeat stress (Rossi et al., 2010).

Electrophysiological recordings have further revealed a marked increase in the firing rate of dorsal raphe 5-HT neurons, desensitization of prefrontocortical 5-$HT_{2A/2C}$ receptors, and enhancement of hippocampal 5-HT_{1A} receptor activity, which are all hallmarks of conventional antidepressant activity (Bambico et al., 2010a). Microdialysis reports have corroborated this facilitation of 5-HT transmission, demonstrating enhanced basal prefrontal 5-HT release and augmented depolarization-induced 5-HT release in

both the PFC and ventral hippocampus (Cassano et al., 2011). FAAH-deficient mice also exhibit pronounced proliferation of hippocampal neural progenitor cells, suggesting that facilitation of AEA tone may have pro-neurogenic effects (Aguado et al., 2005). Together, these data argue that genetic deletion of the enzyme responsible for the degradation of AEA (thereby promoting increased tonic endocannabinoid activity) elicits a phenotype that is in stark contrast to mice lacking the CB_1 receptor. Moreover, these mice exhibit antidepressant and anxiolytic responses in preclinical paradigms, coupled to alterations in 5-HT transmission and hippocampal cell proliferation that are characteristic of conventional somatic and pharmacotherapeutic interventions.

Preclinical research implementing pharmacological inhibitors of FAAH have provided extensive support for the notion that alterations in AEA signaling may be functionally implicated in the pathophysiology and treatment of mood disorders. As expected from studies using FAAH knockout mice, systemic administration of the FAAH inhibitor URB597 has been shown to elicit antidepressant and anxiolytic effects and enhance aversive (but not appetitive) memory extinction in rodents (Kathuria et al., 2003; Gobbi et al., 2005; Patel and Hillard, 2006; Hill et al., 2007b; Naidu et al., 2007; Varvel et al., 2007; Moreira et al., 2008; Scherma et al., 2008; Manwell et al., 2009). Additionally, systemic or intracerebroventricular administration of URB597 confers resilience to the anxiogenic phenotype induced by social defeat stress (Rossi et al., 2010). Recent studies employing intracerebral microinjection techniques have further demonstrated a role for FAAH/AEA signaling in the PFC, as local administration of URB597 into the ventromedial PFC has also been shown to promote anxiolytic responses at low doses (Rubino et al., 2008b). Conversely, lentivirus-mediated overexpression of

FAAH locally within the ventromedial PFC has been shown to elicit an anxiogenic profile (Rubino et al., 2008b). These convergent findings have been extended to show that long-term inhibition of AEA hydrolysis reverses the weight gain and development of anhedonia following chronic stress exposure in a manner comparable to the antidepressant imipramine (Bortolato et al., 2007). This antidepressant-like response also occurs in tandem with enhanced AEA signaling in the midbrain, striatum, and thalamus (Bortolato et al., 2007). Furthermore, both acute and chronic URB597 administration induces a CB_1 receptor-dependent enhancement of 5-HT and NA firing from the dorsal raphe and locus coeruleus, respectively (Gobbi et al., 2005), and increases both tonic activity of $5\text{-}HT_{1A}$ hippocampal heteroreceptors (Bambico et al., 2010a) and hippocampal cell proliferation (Aguado et al., 2005). Therefore, pharmacological inhibition of FAAH mirrors the effects of conventional antidepressant treatments in preclinical animal models, suggesting that AEA/CB_1 signaling may represent a viable target for the discovery of novel, more efficacious antidepressants.

1.8. The Endocannabinoid System and Regulation of Stress

1.8.1. Endocannabinoid Signaling and the Acute Stress Response

Multiple lines of evidence suggest that endocannabinoid signaling in the brain regulates neuroendocrine and behavioral responses to psychological stress, while genetic or pharmacological blockade of the CB_1 receptor profoundly disrupts these processes. For instance, transgenic mice lacking the CB_1 receptor express increased CRH mRNA in the PVN, decreased glucocorticoid receptor mRNA in the CA1 region of the hippocampus, elevated corticosterone concentrations at the onset of the active cycle, and exaggerated ACTH secretion in response to CRH or forskolin challenge (Cota et al., 2007). CB_1 receptor knockout mice also exhibit enhanced ACTH and corticosterone

secretion in response to acute restraint stress (Barna et al., 2004; Haller et al., 2004a; Aso et al., 2008; Steiner et al., 2008a; Steiner and Wotjak, 2008). Accordingly, acute CB_1 receptor blockade also increases basal and stress-induced levels of circulating corticosterone in rodents, while systemic activation of CB_1 receptors or inhibition of endocannabinoid uptake or metabolism suppresses HPA axis activation at low doses (Barna et al., 2004; Patel et al., 2004; Wade et al., 2006; Steiner et al., 2008a; Steiner and Wotjak, 2008).

Endocannabinoid signaling is now known to be tightly regulated by glucocorticoid hormones. In the hypothalamus, CB_1 receptors are present on glutamatergic neurons that serve to activate CRH neurosecretory cells at the level of the PVN, and as such, are ideally positioned to gate excitatory activity of the HPA axis (Di et al., 2003). Furthermore, the rapid behavioral responses induced by glucocorticoid administration in *vivo* are blocked by administration of CB_1 receptor antagonists (Coddington et al., 2007; Campolongo et al., 2009). Glucocorticoids have also been shown to rapidly stimulate synthesis of both AEA and 2-AG in the PVN via a $G\alpha_s$–cAMP-protein kinase A (PKA)-dependent pathway (Malcher-Lopes et al., 2006), while local bilateral injection of glucocorticoids into the PVN results in suppression of stress-induced HPA axis activation in a CB_1 receptor-dependent manner via a putative membrane-bound glucocorticoid receptor (Evanson et al., 2010). Interestingly, repeated immobilization stress in juvenile rats that do not exhibit proper stress habituation causes a glucocorticoid-dependent functional downregulation of CB_1 receptors in the PVN that impairs both activity and receptor-dependent endocannabinoid signaling at glutamatergic synapses in this region (Wamsteeker et al., 2010). These studies collectively suggest that

the rapid glucocorticoid-mediated suppression of HPA axis activity in the hypothalamus is mediated by synthesis of endocannabinoids and activation of CB_1 receptors on glutamatergic synapses in PVN (see Tasker and Herman, 2011 for review).

An extensive extrahypothalamic network of inhibitory and excitatory inputs also potently modulates HPA axis activity, including stimulatory inputs from the amygdala and inhibitory inputs from the hippocampus (Jankord and Herman, 2008). In the amygdala, acute exposure to restraint stress has been shown to enhance FAAH-mediated hydrolysis of AEA, resulting in a suppression of tonic AEA/CB_1 receptor signaling and a facilitation of HPA axis activation (Hill et al., 2009b). Accordingly, acute systemic administration of corticosterone has been shown to increase tissue content of AEA in the amygdala at 10 min, but not 1 hr post-administration (Hill et al., 2010a). In the hippocampus, a similar enhancement of AEA content has been demonstrated immediately following corticosterone administration (Hill et al., 2010a), however, a reduction in hippocampal AEA content has been observed 18 hr following corticosterone exposure (Hill et al., 2008a), suggesting that the acute effects of systemic glucocorticoid administration on AEA signaling are region-specific and time-dependent. Moreover, acute stress has recently been shown to produce a delayed increase in 2-AG content in the hippocampus at 30 min post-stress exposure, coupled to an enhanced modulation of GABA release as measured by whole-cell voltage clamp of inhibitory postsynaptic currents in hippocampal CA1 pyramidal cells (Wang et al., 2011). This endocannabinoid-mediated depolarization-induced suppression of inhibition (DSI) was further shown to be glucocorticoid receptor dependent and mimicked by both in *vitro* and in *vivo* corticosterone treatment (Wang et al., 2011).

Collectively, these data suggest that under basal steady-state conditions, endocannabinoid signaling tonically regulates hypothalamic and extrahypothalamic (amygdalar and hippocampal) activation, thus constraining the persistent drive of the HPA axis. Upon exposure to stress, AEA content experiences a decline in these stress-responsive brain regions, likely via an enhancement of enzymatic hydrolysis, thus allowing for the cascade of neuroendocrine and behavioral responses induced by stress. If AEA levels are maintained prior to stress induction, this steady-state inhibition is preserved and HPA axis activation is attenuated, but if endocannabinoid signaling is disrupted, HPA axis activation and neuroendocrine output becomes potentiated. A delayed glucocorticoid-dependent increase in 2-AG mobilization occurs next, which functions to modulate synaptic strength and participates in feedback inhibition of the HPA axis, thereby promoting recovery to homeostasis. This reciprocal crosstalk between endocannabinoids and the HPA axis is particularly relevant for understanding the pathogenesis of major depression, especially given the regulatory role for endocannabinoid signaling in constraining HPA axis hyperactivation. Over time, prolonged exposure to stress downregulates AEA/CB_1 receptor signaling, allowing for exaggerated HPA axis responses, hypersecretion of glucocorticoids, and maladaptive stress coping responses, which are all hallmark symptoms of melancholic depression.

1.8.2. Endocannabinoid Signaling and Stress Adaptation

Chronic exposure to homotypic stress typically involves some form of habituation, which serves to dampen the detrimental effects of a prolonged neurochemical stress response and increases the psychological tolerability of the stressor. Such adaptation is fundamentally important to the survival of an organism. Accumulating

evidence suggests that the endocannabinoid system is integrally involved in this process. In mice, repeated exposure to a homotypic stressor such as restraint stress produces an increase in 2-AG content within the limbic forebrain, amygdala, hippocampus, and hypothalamus, and a decrease in 2-AG content in the ventral striatum (Patel et al., 2005b; Rademacher et al., 2008). AEA on the other hand, undergoes a progressive reduction in the amygdala with a concurrent increase in the ventral striatum that is coupled to corresponding changes in FAAH in these same regions (Rademacher et al., 2008). These alterations in endocannabinoid ligand content are important for stress habituation processes, as acute CB_1 receptor blockade during the fifth restraint exposure session reinstates active escape behaviors that had previously undergone habituation in this paradigm (Patel et al., 2005b).

Our laboratory has shown that in response to repeated restraint stress, AEA content is downregulated throughout the corticolimbic stress circuit (hypothalamus, amygdala, PFC, and hippocampus), while 2-AG is exclusively elevated in the amygdala in a stress-dependent manner (Hill et al., 2010b). The decline in corticolimbic AEA signaling contributes to chronic stress-induced basal hypersecretion of corticosterone, while the increase in amygdalar 2-AG content participates in the habituation of the peak corticosterone response to repeated stress (Hill et al., 2010b). Whereas the decline in corticolimbic AEA signaling is persistent, the 2-AG response in the amygdala is temporally restricted, with elevations found within 20–30 min following the onset of stress, but dissipating within one hr after stress onset and reversing completely within 24 hr (Patel et al., 2009; Hill et al., 2010b). Based on these data, it has been suggested that repeated stress exposure results in an enhanced capacity to elevate amygdalar 2-AG

content upon subsequent stressor exposures, and this increase in 2-AG in turn mediates habituation to stress (Hill et al., 2010c).

This hypothesis is supported by studies on fear extinction. For instance, mice lacking CB_1 receptors do not show proper habituation to a fear-conditioned stimulus (e.g., tone) during trials where the tone is repeatedly presented in the absence of the shock (Marsicano et al., 2002; Fride et al., 2005; Kamprath et al., 2006). Moreover, during extinction training, but not initial fear conditioning, 2-AG levels in the amygdala are elevated in wild-type animals (Marsicano et al., 2002), again demonstrating the importance of amygdalar 2-AG signaling in proper adaptation to aversive stimuli.

Given the fact that endocannabinoid signaling in the BLA is known to gate glutamatergic inputs to principal neurons (Azad et al., 2003; Kodirov et al., 2010), it is likely that transient augmentation of 2-AG signaling following repeated stressor exposure dampens excitatory inputs to the BLA, thereby decreasing outflow of the amygdala, resulting in blunted HPA axis activation. This theory is corroborated by a recent report showing that corticosterone inhibits glutamatergic inputs to the BLA in an endocannabinoid-dependent fashion, but only in rodents with a previous history of stress exposure (Karst et al., 2010). In line with this finding, conditional mutants lacking CB_1 receptors specifically on cortical glutamatergic neurons do not express acute fear adaptation (Kamprath et al., 2009). Thus, CB_1 receptors regulating cortical glutamatergic inputs to BLA neurons mediate the expression of both neuroendocrine and behavioural responses to stress and fear, respectively (Hill et al., 2010c).

Endocannabinoid signaling at GABAergic synapses in the BLA may also participate in the neuroadaptations that occur in response to chronic stress. For example,

endocannabinoid-mediated synaptic suppression in the form of DSI is significantly enhanced in BLA neurons of rodents exposed to 10 days (but not one day) of restraint stress (Patel et al., 2009). Intriguingly, the anxiety-like phenotype induced by chronic stress occurs in tandem with enhanced 2-AG-mediated long-term depression at inhibitory GABAergic synapses (LTDi) in the BLA, which is mediated in part by functional downregulation of the 2-AG-degrading enzyme MGL (Sumislawski et al., 2011).

Endocannabinoid signaling at GABAergic synapses in the BLA may also facilitate aversive memory consolidation. Glucocorticoids are known to enhance long-term consolidation of emotionally arousing experiences via activation of intracellular signaling cascades in the BLA (Roozendaal, 2000; McGaugh, 2002). Evidence now suggests that glucocorticoids recruit endocannabinoid signaling in the BLA to modulate aversive memory consolidation. Post-training infusion of a CB_1 receptor agonist into the BLA is capable of enhancing inhibitory avoidance retention in a manner similar to that witnessed following post-training administration of glucocorticoids (Campolongo et al., 2009). Moreover, intra-BLA CB_1 blockade inhibits the ability of glucocorticoids to facilitate aversive memory consolidation (Campolongo et al., 2009). Previous research has demonstrated that inhibition of GABA activity in the BLA facilitates aversive memory consolidation by increasing the release of NA (Hatfield and McGaugh, 1999; Hatfield et al., 1999). Based on these and other findings, a hypothetical model has been proposed, whereby corticosterone binds to membrane-bound glucocorticoid receptors to rapidly induce the synthesis of 2-AG in the BLA. 2-AG is then released into the synapse where it binds to CB_1 receptors on GABAergic inhibitory neurons. Activation of CB_1 receptors leads to inhibition of GABA release, thereby disinhibiting NA release which

activates postsynaptic ß-adrenreceptors expressed on BLA pyramidal neurons, ultimately resulting in consolidation of the aversive memory (see Hill and McEwen, 2009 for description of this model).

As a whole, these data suggest that enhanced 2-AG/CB_1 receptor signaling modulates both excitatory and inhibitory synapses in the BLA in response to repeated stress, albeit with dissociable effects. Specifically, enhanced 2-AG signaling at glutamatergic synapses in the BLA promotes fear extinction (Marsicano et al., 2002) and dampens HPA axis activation in a corticosterone-dependent manner (Patel et al., 2009; Hill et al., 2010b), while enhanced 2-AG signaling at GABAergic synapses in the BLA induces LTDi, which coincides with the emergence of anxiety-like behavior (Sumislawski et al., 2011) and the consolidation of emotionally aversive memories (Campolongo et al., 2009). The net effect of changes in CB_1 receptor activation on both glutamatergic and GABAergic synapses evidently determines the magnitude of BLA activation and the resultant behavioral effect. Regardless, it is important to note that chronic systemic inhibition of MGL is capable of preventing the enhanced LTDi in the BLA and the emergence of anxiety following exposure to chronic stress (Sumislawski et al., 2011). Thus, inhibiting 2-AG degradation may preclude the development of anxiety and the synaptic adaptations that occur in response to chronic stress.

1.9. Endocannabinoid Signaling in Animal Models of Depression

Our current understanding of the pathophysiological development of major depression largely stems from research employing preclinical paradigms that model the depressive-like phenotype in rodents, as these paradigms are known to elicit a symptom profile that closely resembles that of major depression in human populations. The

chronic unpredictable stress (CUS) and olfactory bulbectomy (OBX) models of depression are two such paradigms that have been extensively implemented for this purpose. Studies utilizing chronic psychosocial stressors (e.g., social conflict and social isolation paradigms) have also offered considerable insight into the neurobiological underpinnings of major depression. In recent years, researchers have begun to examine neurobiological alterations in the endocannabinoid system that occur following exposure to these animal models with some rather intriguing results. The following section will review the biochemical and physiological changes in endocannabinoid signaling parameters induced by regimens that model the pathological development of depressive-like symptoms (see Table 1.1) and discuss their implications in the context of major depression.

Table 1.1. Alterations in endocannabinoid signaling following exposure to various animal models of depression

Animal Model	Brain Region	AEA	2-AG	CB_1R	Reversal by Antidepressants	Reference
CUS	Prefrontal Cortex	↓	↔	↑	CB_1 – Yes AEA – No	Hillard et al., 2006; Hill et al., 2008
	Hippocampus	↓	↓	↓♂ ↑♀	CB_1 ♂ – No AEA – No	Perez-Rial et al., 2004; Hill et al., 2005; Hill et al., 2008; Reich et al., 2009
	Hypothalamus	↓	↑	↓	CB_1 – Yes AEA, 2-AG – No	Hill et al., 2008
	Amygdala	↓	↔	↔	AEA – No	Hill et al., 2008
	Midbrain	↓	↑	↔	AEA, 2-AG – No	Hill et al., 2008
	Ventral Striatum	↓	↔	↓	CB_1 – Yes AEA – No	Hill et al., 2008; Wang et al., 2010
OBX	Prefrontal Cortex	n.d.	n.d.	↑	CB_1 – Yes	Rodríguez-Gaztelumendi et al., 2009
	Hippocampus	↔	↔	↔	--	Eisenstein et al., 2010
	Amygdala	↓	↔	↔	n.d.	Eisenstein et al., 2010
	Ventral Striatum	↓	↓	↔	n.d.	Eisenstein et al., 2010
SI	Prefrontal Cortex	↔	↑	↔	n.d.	Malone et al., 2008; Sciolino et al., 2010
	Hippocampus	↔	↔	↔	--	Malone et al., 2008; Sciolino et al., 2010
	Hypothalamus	↔	↔	↓	n.d.	Sciolino et al., 2010
	Thalamus	↔	↔	↓	n.d.	Sciolino et al., 2010
	Amygdala	↔	↔	↓	n.d.	Malone et al., 2008; Sciolino et al., 2010
	Ventral Striatum	↔	↔	↑, ↓	n.d.	Malone et al., 2008; Sciolino et al., 2010
	Caudate Putamen	↔	↔	↑, ↓	n.d.	Malone et al., 2008; Sciolino et al., 2010
SD	Striatum	n.d.	n.d.	↓	CB_1 – Yes (via FAAH Inhibition)	Rossi et al., 2008; 2010

AEA: Anandamide, *2-AG*: 2-Arachidonylglycerol, *CB_1R*: CB_1 Receptor, *CUS*: Chronic Unpredictable Stress, *OBX*: Olfactory Bulbectomy, *SI*: Social Isolation, *SD*: Social Defeat, ↑:Increase, ↓:Decrease, ↔: No Change, n.d.: Not determined, -- : Not applicable

1.9.1. Chronic Unpredictable Stress

The CUS model of depression is often regarded as one of the strongest animal models of melancholic depression, due to its relatively high levels of face validity (symptom profile), construct validity (theoretical rationale), and predictive validity (pharmacological profile) (Willner, 2005). CUS exposure induces hypersecretion of glucocorticoid stress hormones, accompanied by profound alterations in hedonic reactivity, reductions in body weight, decreased grooming behaviors, and notably, a lack of habituation to stress, all of which are ameliorated by chronic antidepressant treatments in a manner consistent with the time course observed in clinically depressed individuals (Willner, 2005).

With respect to endocannabinoid signaling, subjecting rodents to this stress regimen has been shown to induce a ubiquitous reduction in AEA content throughout the corticolimbic stress circuit, as well as a reduction in the maximal binding site density of CB_1 receptors in subcortical limbic structures such as the hippocampus, hypothalamus, and ventral striatum, while exerting an opposite pattern on CB_1 receptors in the PFC (Hill et al., 2005; Hillard et al., 2006; Hill et al., 2008b). Similarly, CUS exposure decreases CB_1 receptor mRNA transcription (Hillard et al., 2006), CB_1 receptor-mediated GTPγS signaling (Perez-Rial et al., 2004), and 2-AG content in the hippocampus (Hill et al., 2005), while increasing CB_1 receptor mRNA transcription in the PFC (Hillard et al., 2006). Thus, CB_1 receptor binding appears to be differentially altered in cortical and subcortical brain regions following CUS exposure. Given the fact that CB_1 receptor knockout mice exhibit a symptom profile that is reminiscent of the phenotype of CUS-exposed rodents and individuals with melancholic depression (Hill and Gorzalka, 2005a),

the most parsimonious explanation posits that the reduction in subcortical CB_1 receptor binding contributes to the development of stress-related pathologies. However, the functional relevance of the increase in CB_1 receptor binding in the PFC of CUS-exposed rodents is less obvious and requires further investigation.

Wang and colleagues have recently measured CB_1 receptor-mediated physiological responses in ventral striatum slices taken from animals exposed to CUS in an effort to capture the dynamic nature of endocannabinoid/CB_1 receptor signaling. In addition to the behavioral changes induced by CUS, this regimen caused persistent downregulation of endocannabinoid-mediated depolarization-induced suppression of excitation (DSE), long-term depression, and CB_1 receptor agonist-induced depression of field excitatory postsynaptic potentials in the nucleus accumbens core, which were reversed by chronic antidepressant administration (Wang et al., 2010). This suggests that CUS exposure induces both biochemical and physiological changes in endocannabinoid signaling within key components of the reward circuitry, which may account for anhedonia, lack of motivation, and other behavioral symptoms of major depression.

Interestingly, CUS-induced alterations in hedonic behavior, AEA content, and CB_1 receptor binding can be ameliorated by concurrently administering compounds that activate the CB_1 receptor or prevent the enzymatic hydrolysis of AEA (Bortolato et al., 2007; Rademacher and Hillard, 2007), thus highlighting the potential therapeutic utility of such compounds in clinically depressed populations. Also, treatment regimens that are effective for treating depression, such as pharmacotherapeutic administration of antidepressants (Hill et al., 2006; Hill et al., 2008b; Hill et al., 2008c), electroconvulsive shock treatment (Hill et al., 2007a), sleep deprivation (Chen and Bazan, 2005), and

voluntary exercise (Hill et al., 2010d) have all been found to increase endocannabinoid signaling in these same limbic structures in rodents. These data collectively suggest that corticolimbic endocannabinoid signaling is functionally compromised in a region-specific manner following CUS, while its restoration coincides with various antidepressant treatment strategies.

1.9.2. Olfactory Bulbectomy

The OBX model of depression involves bilateral removal of the olfactory bulbs, which produces behavioral, structural, and neurochemical changes that are akin to those observed in the clinical population (Song and Leonard, 2005). Furthermore, these alterations are normalized by chronic (but not acute) administration of antidepressants in a time frame that closely resembles the temporal dynamics of these compounds in the clinic (Song and Leonard, 2005). Mounting evidence suggests that endocannabinoids may be functionally implicated in the development of these depressogenic and anxiogenic effects. For instance, OBX produces significant increases in maximal CB_1 receptor binding and CB_1 receptor-mediated GTPγS signaling in the PFC in a manner similar to that observed following CUS exposure (Rodriguez-Gaztelumendi et al., 2009). Moreover, chronic antidepressant treatment prevents OBX-induced hyperactivity and the increase in CB_1 receptor functionality in the PFC (Rodriguez-Gaztelumendi et al., 2009).

OBX animals also exhibit reduced levels of AEA and 2-AG in the ventral striatum, although no significant changes in CB_1 receptor density were observed in this region (Eisenstein et al., 2010). Furthermore, 2-AG levels in OBX rats were shown to negatively correlate with distance traveled in the habituation phase of open field exposure, while CB_1 receptor blockade further increased the distance traveled during this

phase (Eisenstein et al., 2010). Thus, downregulation of endocannabinoid signaling in the ventral striatum may be implicated in the anxiogenic response induced by OBX.

1.9.3. Social Defeat

Repeated exposure to inter-male confrontations where defeat ensues has also been reliably shown to promote the development of anxiogenic and depressive-like neurobiological and behavioral symptoms in rodents (Avgustinovich et al., 2005). With respect to endocannabinoid signaling, Rossi and coworkers have demonstrated that chronic social defeat stress progressively alters CB_1 receptor-mediated control of GABAergic synaptic transmission in the striatum in a glucocorticoid receptor-dependent manner (Rossi et al., 2008). Moreover, recovery of these synaptic deficits was encouraged when stressed rats were given access to rewarding stimuli such as a running wheel, sucrose, or an injection of cocaine (Rossi et al., 2008). In a follow-up study, this group further revealed that genetic deletion of FAAH or repeated administration of a FAAH inhibitor prevented the anxious phenotype of mice exposed to social defeat stress in a CB_1 receptor-dependent fashion (Rossi et al., 2010). Remarkably, this effect was also associated with preserved activity of CB_1 receptors regulating GABA activity in the striatum (Rossi et al., 2010). Together, these findings argue that alterations in striatal endocannabinoid signaling induced by chronic psychosocial stress may have functional consequences that manifest as disturbances in motor, cognitive, and emotional function. Furthermore, inhibiting FAAH-mediated degradation of AEA prevents these disturbances by preserving CB_1 receptor-mediated GABA control in the striatum.

1.9.4. Social Isolation

Rearing rats in isolation post-weaning is an animal model of social deprivation that recapitulates many features of pathological mood disorders in humans. Social isolation produces long-term changes characteristic of emotional disorders including; anxiety, neophobia, cognitive rigidity, aggression, hypofunction of the mesocortical DA system, reduced PFC volume, compromised 5-HT function, and decreased cortical and hippocampal synaptic plasticity (Fone and Porkess, 2008). Immunohistochemical examinations have shown that isolation-reared rats display reduced CB_1 receptor expression in the caudate putamen and amygdala, along with increased FAAH expression in the caudate putamen and ventral striatum (Malone et al., 2008). However, recent biochemical analyses have shown a different pattern, demonstrating increased CB_1 receptor binding in the caudate putamen, dorsal and ventral striatum, hypothalamus, and thalamus, in addition to increased 2-AG levels in the PFC (Sciolino et al., 2010). It is interesting to note that the increase in PFC 2-AG content observed in isolation-reared rats mirrors the increase in 2-AG found in the PFC of rodents exposed to chronic restraint stress (Patel et al., 2005b). Similarly, Sciolino and coworkers were able to show increased PFC 2-AG content in handled vs. non-handled rats (Sciolino et al., 2010). Like repeated restraint, handling could also be considered an initially stressful stimulus that is subject to habituation and consequently loses its aversive quality over time. Thus, the increase in prefrontocortical 2-AG content could participate in stress habituation processes, which are conspicuously absent in CUS-exposed rodents and individuals afflicted with melancholic depression.

1.10. Endocannabinoid Signaling in Major Depression

Accumulating evidence from post-mortem analyses, genetic studies, and clinical trials complements the preclinical data and further supports the notion that endocannabinoid signaling may be compromised in humans suffering from major depression. Hungund and colleagues have reported elevated CB_1 receptor density and functionality in the dorsolateral PFC of depressed suicide victims compared to healthy controls (Hungund et al., 2004). These observations were also replicated in chronic alcoholics who died by suicide when compared with matched alcoholic controls that died by other causes (Vinod et al., 2005). Although these changes were observed in the dorsolateral PFC, it should be noted that this increase in PFC CB_1 receptor functionality is in agreement with preclinical studies demonstrating an increase in PFC CB_1 receptor binding in CUS- and OBX-exposed rodents (Hill et al., 2008b; Rodriguez-Gaztelumendi et al., 2009).

In clinical patient populations, our laboratory has shown that circulating levels of endocannabinoid ligands were significantly reduced in two independent samples of depressed women (Hill et al., 2008d; Hill et al., 2009c). Additionally, recent genetic studies have revealed that individuals with different variants of the CB_1 receptor gene (CNR1) are characterized by higher levels of neuroticism and low agreeableness, and display increased vulnerability to depression following adverse life events (Juhasz et al., 2009). Additionally, individuals suffering from recurrent major depressive episodes exhibit a significantly higher frequency of the mutant allele of the CNR1 gene compared to healthy controls (Monteleone et al., 2010). These individuals also exhibit resistance to antidepressant treatment and display weaker striatal and thalamic activation in response

to emotional stimuli (Domschke et al., 2008). Similarly, patients with Parkinson's disease who possess long alleles in the CNR1 gene may actually be less susceptible to major depression (Barrero et al., 2005).

Intriguing results from clinical trials implementing the CB_1 receptor antagonist rimonabant for the treatment of obesity have revealed that a significant proportion of individuals taking the drug spontaneously developed increased anxiety, adverse depressive-like symptoms, and suicidal ideations that inevitably led to the suspension of clinical trials in both North America and Europe (Christensen et al., 2007; Hill and Gorzalka, 2009b). Moreover, these effects were also observed in clinical trials for rimonabant in the treatment of atherosclerosis (the STRADIVARIUS, or Strategy to Reduce Atherosclerosis Development Involving Administration of Rimonabant – The Intravascular Ultrasound Study) (Nissen et al., 2008). In these trials, 43% of individuals taking rimonabant developed adverse mood and anxiety responses, compared to 28% in the placebo condition (Nissen et al., 2008). In fact, the emergence of these symptoms was sufficient for one in 13 individuals to discontinue use of rimonabant, compared to one in 47 individuals who discontinued placebo treatment for these same reasons (Nissen et al., 2008). A likely explanation for the strikingly high incidence of adverse depressive-like symptoms observed in these trials is the non-exclusion of patients with prior psychiatric disorders. This inevitably resulted in a less selected study population that more closely reflects the risks of depression and anxiety with rimonabant treatment in routine clinical practice (Rumsfeld and Nallamothu, 2008).

The neural substrates responsible for the increased incidence of depressive symptoms following rimonabant administration is still speculative, but may be due to

interactions with 5-HT systems. Under conditions of high 5-HT activity, electrophysiology studies have shown that a low dose of rimonabant typically reduces 5-HT firing rates, but when 5-HT activity is within the normal range, a high dose of rimonabant further decreases 5-HT firing rates below optimal levels, which could have implications for the spontaneous development of depressive-like symptoms (Gobbi et al., 2005; Bambico et al., 2007). Regardless of the mechanism responsible, it is evident that pharmacological or genetic disturbances in CB_1 receptor signaling have profound consequences for the instantiation of major depression in clinical populations, thus converging with the preclinical data described above. Therefore, disruption of endocannabinoid signaling not only has negative implications in preclinical animal models, but in human populations as well.

1.11. Endocannabinoid Signaling in the Prefrontal Cortex

The body of evidence described thus far persuasively argues that the endocannabinoid system is an important regulator of stress and emotional states, and may be an appropriate target for the treatment of melancholic depression. Although these findings are encouraging to say the least, the role of endocannabinoids within discrete corticolimbic brain structures is considerably complex. To fully understand the mechanisms by which endocannabinoids elicit alterations in stress responsivity and emotional behavior, a neuroanatomical approach must be employed to further examine the discrete neurobiological effects of endocannabinoid manipulations within key brain regions that are known to mediate these phenomena.

As described above, the PFC is fundamentally important in this respect, exerting complex hierarchical control over a range of cognitive, behavioral, and neuroendocrine

processes that are necessary to plan, control, and direct behavior according to shifting environmental demands. The PFC is particularly susceptible to the detrimental impact of chronic stress, exhibiting morphological and functional disturbances in both depressed individuals and rodents exposed to chronic stress. Intriguingly, CB_1 receptor signaling is uniquely up-regulated in the CUS and OBX models of depression, as well as in post-mortem tissue obtained from depressed suicide victims. However, the functional relevance of these alterations has yet to be empirically evaluated and is fertile ground for future scientific investigations. Moreover, despite the established role of the medial PFC and the endocannabinoid system in regulating the neuroendocrine response to acute stress, the role of endocannabinoid signaling in the medial PFC has been virtually unexplored in this domain. Therefore, the following sections will provide an overview of the localization and function of the endocannabinoid system within the PFC and outline the current body of evidence regarding the role of endocannabinoid signaling in the PFC in emotionality, chronic stress, and major depression.

1.11.1. CB_1 Receptor Localization and Function in the PFC

Autoradiography studies have illustrated a distinct laminar distribution of CB_1 receptor binding throughout the neocortex, with the highest levels of expression in GABA-expressing layers II/III and diffuse localization within layer V, the major output layer of the PFC (Egertova et al., 2003). Immunohistochemistry and microscopy studies have confirmed this and further revealed that CB_1 receptors are present on cell bodies, axons, and dendrites in the neocortex of rodents (Tsou et al., 1998) and primates (Ong and Mackie, 1999), with greater densities of CB_1-immunoreactive axons in affective association areas such as the prefrontal and cingulate cortices compared to primary motor

and sensory cortices (Sim-Selley et al., 2002; Eggan and Lewis, 2007). Accordingly, CB_1 receptor mRNA has also been detected in these cortical areas using in *situ* hybridization (Mailleux et al., 1992; Mailleux and Vanderhaeghen, 1992; Matsuda et al., 1993; Marsicano and Lutz, 1999).

Neurons in the neocortex and associated limbic regions that express high levels of CB_1 receptor mRNA have also been found to express mRNA for glutamic acid decarboxylase 65, a synthesizing enzyme of GABA (Marsicano and Lutz, 1999). CB_1 receptors primarily exist on calbindin-positive GABAergic interneurons in the medial PFC, which are known to influence the firing of apical dendrites on pyramidal neurons (Wedzony and Chocyk, 2009). Furthermore, our laboratory has recently used electron microscopy to demonstrate that CB_1 receptors are present predominantly on inhibitory GABA terminals impinging upon pyramidal output neurons in layer V of the medial PFC (Hill et al., 2011b). This has been corroborated by additional studies showing colocalization of CB_1 receptors and D_2 receptors at terminals of GABAergic synapses in the medial PFC (Chiu et al., 2010). Electrophysiology experiments have further revealed that CB_1 receptor activation suppresses GABA release onto pyramidal cells in layer V (Chiu et al., 2010) and reduces the amplitude of inhibitory post-synaptic currents (IPSCs) in layer II/III of neocortical slices (Trettel et al., 2004; Bodor et al., 2005). It appears as though endocannabinoids and DA actually work in tandem at presynaptic CB_1 and D_2 receptors, respectively, to trigger LTDi at GABAergic synapses in the medial PFC (Chiu et al., 2010). In agreement with these data, microdialysis studies have demonstrated that systemic administration of CB_1 receptor agonists decreases extracellular levels of GABA and increases levels of glutamate and DA in the rodent PFC (Ferraro et al., 2001a;

Ferraro et al., 2001b; Pistis et al., 2002). These congruent findings argue that CB_1 receptors, with the participation of presynaptically-located D_2 receptors, potently regulate GABA-mediated inhibition of pyramidal output neurons in the medial PFC, which are known to mediate behavioral and neuroendocrine responses to stress.

Although CB_1 receptors are more prominently expressed in GABAergic neurons relative to glutamatergic principal neurons, electron microscopy analyses have revealed that presynaptic CB_1 receptors also face metabotropic glutamate receptor 5 (mGluR5) in layers 5/6 of the mouse prelimbic cortex, while DGL, the enzyme responsible for synthesis of 2-AG, is also expressed in dendrites containing mGluR5 (Lafourcade et al., 2007). Accordingly, CB_1 receptor activation has been shown to presynaptically inhibit glutamatergic synaptic transmission in cortical slices (Auclair et al., 2000). Inducing activity in layer V projection neurons also leads to endocannabinoid-mediated DSE, suggesting that endocannabinoid signaling in layer V neurons also directly inhibits excitatory transmission in *vivo* (Fortin and Levine, 2007). Moreover, long-term depression in layer V/VI excitatory inputs has been shown to depend on 2-AG/CB_1 receptor signaling at postsynaptic mGluR5, as this form of long-term synaptic plasticity can be blocked by inhibiting 2-AG synthesis and augmented by inhibiting enzymatic degradation of 2-AG (Lafourcade et al., 2007). Conditional mutagenesis studies have demonstrated that CB_1 receptors in glutamatergic and GABAergic neurons have functionally dissociable roles, with CB_1 receptors expressed on cortical glutamatergic neurons being important for the behavioral effects induced by high-dose THC exposure (Monory et al., 2007), neuroprotection from excitotoxic seizures (Monory et al., 2006), and appropriate behavioral and neuroendocrine responses to stress (Steiner et al., 2008b).

In addition to the regulatory role of CB_1 receptor activation on cortical GABAergic and glutamatergic activity, endocannabinoid signaling also appears to modulate the release of NA and 5-HT from the locus coeruleus and dorsal raphe, respectively. With respect to NA, both systemic administration and local activation of CB_1 receptors within the frontal cortex with a CB_1 receptor agonist stimulates NA release and increases the firing rate of NA neurons in the coeruleo-frontal pathway (Oropeza et al., 2005; Muntoni et al., 2006; Page et al., 2008). In the context of 5-HT, Gobbi and coworkers have confirmed that both systemic administration and local activation of CB_1 receptors specifically within the ventromedial region of the PFC increases the firing rate of dorsal raphe 5-HT neurons (Bambico et al., 2007). Together, these findings suggest that the modulatory effects of CB_1 receptor signaling in the medial PFC are widespread, with a substantial role in regulating neuroendocrine and monoaminergic neural networks that have long been the target of conventional treatments for major depression.

1.11.2. Endocannabinoids, the Prefrontal Cortex and Emotionality

As described above, deep brain stimulation of the ventromedial region of the PFC has emerged as a particularly promising therapeutic strategy for combating depressive-like symptoms in both rats (Hamani and Nobrega, 2010; Hamani et al., 2010c) and humans (Mayberg et al., 2005). In rodents, these responses were shown to be dependent on the integrity of the 5-HT system, thus stressing the importance of crosstalk between the ventromedial PFC and midbrain 5-HT neuronal activity in regulating emotional responses. Microinfusion studies employing local activation of ventromedial PFC CB_1 receptors have illustrated a similar antidepressant-like mechanism, as local CB_1 receptor activation in the ventromedial (but not lateral) PFC has been shown to elicit an

antidepressant-like response in the FST by enhancing 5-HT output from the dorsal raphe (Bambico et al., 2007). In the realm of anxiety, Parolaro and colleagues have reported a CB_1 receptor-dependent anxiolytic profile in rodents receiving discrete microinjections of a low dose of THC (Rubino et al., 2008a), methanandamide (a metabolically stable analog of AEA) (Rubino et al., 2008b), or the FAAH inhibitor URB597 (Rubino et al., 2008b) directly into the medial PFC. Accordingly, lentivirus-mediated local overexpression of FAAH in the medial PFC (which elicits a marked decrease in AEA in this region) was shown to elicit an anxiogenic response in the elevated plus maze (Rubino et al., 2008b).

The amygdala is intimately linked to the generation and maintenance of fear and anxiety states, which it accomplishes via crosstalk with the medial PFC (Shin and Liberzon, 2010). In particular, the BLA is implicated in emotional processing and the encoding of associative memories with an affective component (Roozendaal, 2000; McGaugh, 2002; McGaugh et al., 2002), which it accomplishes via direct reciprocal connections with the medial PFC (Rosenkranz and Grace, 2002, 2003; Rosenkranz et al., 2003; Laviolette et al., 2005; Laviolette and Grace, 2006a, b). Individuals suffering from mood disorders characterized by glucocorticoid hypersecretion typically display elevated resting metabolism within the amygdala and exaggerated hemodynamic responses to negative stimuli (Price and Drevets, 2010). Metabolic activity in the PFC and amygdala is often inversely correlated in these clinical populations (Davidson, 2002; Urry et al., 2006; van Reekum et al., 2007; Drevets et al., 2008a), lending further support to the notion that imbalances within this corticoamygdalar pathway contribute to impairments in emotional behavior.

An elegant series of experiments from Laviolette and colleagues suggests that CB_1 receptors may be involved in this phenomenon. First, these researchers showed that systemic blockade of CB_1 receptors abolished long-term potentiation along the BLA-PFC pathway and prevented the behavioral acquisition of conditioned fear memories (Tan et al., 2010). Moreover, asymmetrical interhemispheric blockade of CB_1 receptor transmission along the BLA-PFC pathway also prevented the acquisition of emotionally salient associative memories (Tan et al., 2010). In accordance with these findings, intra-BLA administration of a CB_1 receptor agonist or endocannabinoid reuptake inhibitor strongly potentiated the emotional salience of normally sub-threshold fear-conditioning stimuli, but not when the medial PFC was inactivated (Tan et al., 2011). Using in *vivo* electrophysiological recordings, these researchers further demonstrated that modulation of BLA CB_1 receptor transmission strongly influences neuronal activity within subpopulations of the medial PFC, as activation of BLA CB_1 receptors produced robust activation of medial PFC pyramidal neurons while blockade of BLA CB_1 receptors inhibited spontaneous firing of these neurons (Tan et al., 2011). Taken together, these studies persuasively argue that CB_1 receptor transmission within the BLA strongly modulates the acquisition and processing of associative fear memory via functional interactions with medial PFC neuronal populations.

With respect to extinction of conditioned fear, Lin and coworkers have shown that direct infusion of a CB_1 receptor agonist, FAAH inhibitor, or endocannabinoid uptake inhibitor into the ventromedial PFC facilitates extinction of a cue-induced fear-potentiated startle response, while infusion of a CB_1 receptor antagonist retarded this form of extinction (Lin et al., 2009). Furthermore, activation of CB_1 receptors within this

region also reduced startle potentiation in the absence of cue presentation, suggesting that these receptors are not only involved in the extinction of conditioned fear, but also in adaptation to aversive situations in general (Lin et al., 2009). Lastly, direct microinjection of cannabidiol, a non-psychotomimetic component of cannabis, into the prelimbic PFC reduced freezing induced by re-exposure to a context previously paired with footshocks (Lemos et al., 2010). However, in the more ventrally located infralimbic region of the PFC, cannabidiol produced an opposite result, increasing the expression of contextual fear conditioning (Lemos et al., 2010). Although the pharmacological mechanisms involved in the effects of cannabidiol are still poorly understood, these results nonetheless illustrate the dissociable role of cannabinergic compounds in dorsal and ventral components of the medial PFC in the expression and extinction of conditioned fear.

Finally, the link between malnutrition and emotional dysfunction has recently garnered attention from the field of neuroscience, and a recent study has implicated synaptic alterations within prefrontocortical CB_1 receptors as a possible neural substrate mediating this link. Lafourcade and colleagues have revealed that life-long deficiency of n-3 polyunsaturated fatty acids, which has been associated with impaired emotional behavior, specifically ablates endocannabinoid-mediated long-term synaptic depression in the prelimbic region of the medial PFC (Lafourcade et al., 2011). Moreover, these CB_1 receptors show reduced coupling to their effector $G_{i/o}$ proteins (Lafourcade et al., 2011). Therefore, a wide range of aversive conditions can produce deficiencies in endocannabinoid signaling within the PFC and these functional alterations may have

profound consequences for emotional behavior and the pathophysiological development of affective illness.

1.11.3. Endocannabinoids, the Prefrontal Cortex and Chronic Stress

As mentioned above, repeated exposure to a homotypic stressor elicits a time-dependent increase in 2-AG content in the PFC, while AEA undergoes a progressive reduction in this region, coupled to a corresponding increase in FAAH activity (Patel et al., 2005b; Rademacher et al., 2008). Notably, CB_1 receptor binding does not appear to be functionally altered in the medial PFC following repeated restraint stress (Rademacher et al., 2008). In contrast, a different pattern of results has been obtained in rodents subjected to chronic *non-habituating* stress. For instance, CUS exposure causes a substantial reduction in AEA content, but increases CB_1 receptor binding (Hill et al., 2008b) and mRNA expression (Hillard et al., 2006; Bortolato et al., 2007) in whole PFC tissue samples. Similarly, four days of immobilization/acoustic stress is sufficient to increase CB_1 receptor mRNA and protein expression in the PFC, which can be prevented by concurrent CB_1 receptor agonist administration (Zoppi et al., 2011).

This unique up-regulation of CB_1 receptor binding in the PFC of rodents subjected to chronic non-habituating stress has also been validated in other preclinical models of depression, namely the OBX model. OBX rats exhibit significant increases in CB_1 receptor density and functionality, coupled to hyperactivity in the open field test (suggestive of an anxiogenic profile), and moreover, these behavioral symptoms and changes in PFC CB_1 receptor activity are reversed following chronic treatment with the antidepressant fluoxetine (Rodriguez-Gaztelumendi et al., 2009). Accordingly, long-term treatment with fluoxetine in intact rodents has been shown to enhance CB_1 receptor-

mediated inhibition of adenylyl cyclase and augment CB_1 receptor coupling to inhibitory G-protein subunits in the PFC (Mato et al., 2010). Thus, increased CB_1 receptor-mediated signal transduction in the PFC represents a characteristic of conventional antidepressant treatment that could mediate the effects of these drugs in preclinical models of depression.

Augmented endocannabinoid signaling in the medial PFC has been argued to represent a compensatory mechanism in response to chronic stress, and increased signaling at CB_1 receptors in this region may circumvent morphological changes following these stress regimens. In support of this theory, chronic exposure to low doses of THC increases the length and branching of dendrites in the medial PFC of rodents (Kolb et al., 2006). With respect to glial cell function, it is interesting to note that endocannabinoid signaling actively promotes biochemical signals resulting in a pro-survival fate for these cells while inducing a selective death in glia-derived tumor cells (Massi et al., 2008). Moreover, under neuropathological conditions, glial cells release an increased amount of endocannabinoids and over-express cannabinoid receptors, which may constitute an endogenous defense mechanism that abrogates further cell damage (Massi et al., 2008). In agreement with this notion, CB_1 receptor knockout mice have recently been shown to exhibit HPA axis dysregulation along with exacerbated excitotoxic/neuroinflammatory responses in the PFC (Zoppi et al., 2011). Moreover, daily treatment with a CB_1 receptor agonist is capable of preventing these stress-induced increases in pro-inflammatory molecules, lipid peroxidation, and decreased glutamate uptake (Zoppi et al., 2011). Given the multifaceted neuroprotective effects of CB_1 receptors in the PFC, it is possible that targeting this subpopulation may represent a

novel therapeutic treatment strategy for combating pathological states that are characterized by HPA axis disturbances and excitotoxic/neuroinflammatory responses, such as major depression.

1.11.4. Endocannabinoids, the Prefrontal Cortex and Major Depression

Although evidence for alterations in prefrontocortical endocannabinoid signaling in humans is sparse, post-mortem analyses have nonetheless demonstrated aberrant CB_1 receptor parameters in cortical brain regions of individuals afflicted with mood disorders. As discussed above, researchers have reported elevated CB_1 receptor density and functionality in the dorsolateral PFC of depressed suicide victims compared to healthy controls (Hungund et al., 2004). These observations were also replicated in chronic alcoholics who died by suicide in comparison with matched alcoholic controls that died by other causes (Vinod et al., 2005). Koethe and colleagues found a significant decrease in glial CB_1 receptor density in the anterior cingulate cortex of patients with major depression from among patients that included manic-depressives and schizophrenics (Koethe et al., 2007). Moreover, several independent studies on schizophrenics, whose negative symptoms overlap with those of melancholic depression, have also revealed increased CB_1 receptor density in the dorsolateral PFC (Dean et al., 2001), as well as in anterior (Zavitsanou et al., 2004) and posterior cingulate cortices (Newell et al., 2006). Although this evidence is preliminary, the increase in CB_1 receptor parameters found in post-mortem cortical tissue from depressed and schizophrenic individuals is in agreement with preclinical studies demonstrating increased PFC CB_1 receptor binding in rodents exposed to models of depression. Therefore, alterations in CB_1 receptor parameters in the PFC observed in preclinical and clinical reports may offer meaningful insight into the

pathological underpinnings of major depression; however, the functional relevance of these changes has yet to be empirically evaluated.

1.12. Summary and Conclusions

Major depression is a debilitating mental illness that is inextricably linked to prolonged exposure to psychoemotional stress and consequent hypersecretion of glucocorticoids. The PFC is a structurally and functionally heterogeneous brain region that is intimately involved in cognitive and executive functioning tasks, and exerts a profound regulatory role on neuroendocrine and behavioral responses to psychological stress. Promising novel antidepressant strategies for the treatment of major depression such as deep brain electrical stimulation typically target the ventromedial region of the PFC, a cortical region sharing dense reciprocal connections with midbrain monoaminergic nuclei and limbic structures known to be implicated in emotionality and feedback inhibition of the HPA axis.

The endocannabinoid system has recently surfaced as a promising pharmacotherapeutic candidate for the treatment of emotional disorders that are precipitated by stress, as converging evidence has revealed a robust bidirectional relationship between endocannabinoid signaling and the neuroendocrine stress response. Chronic stress induces a ubiquitous and progressive reduction in corticolimbic AEA content coupled with an up-regulation of CB_1 receptors in the PFC that is in stark contrast to changes in subcortical structures such as the hippocampus, hypothalamus, and amygdala where AEA/CB_1 receptor signaling is unanimously downregulated. Preclinical data suggests that augmentation of AEA signaling is capable of producing all of the major behavioral and neurochemical changes elicited by conventional antidepressants.

Furthermore, these compounds appear to exert these effects without producing the secondary side effects of conventional antidepressants or the addiction liability associated with exogenous CB_1 receptor agonists (Justinova et al., 2008; Bambico et al., 2009).

Within the medial PFC, CB_1 receptors are predominantly located on GABAergic interneurons that control the output of pyramidal projection neurons. AEA/CB_1 receptor signaling tonically suppresses this GABAergic inhibition in the ventromedial PFC and produces anxiolytic and antidepressant-like behavioral responses in preclinical paradigms, possibly via trans-synaptic activation of dorsal raphe 5-HT neurons (Bambico et al., 2007). The current body of literature suggests that local facilitation of endocannabinoid signaling within the ventromedial PFC (effectively reducing high GABAergic inhibitory tone in this region) may jumpstart an underactive ventromedial PFC in depressed individuals and rodents subjected to various stress regimens, thereby promoting activity of principal output neurons, enhancing feedback inhibition of the HPA axis, and increasing monoaminergic neurotransmission. These changes ultimately result in more effective behavioural, neuroendocrine, and neuroimflammatory responses during a stressful challenge. Considering the paucity of effective pharmacotherapeutic interventions for stress-related disorders including major depression, this collective body of literature provides the impetus to further investigate how functional disturbances in prefrontocortical endocannabinoid signaling are implicated in responses to acute and chronic stress, as this knowledge may offer valuable insight into the pathological development of these devastating disorders.

1.13. Objectives and Hypotheses

The studies described in Chapters 2, 3, and 4 were designed with the intent of providing a general framework for how endocannabinoid signaling in the medial PFC is biochemically altered by acute and chronic stress regimens. Furthermore, we sought to elucidate the functional relevance of changes in prefrontocortical endocannabinoid signaling with regard to recovery from stress and the adoption of coping strategies in preclinical models of emotionality (FST) and major depression (CUS). The overarching hypothesis of this collective body of work is that endocannabinoid signaling in the medial PFC critically mediates neuroendocrine and behavioral responses to both acute and chronic stress. With that said, the objectives of the proposed research are as follows.

1. The primary objective of Chapter 2 was to examine whether acute restraint stress elicits alterations in endocannabinoid content in the medial PFC at sequential time intervals following stress exposure, and to determine whether genetic deletion or local pharmacological blockade of CB_1 receptors in the medial PFC affects stress-induced alterations in corticosterone secretion and recovery. Our hypothesis was that acute stress would increase 2-AG content in the medial PFC, and that this would coincide with corticosterone recovery. Moreover, we expected that genetic or pharmacological disruption of CB_1 receptors in the medial PFC would delay stress recovery in a glucocorticoid-dependent manner. **A secondary objective of this chapter was to determine the precise localization and function of CB_1 receptors in the medial PFC using immunohistochemistry and in *vitro* electrophysiology, respectively, in an effort to examine the mechanism by which medial prefrontocortical endocannabinoid signaling exerts its effects.** We hypothesized that

bath application of a CB_1 receptor antagonist to medial PFC slices would impair synaptic plasticity at GABA synapses in this region and moreover, we expected that glucocorticoids would depress GABA-mediated currents in layer V pyramidal output neurons of the medial PFC via recruitment of endocannabinoid signaling.

2. The objective of Chapter 3 was to examine whether forced swim exposure alters endocannabinoid ligand content in the medial PFC, and to determine whether local facilitation of endocannabinoid signaling (via FAAH inhibition) affects coping responses in the FST. Based on previous literature, we hypothesized that forced swim stress would rapidly suppress AEA content in the medial PFC, while local pharmacological facilitation of AEA within this region would prevent the adoption of passive, despair-like coping responses in the FST. **A secondary objective of this chapter was to further explore the involvement of 5-HT neurotransmission in this phenomenon using a combination of pharmacological and in *vivo* electrophysiological techniques.** We predicted that if local FAAH inhibition within the medial PFC were successful in promoting active coping responses, then the mechanism by which this occurs would involve an enhancement of dorsal raphe 5-HT firing.

3. The objective of Chapter 4 was to first determine whether CB_1 receptor binding is differentially altered in dorsal and ventral subregions of the medial PFC following CUS, a valid and reliable preclinical model of depression. Given the established role of endocannabinoid signaling in the ventromedial PFC with respect to stress coping and recovery, we hypothesized that CUS exposure would increase the maximal binding site density of CB_1 receptors predominantly within the ventromedial PFC as opposed to the dorsomedial PFC. **Additionally, we further sought to clarify**

whether CUS-induced alterations in prefrontocortical CB_1 receptor binding represent a maladaptive consequence of CUS exposure or alternately, a compensatory response engaged to dampen the detrimental effects of CUS exposure. We predicted that local pharmacological blockade of CB_1 receptors within the ventromedial PFC following 21-day CUS exposure would greatly exacerbate the expression of despair-like responses in the FST, thereby providing evidence that up-regulated CB_1 receptor binding in this subregion affords a compensatory role by constraining adverse changes in stress coping strategies.

2. Recruitment of Prefrontocortical Endocannabinoid Signaling by Glucocorticoids Contributes to Termination of the Stress Response[1]

2.1. Introduction

Exposure to stressful stimuli evokes a well-characterized activation of the HPA axis that results in the secretion of glucocorticoids into the circulation (Pecoraro et al., 2006). In the short term, glucocorticoids optimize physiological and metabolic conditions such that an organism can appropriately respond to the threat at hand by mobilizing glucose stores, trafficking leukocytes and enhancing vigilance and attention (McEwen et al., 1997; Pecoraro et al., 2006). However, persistent glucocorticoid secretion can produce detrimental effects on cardiovascular, metabolic and neural systems and is associated with many disease states such as hypertension, type II diabetes and mood disorders (McEwen, 2008; Chrousos, 2009). Accordingly, secretion of glucocorticoids is tightly regulated by neural and hormonally-mediated negative feedback processes which limit the magnitude and duration of HPA axis activity through both rapid and delayed processes. Rapid feedback inhibition of the HPA axis by glucocorticoids is accomplished by local actions of glucocorticoids at the pituitary and the hypothalamic PVN, but the long-loop feedback inhibition of HPA axis activity is driven by upstream corticolimbic structures that communicate with the hypothalamus (Herman et al., 2003; Pecoraro et al., 2006).

[1]A version of this chapter has been published. Hill MN*, McLaughlin RJ*, Pan B, Fitzgerald ML, Roberts CJ, Lee TT, Karatsoreos IN, Mackie K, Viau V, Pickel VM, McEwen BS, Liu QS, Gorzalka BB, Hillard CJ. (2011). Recruitment of prefrontal cortical endocannabinoid signaling by glucocorticoids contributes to termination of the stress response. *J Neurosci*, *31(29)*, 10506-15. Article content reproduced here with permission from Society of Neuroscience. Copyright 2011. * denotes co-first author status.

Neuroendocrine and neuroanatomical studies have identified the medial PFC as an essential site of action for glucocorticoid-mediated termination of HPA axis activity following exposure to stress. Glucocorticoid receptors are present within the medial PFC, and corticosterone implants within this region dampen stress-induced activation of the HPA axis and accelerate the return of circulating glucocorticoid concentrations to baseline (Diorio et al., 1993). Lesions of the medial PFC, in particular the prelimbic region of the medial PFC, impair termination of HPA axis activity following cessation of stress (Diorio et al., 1993; Figueiredo et al., 2003; Spencer et al., 2005; Radley et al., 2006a; Radley et al., 2009). Elegant anatomical work has delineated the circuit subserving prefrontal cortical regulation of the HPA axis. This circuit involves activation of glutamatergic afferents from the prelimbic region of the medial PFC, which then activate inhibitory relays to the PVN in the BNST, and possibly the peri-PVN region (Spencer et al., 2005; Radley et al., 2006a; Radley et al., 2009; Ulrich-Lai and Herman, 2009). Despite the mapping of this circuit, surprisingly little is known about the mechanisms by which glucocorticoids influence medial PFC neuronal activity to promote activation of output projections that contribute to termination of HPA axis activity.

Several lines of evidence suggest that the endocannabinoid system could be involved in coordinating the effects of glucocorticoids on medial PFC neuronal activation. First, the endocannabinoid ligands AEA and 2-AG modulate the balance of excitation and inhibition within a given neural circuit through their ability to inhibit synaptic release of neurotransmitters via activation of presynaptic cannabinoid CB_1 receptors (Freund et al., 2003). Second, both in *vitro* and in *vivo* studies have demonstrated that glucocorticoids induce endocannabinoid signaling (Di et al., 2005;

Malcher-Lopes et al., 2006; Hill et al., 2010a). Third, disruption of endocannabinoid/CB_1 receptor signaling promotes activation of the HPA axis, indicating that this system negatively regulates activation of the HPA axis (Patel et al., 2004; Steiner and Wotjak, 2008; Hill and McEwen, 2010). Taken together, these data suggest that glucocorticoids recruit endocannabinoid signaling to increase the excitability of principal neurons in the prelimbic region of the medial PFC, which initiate inhibitory relays that terminate HPA axis activation.

2.2. Materials and Methods

2.2.1. Neuroendocrine Studies

2.2.1.1. Subjects

Seventy-day-old male Sprague-Dawley rats (300 g; Charles River, Montreal, Canada) were used to determine the role of CB_1 receptor signaling within the medial PFC. Rats were pair-housed (except following surgical procedures, when they were individually housed) in standard maternity bins lined with contact bedding. Colony rooms were maintained at 21 ºC, and on a 12 hr light/dark cycle, with lights on at 0900hr. All rats were given free access to Purina Rat Chow and tap water. All protocols were approved by the Canadian Council for Animal Care and the Animal Care Committee of the University of British Columbia. All studies occurred during the first third of the light cycle, during the daily nadir of HPA axis activity.

2.2.1.2. Surgical Cannula Implantation

For microinjection studies, animals were subjected to stereotaxic surgery. Rats were anesthetized with 100 mg/kg of ketamine hydrochloride and 7 mg/kg xylazine, and implanted with bilateral 23 gauge stainless-steel guide cannula. Cannula were surgically

implanted bilaterally into the medial PFC [coordinates from bregma: anterior-posterior (AP) +3.0 mm; medial-lateral (ML) +/- 0.7 mm; dorsal-ventral (DV) -3.4 mm from dura mater; Paxinos and Watson, 1998]. Four steel screws and dental acrylic were used to permanently affix the guide cannula to the skull. Stainless steel stylets (30-gauge) were inserted into the guide cannula until the time of infusion. Immediately following surgery, antibiotic ointment was applied to the skull and surrounding incision. All rats were allowed one week of recovery before testing and were individually housed during this recovery period.

2.2.1.3. Acute Restraint Stress

For stress testing, subjects were put into a polystyrene tube (diameter 6 cm, length 20 cm) with breathing holes. Tubes were long enough to completely encase the rat and too narrow for turning or other large movements. Rats were left in the tubes for 30 min, then removed and returned to their home cage. Blood samples were drawn via a small nick made at the tip of the tail from which 100 µl of blood were collected for corticosterone analysis, immediately at stress offset, and following both 30 and 60 min. Subjects were randomly divided to receive either AM251 (0.28 ng/side) or vehicle (one part dimethyl sulfoxide; DMSO: 9 parts 0.9% sterile saline) and animals received bilateral infusions of either solution 10 min prior to the initiation of restraint stress. A 30-gauge injection cannula extending 0.8 mm below the tips of the guide cannula was used for infusions. Drug solutions or vehicle were delivered at a rate of 0.2 µl/28 s using a microsyringe pump (Sage Instruments Model 341). Injection cannula were left in place for an additional one min to allow for diffusion. Following infusions, animals were returned to their home cages for 10 min prior to stress induction. Blood samples

(approximately 100 µl) were taken immediately at stress offset and then at 30 and 60 min following the cessation of stress. A separate cohort of animals was prepared in an identical fashion and received infusions of AM251 or vehicle under identical conditions, except that they were not exposed to restraint stress. These animals were bled at identical time points following AM251 administration to determine if AM251 administration under basal conditions activated the HPA axis. All rats were killed in a carbon dioxide chamber 24 hr following testing. Brains were removed and fixed in a 4% formalin solution. The brains were frozen and sliced in 50 µm sections and mounted. Placements were verified with reference to the atlas of Paxinos and Watson (1998) and histological analysis demonstrated that approximately 85% of cannula placements were in boundaries of the nuclei of interest (see Fig. 1A). Subjects with cannula outside of the desired structure were excluded from subsequent analyses.

2.2.1.4. CB_1 Receptor Knockout Mice

Male ICR mice, aged 9-12 weeks, were used to determine the course of HPA axis recovery following stress in CB_1-receptor-deficient mice. Mice were maintained on a 12 hr light:dark cycle with lights on at 0600hr, and food and water were available ad libitum. CB_1 receptor null mice were bred in house from a founder line generously provided by Roche Laboratories and back-crossed for 9 generations onto the ICR strain (Pan et al., 2008). Wild type mice derived from the same backcrossing were used as controls in those studies. Genotypes were determined by polymerase chain reaction using deoxyribonucleic acid (DNA) isolated from ear tissue obtained at weaning. All procedures carried out with mice were approved by the Institutional Animal Use and Care Committee of the Medical College of Wisconsin. Mice were restrained for 30 min

by anchoring the proximal portion of the tail to a lab bench top with strips of cloth tape. Blood (approximately 10 µl) was collected from a tail nick into a hematocrit tube immediately prior to restraint, immediately after the restraint, and at various time points during recovery.

2.2.1.5. Corticosterone Measurements

For all neuroendocrine studies, blood samples were centrifuged (for rat 3000 x g for 10 min and for mouse 10000 x g for 1 min), after which plasma was removed and stored at -80 ºC. Corticosterone (5 µl for rat and 2 µl for mouse) was measured in duplicate using commercial RIA kits (MP Biomedicals, Costa Mesa, CA). Samples were diluted 1:100 and 1:200 for basal and stress conditions, respectively, to render hormone detection within the linear part of the corticosterone standard curve. [^{125}I]-labeled corticosterone was used as tracer; the corticosterone antibody cross-reacts slightly with desoxycorticosterone (0.34%) and testosterone and cortisol (0.10%).

2.2.2. Biochemical Studies

2.2.2.1. Tissue Collection

Male rats were used for biochemical studies and housing conditions were identical to those described above. Subjects were randomly assigned to one of four conditions: vehicle (1:1 ratio of 0.9% saline:propylene glycol)/no stress; the glucocorticoid receptor antagonist RU486 (20 mg/kg; Sigma, Canada)/no stress; vehicle/stress (30 min restraint stress); RU486/stress. Stress procedures were identical as those described above for the rat studies. Given that the effects of intra-medial PFC administration of the CB_1 receptor antagonist AM251 on stress-induced corticosterone secretion emerged at 30 min post stress cessation, we used this time point for

biochemical analysis of endocannabinoid content. As such, animals in the stress condition were returned to their home cage for 30 min following the conclusion of the 30 min restraint session before being terminated. RU486 injections occurred 30 min prior to stress onset, and time points for termination in vehicle and RU486 conditions with no stress were performed at comparable times following injection as those in the stress condition. All subjects were rapidly decapitated. The medial PFC was dissected as a tissue block that was anatomically defined as the area dorsal to the anterior olfactory nucleus, ventral to the motor cortex and medial to the corpus callosum and claustrum formation, frozen in liquid nitrogen within 5 min of decapitation and stored at -80 ºC until analysis. Once we had established the effect of stress on endocannabinoids in the medial PFC, we sought to determine if this response occurred throughout the entire PFC or was restricted to regions that were known to be involved in HPA axis regulation (Radley et al., 2006a). A separate cohort of rats was exposed to 30 min restraint stress and then returned to their home cage for 30 min, or acted as cage controls, after which they were rapidly decapitated and a dorsal region of the frontal cortex was collected for analysis.

2.2.2.2. Endocannabinoid Content Analysis

Brain regions were subjected to a lipid extraction process as described previously (Patel et al., 2003). Tissue samples were weighed and placed into borosilicate glass culture tubes containing two ml of acetonitrile with 84 pmol of [$^2H_8$]AEA and 186 pmol of [$^2H_8$]2-AG. Tissue was homogenized with a glass rod and sonicated for 30 min. Samples were incubated overnight at -20°C to precipitate proteins, then centrifuged at 1,500 x g to remove particulates. The supernatants were removed to a new glass tube and

evaporated to dryness under N_2 gas. The samples were resuspended in 300 µl of methanol to recapture any lipids adhering to the glass tube, and dried again under N_2 gas. Final lipid extracts were suspended in 20 µl of methanol, and stored at –80^o C until analysis.

The contents of the two primary endocannabinoids AEA and 2-AG within lipid extracts in methanol from brain tissue were determined using isotope-dilution, liquid chromatography-mass spectrometry as described previously (Patel et al., 2005a). Samples (5 µl) were separated on a reverse-phase C_{18} 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 [$^2H_8$]AEA (m/z 356; retention time = 13.7 min), AEA (m/z 348; retention time = 13.9 min), [$^2H_8$]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), so the area of both peaks was combined to yield total 2-AG. Endocannabinoid contents were normalized to wet tissue weight.

2.2.3. Immunofluorescence Studies

2.2.3.1. Subjects

For immunofluorescence studies of CB_1 receptor expression in the medial PFC, we employed both rats and mice. Male Sprague-Dawley rats were obtained and housed as described for the neuroendocrine and biochemical studies above. Male C57/Bl6 mice (10 weeks of age) were obtained from Charles River (Wilmington MA, USA) and housed in groups of 5 per cage under a 12:12 hr light cycle with lights off at 0700hr and food and water available ad libitum. All procedures involving these mice were approved by the Institutional Animal Use and Care Committee of the Rockefeller University.

2.2.3.2. Tissue Preparation and Staining

To obtain tissue for immunofluorescence staining, both rats and mice were transcardially perfused with 4% paraformaldehyde and whole brains were removed and postfixed in 4% v/v formaldehyde overnight and then in 30% w/v sucrose in phosphate-buffered saline (PBS) at 4°C for 72 hr. Brains were sliced into 40 μm slices on a freezing microtome and free floating slices of the PFC (from 3.2 to 2.7 mm rostral to bregma) were processed for expression of CB_1 receptors. Free floating slices were washed in PBS and then blocked in PBS containing 5 % normal horse serum and 0.1 % triton X (PBS-T) for 1 hr. Tissue slices were then incubated for 24 hr at room temperature in a cocktail of PBS with 5 % normal donkey serum, 0.1% triton X and a guinea pig anti-CB_1 C-terminus antibody (1:1000 dilution) (Berghuis et al., 2007). Following incubation, tissue slices were washed repeatedly in PBS and then incubated in PBS with 5% normal horse serum, 0.1% triton x and Alexa Fluor® 680 conjugated donkey anti-guinea pig IgG (H+L; 1:200 dilution) for 2 hr at room temperature. Tissue slices were then washed in

PBS repeatedly and mounted on gel-coated slides and coverslipped. Immunofluorescence was examined within the PFC using a fluorescence microscope.

2.2.4. In Vitro Electrophysiology Studies

Male, ICR mice (Harlan laboratories, Madison, WI) were sacrificed and coronal slices containing the medial PFC were prepared and placed into artificial cerebrospinal fluid (ACSF) as described previously (Pan et al., 2008). All procedures carried out with mice were approved by the Institutional Animal Use and Care Committee of the Medical College of Wisconsin. Pyramidal neurons (which represent principal neurons) in layer V were identified visually based upon pyramidal shaped soma and ascending apical dendrites. Whole-cell patch clamp recordings were made from pyramidal neurons whose identity was confirmed by examination of firing characteristics in response to the injection of depolarizing and hyperpolarizing currents. The pipette solution contained (in mM): K-gluconate 100, KCl_2 50, HEPES 10, EGTA 0.2, $MgCl_2$ 2, MgATP 4, Na_2GTP 0.3, and Na_2-phosphocreatine 10 at pH 7.2 (with KOH). The glutamate receptor antagonists CNQX (10-20 μM) and AP-5 (50 μM) were added to inhibit excitatory responses. For recording of evoked IPSCs, pyramidal neurons were voltage-clamped at -60 mV and IPSCs were evoked at 0.1 Hz by a tungsten stimulation electrode placed near the apical dendrites. In some studies, DSI was induced by depolarization from -60 mV to 0 mV for 5 s. For the studies of the effects of corticosterone, slices were allowed to recover from harvest for 1 hr, then were incubated with 100 nM corticosterone in ethanol (0.001% final concentration) or ethanol alone at 32°C. Twenty min after the addition of corticosterone, the slices were washed and stored in normal ACSF (without corticosterone or ethanol) for at least 1 hr (and up to 4 hr) at room temperature before

being transferred into the recording chamber. For studies examining interactions between corticosterone application and the endocannabinoid system, AM251 (2 μM in 0.05% DMSO) was applied at the same time as corticosterone and was also present in the incubation media during patch clamp recordings. Spontaneous miniature IPSCs (mIPSCs) were recorded from the pyramidal neurons at a holding potential of -70 mV. Action potential generation was blocked with tetrodotoxin (TTX, 0.5 μM). In paired pulse ratio (PPR) paradigm, IPSCs were evoked at 0.05 Hz using a bipolar, tungsten electrode placed adjacent to the recorded neuron. Paired-pulse stimulation with a 100 ms inter-stimulus interval was applied. TTX was not present in the incubation media.

2.2.5. Statistics

Data for the effects of intra-medial PFC administration of AM251 or CB_1 receptor-deficient mice on stress-induced corticosterone secretion was analyzed with a repeated measures analysis of variance (ANOVA) with time being the within factor and either drug treatment or genotype being the between factor. For endocannabinoid ligand analysis, a univariate ANOVA was used with both stress and drug treatment as fixed factors.

2.3. Results

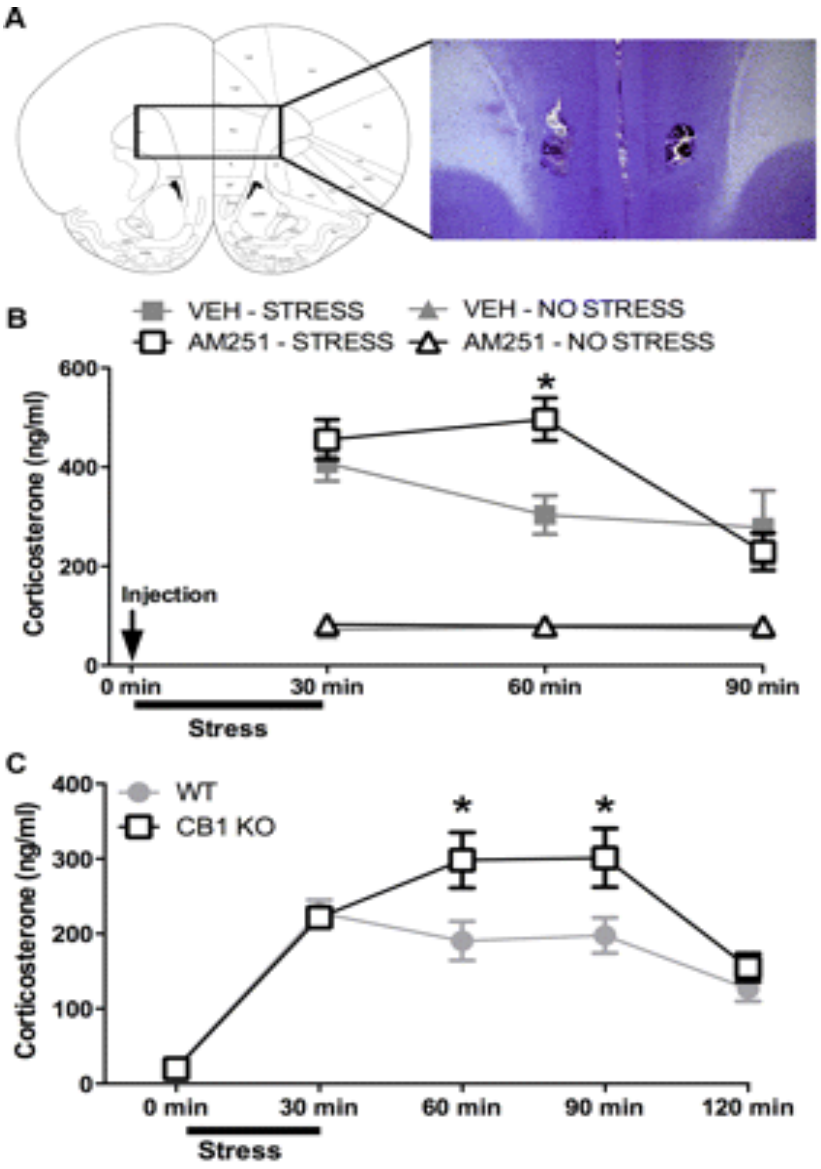
2.3.1. CB_1 Receptor Signaling is Required For Termination of HPA Axis Activity Following Cessation of Stress

We examined the effects of local antagonism of CB_1 receptors within the medial PFC to determine if CB_1 receptor signaling in this region regulates HPA axis activity following exposure to stress. Bilateral administration of the CB_1 receptor antagonist AM251 (0.28 ng/side; see Fig. 2.1A for representative histological cannula placement)

into the medial PFC resulted in a significant interaction between AM251 administration and time following stress exposure on levels of corticosterone [$F_{(2, 20)} = 3.49$, $p < 0.05$; Fig. 2.1B]. Post hoc analysis revealed that AM251 administration did not alter peak levels of corticosterone when examined immediately at stress offset ($p > 0.05$). However, at 30 min following cessation of stress, local antagonism of CB_1 receptors resulted in significantly elevated levels of corticosterone relative to vehicle infused animals ($p < 0.05$). There was no interaction between intra-medial PFC AM251 administration and time on corticosterone secretion in animals that were not exposed to stress [$F_{(2, 20)} = 0.07$, $p > 0.05$; Fig. 2.1B]. There was no main effect of either AM251 administration [$F_{(1, 20)} = 0.14$, $p > 0.05$] or time [$F_{(1, 20)} = 0.03$, $p > 0.05$] on circulating levels of corticosterone.

We also examined levels of corticosterone over time following stress exposure in mice lacking the CB_1 receptor (CB_1R-/-). Statistical analysis revealed a significant interaction between genotype and time on circulating levels of corticosterone [$F_{(4, 140)} = 4.41$, $p < 0.01$; Fig. 2.1C]. Post hoc analysis showed that there was neither a difference in basal levels of corticosterone ($p > 0.05$; wild type: 22.03 +/- 3.44 ng/ml vs. CB_1R-/-: 20.16 +/- 3.53 ng/ml) nor in corticosterone secretion immediately after stress offset ($p > 0.05$) between wild type and CB_1R-/- mice. However, the CB_1R-/- mice exhibited a significantly prolonged corticosterone response compared to the wild type at 30 ($p < 0.01$) and 60 ($p < 0.01$) min after stress offset. These findings mirror what was seen following intra-medial PFC administration of AM251 to rats and support the hypothesis that CB_1 receptor activation is required for appropriate termination of glucocorticoid secretion following the cessation of stress.

Figure 2.1. CB_1 receptor signaling within the medial PFC is required for the decline of corticosterone levels following cessation of stress exposure.

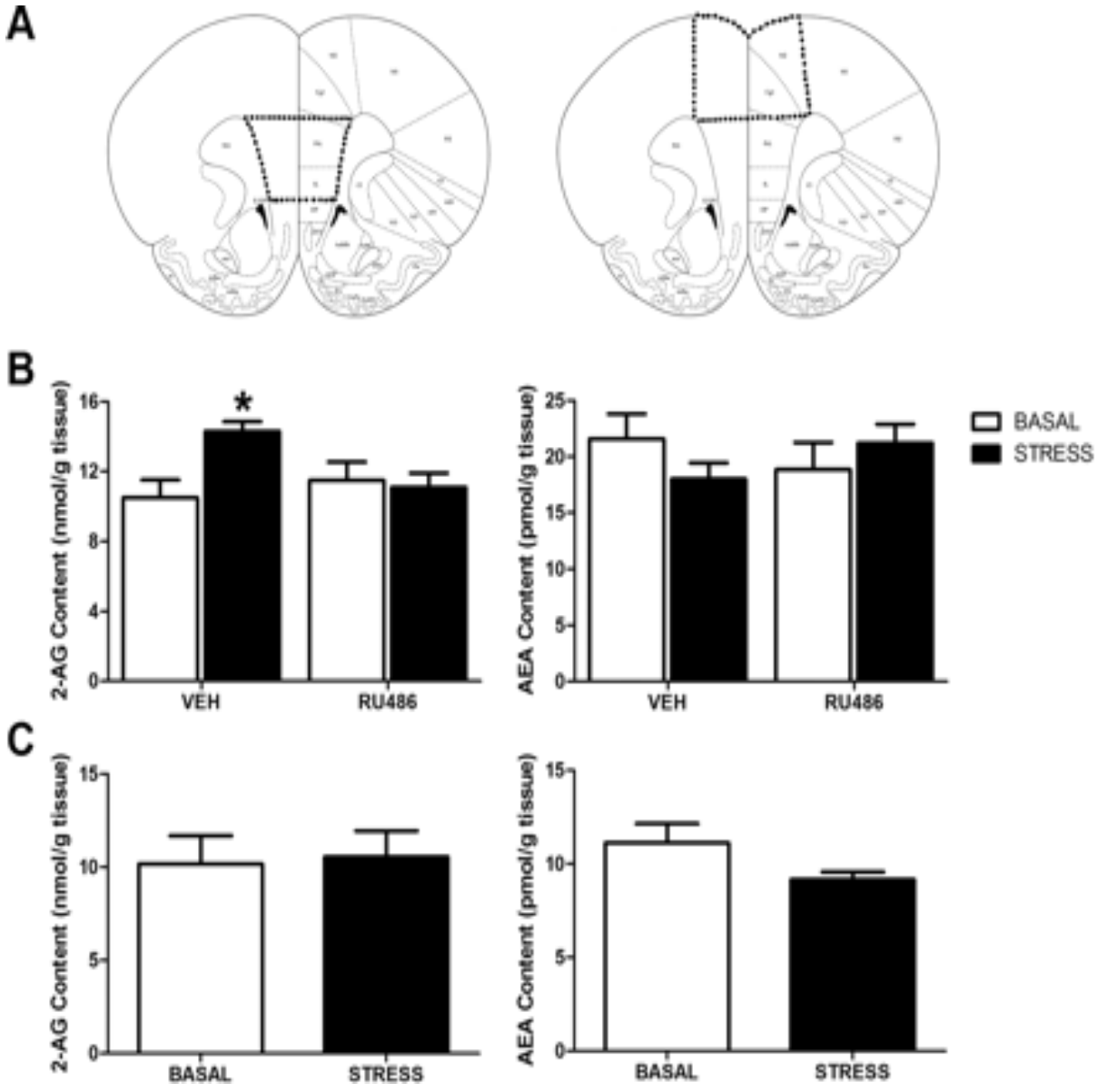


A. Representative photomicrograph of a cannula track terminating in the medial PFC. **B.** Local administration of the CB_1 receptor antagonist AM251 (0.28 ng/side) into the medial PFC of male Sprague Dawley rats prolonged corticosterone secretion following exposure to 30 min restraint stress (n = 6/condition; *p < 0.05 indicates a significant difference between AM251- and vehicle (VEH)-treated animals that have been exposed to stress). Animals exposed to stress are identified by square symbols, and animals not exposed to stress are identified by triangle symbols. Intracortical injections of VEH or AM251 occurred 10 min before stress induction. **C**. ICR mice lacking the CB_1 receptors (CB1KO) exhibited a prolongation in corticosterone secretion following exposure to 30 min of restraint stress compared to control (WT) (n = 8–9/condition; *p < 0.05 indicates a difference between CB1KO and WT mice).

2.3.2. Exposure to Stress Mobilizes 2-AG Within the Medial PFC

The requirement of CB_1 receptor activation in the medial PFC for HPA axis recovery following stress suggests that stress recruits endocannabinoid signaling in this brain region. To test this hypothesis, we determined the effects of stress exposure on endocannabinoid content within the medial PFC (composed of prelimbic and infralimbic cortices). Since the effects of intra-medial PFC AM251 treatment to delay stress recovery became apparent at 30 min post stress cessation, we analyzed endocannabinoid content at 30 min following stress cessation as well. The role of glucocorticoid receptor activation was determined in rats pre-treated with the glucocorticoid receptor antagonist, RU486 (20 mg/kg). Analysis of lipid extracts of medial PFC sections for endocannabinoid concentrations (see Fig. 2.2A for region of tissue analyzed) revealed a significant interaction between stress exposure and RU486 treatment on 2-AG content [$F_{(1, 23)} = 5.98$, $p < 0.03$; Fig. 2.2B]. Post hoc analysis demonstrated that tissue content of 2-AG within the medial PFC was elevated 30 min following the cessation of stress ($p < 0.01$). Systemic pretreatment with RU486 prevented the increase in 2-AG produced by stress. There was no main effect of either stress [$F_{(1, 23)} = .10$, $p > 0.05$] or RU486 treatment [$F_{(1, 23)} = 0.01$, $p > 0.05$] and no interaction [$F_{(1, 23)} = 2.25$, $p > 0.05$; Fig 2.2C] between stress exposure and RU486 treatment on AEA content. To determine the regional specificity of the stress effect on endocannabinoids within PFC subregions, we also assessed the effect of stress on endocannabinoid tissue contents within the dorsomedial PFC, composed of the motor and cingulate cortices (see Fig. 2.2A for region of tissue analyzed). Stress exposure did not significantly affect either 2-AG [$t_{(12)} = 0.86$, $p < 0.05$; Fig. 2.2C] or AEA [$t_{(12)} = 1.78$, $p < 0.05$; Fig. 2.2C] tissue content in the dorsomedial PFC.

Figure 2.2. Stress-induced mobilization of endocannabinoid content within the medial PFC depends on the glucocorticoid receptor.

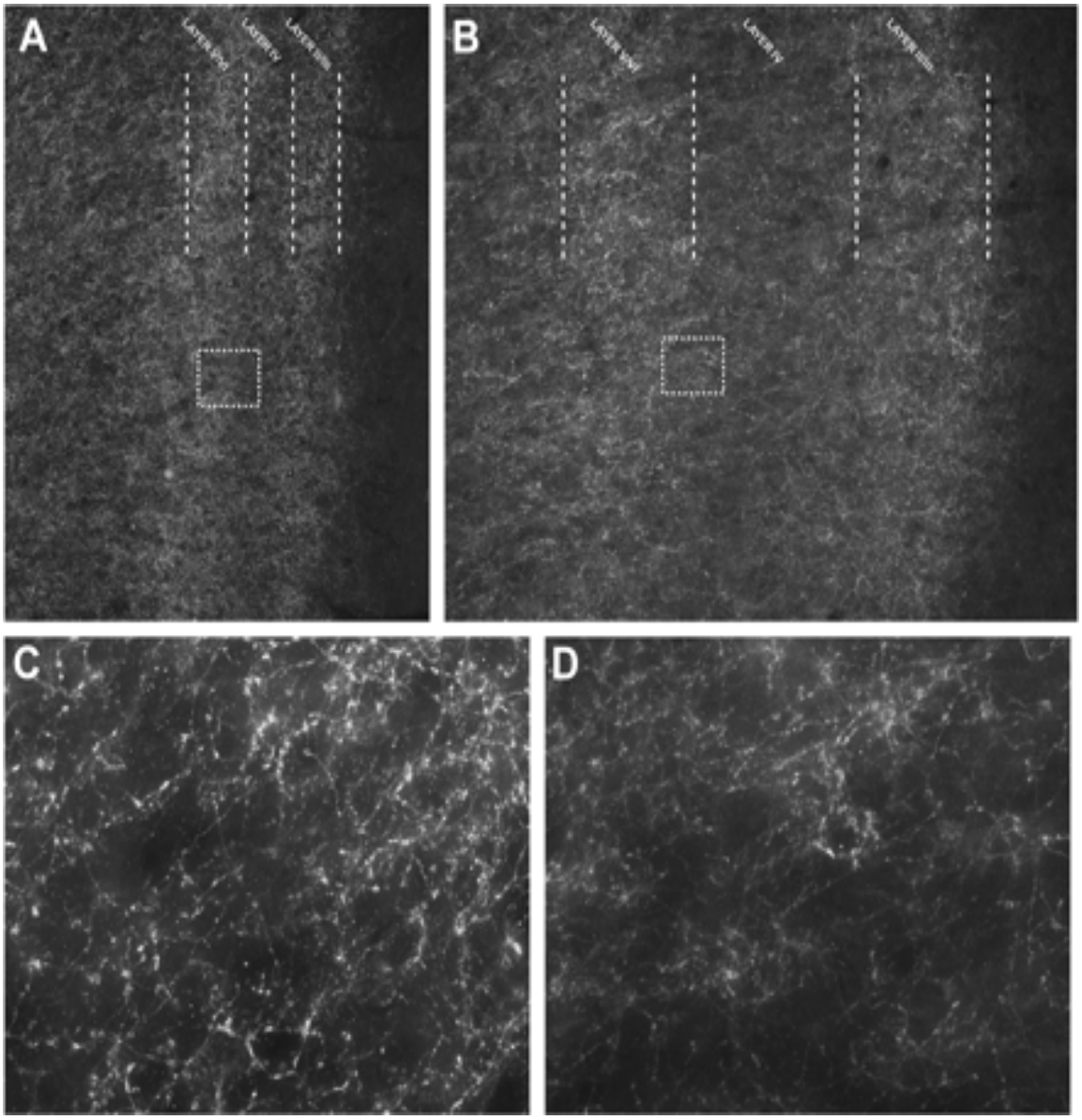


A. Diagrammatic representation of the regions of the frontal cortex, which were dissected out for AEA and 2-AG analysis: left, medial PFC; right, dorsal frontal cortex. **B.** The tissue content of 2-AG, but not AEA, was elevated within the medial PFC of male Sprague Dawley rats 30 min following a 30 min exposure to restraint stress. This effect was blocked by systemic pre-administration of RU486 (20 mg/kg), a glucocorticoid receptor antagonist (n = 7–8/condition; *$p < 0.05$). **C.** Restraint stress had no effect on 2-AG or AEA content within the dorsal region of the frontal cortex, primarily composed of motor cortex (n = 8/condition).

2.3.3. CB_1 Receptor Activation Inhibits GABA Release At Synapses With Principal Neurons Within Layer V of the Medial PFC

Increased activity of projection neurons from the medial PFC is required for termination of the HPA axis (Radley et al., 2006a; Radley et al., 2009) and CB_1 receptor activation is known to inhibit GABA release in many brain regions, including the PFC (Chiu et al., 2010). Thus, we explored the hypothesis that stress-induced endocannabinoid signaling disinhibits pyramidal neurons in the medial PFC via inhibition of GABA release. Our first objective was to examine CB_1 receptor expression in the medial PFC of both rats and mice since both species respond similarly to loss of CB_1 receptor signaling. Immunofluorescence studies using a CB_1 receptor polyclonal antibody revealed dense CB_1 receptor expression throughout layers II/III and V of the prelimbic region of the medial PFC in both rats (Fig. 2.3A) and mice (Fig. 2.3B), a region of the medial PFC particularly important for glucocorticoid-mediated regulation of the HPA axis and termination of stress-induced corticosterone secretion (Diorio et al., 1993; Radley et al., 2006a; Radley et al., 2009).

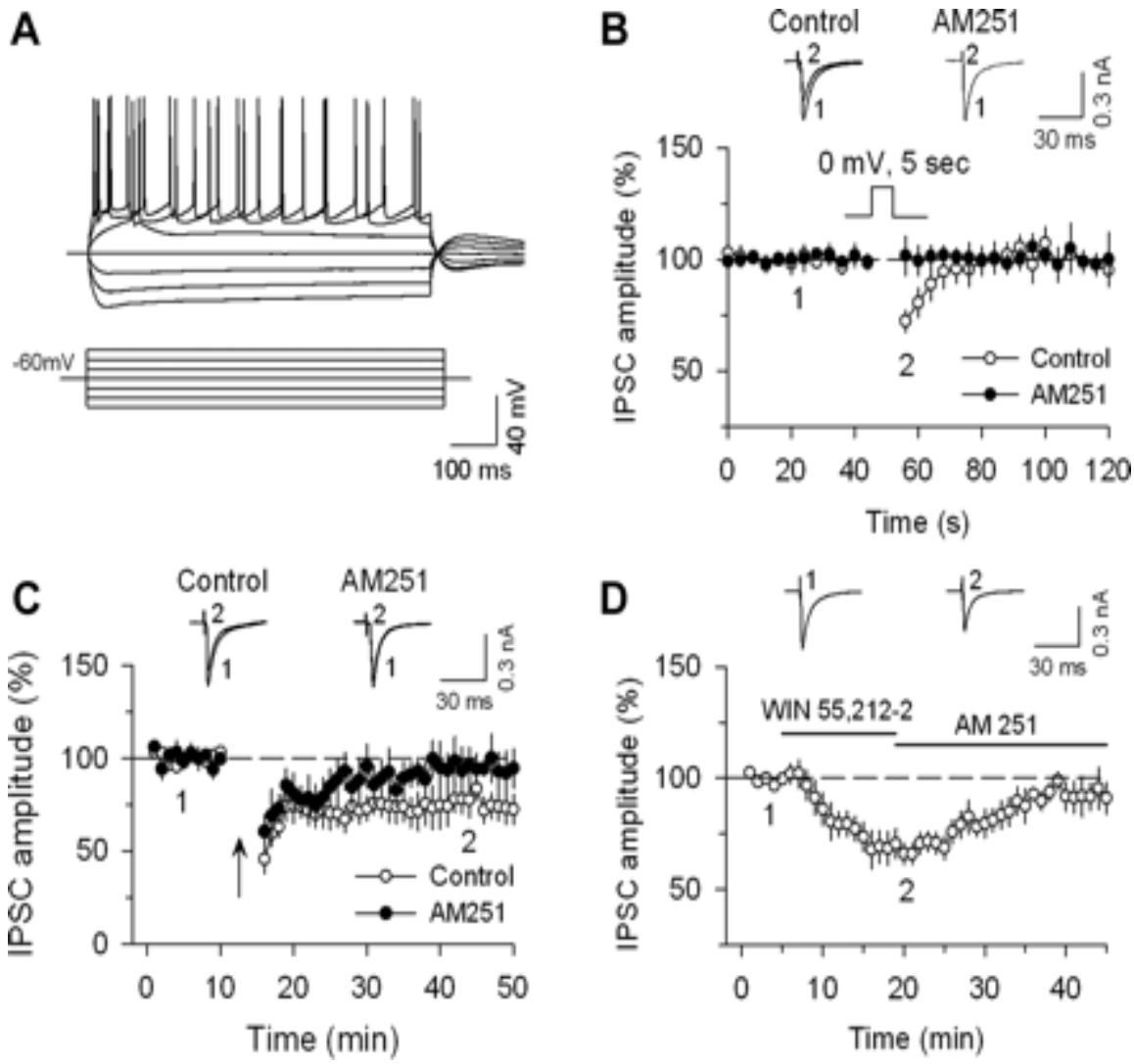
Figure 2.3. Distribution of CB_1 receptors within the prelimbic region of the medial PFC.



A and B. Immunofluorescence of CB_1 receptors within the prelimbic region of the medial PFC (at 10× magnification) demonstrates prominent expression of the CB_1 receptor within both layers II/III and layer V of the PFC of male C57BL/6J mice (**A**) and male Sprague Dawley rats (**B**). **C and D**. High magnification (60× magnification) of CB_1 receptor expression within layer V of the prelimbic region of the PFC reveals a punctate network of CB_1 receptor expression around cell bodies in both mouse (**C**) and rat (**D**).

Whole cell patch clamp electrophysiology in slices from adult male mice was used to examine CB_1 receptor regulation of GABA-mediated IPSCs in layer V principal neurons in the medial PFC. Glutamate receptor antagonists CNQX (20 μM) and D-AP-5 (20 μM) were included in the ACSF. Visually identified principal neurons exhibited spike frequency adaptation (Figure 2.4A), a common feature of pyramidal neurons. We recorded three types of endocannabinoid/CB_1 receptor-mediated responses, DSI, LTDi, and CB_1 receptor agonist-induced depression of IPSCs. Depolarization of principal neurons to 0 mV for 5 s induced a transient suppression of evoked IPSCs (DSI), which was abolished by 2 μM AM251 (Figure 2.4B). Following baseline recordings of evoked IPSCs at 0.1 Hz, a 10 Hz stimulation was applied for 5 min to induce LTDi. This stimulation protocol induced a long-lasting depression of IPSCs in medial PFC slices, which was blocked by 2 μM AM251 (Figure 2.4C). This is consistent with LTDi described in other brain regions, as well as a recent report of a comparable phenomenon in the frontal cortex, which is influenced by dopaminergic signaling (Edwards et al., 2006; Chiu et al., 2010). Bath application of the CB_1 receptor agonist WIN55212-2 (2 μM) induced significant depression of evoked IPSCs, which was blocked by the CB_1 receptor antagonist AM251 (4 μM; Figure 2.4D). Thus, GABAergic synapses impinging on principal neurons in layer V of the prelimbic region of the medial PFC exhibit characteristics of endocannabinoid/CB_1 receptor-mediated regulation and synaptic plasticity.

Figure 2.4. CB_1 receptor regulation of GABA-mediated currents within layer V principal neurons of the medial PFC.

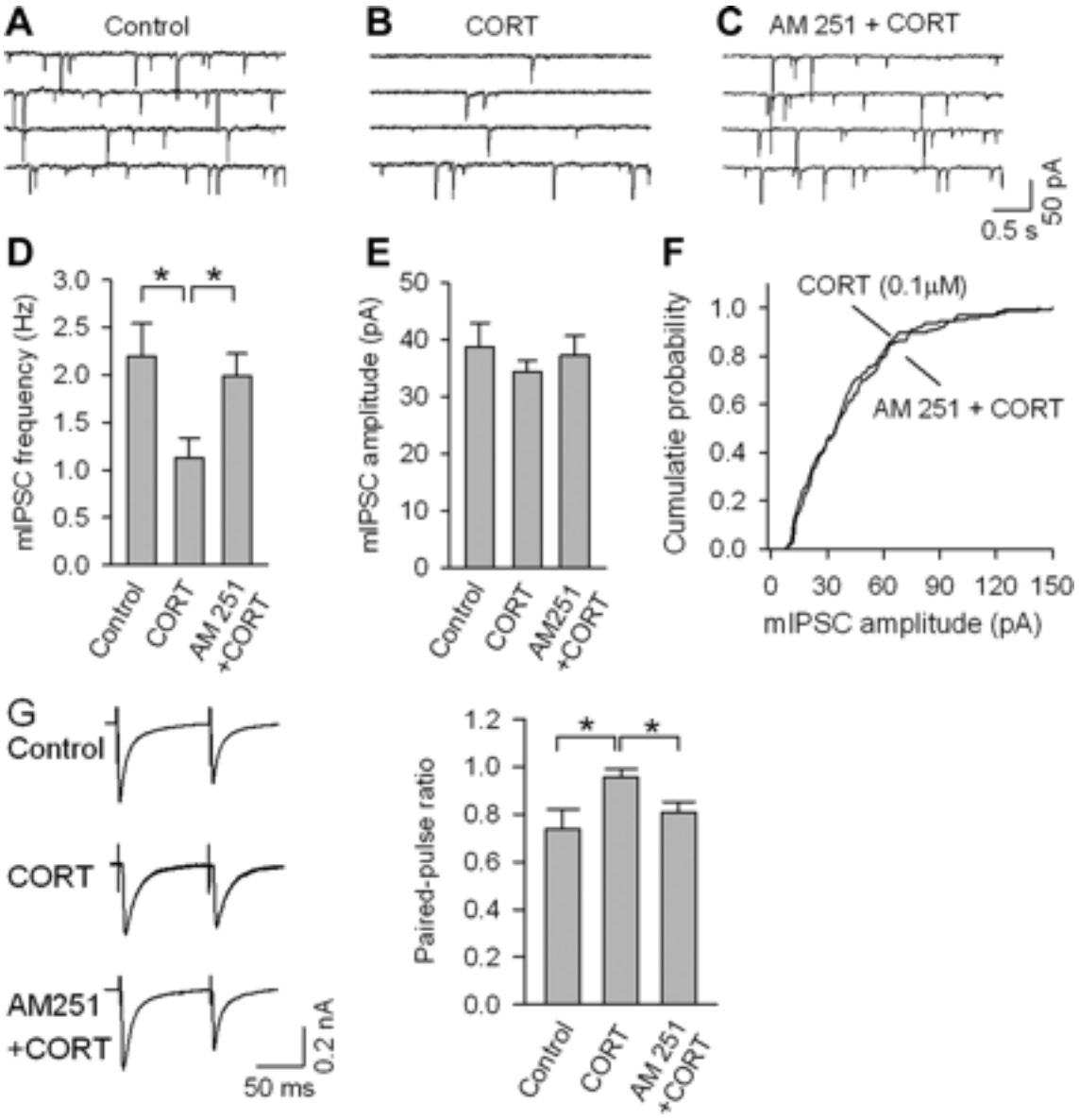


A. Current-clamp recordings from a pyramidal neuron within layer V of the prelimbic region of the medial PFC of ICR mice at a membrane potential of −60 mV during injection of depolarizing and hyperpolarizing current pulses of different intensities (pulses are shown in inset below the responses). A typical pyramidal neuron shows spike frequency adaptation. **B.** Depolarization from −60 to 0 mV for 5 s induced DSI (n = 11), which was blocked by the CB_1 receptor antagonist AM251 (2 μM; n = 8; $p < 0.05$ vs. control). **C.** Repetitive stimulation (10 Hz for 5 min; indicated by arrow) of synaptic afferents induced LTDi (n = 7, $p < 0.05$ vs. baseline), which was blocked by AM251 (n = 7, $p > 0.05$ vs. baseline). **D.** Bath application of the CB_1 receptor agonist WIN 55212–2 (2 μM) depressed evoked IPSC amplitude, and this depression was reversed after addition of AM251 (4 μM, n = 7).

2.3.4. Glucocorticoids Suppress GABA Release in the Medial PFC Via an Endocannabinoid Mechanism

We hypothesized that stress-induced increases in corticosterone levels activate principal neuron outflow via CB_1 receptor-mediated disinhibition of principal neurons in layer V of the prelimbic region of the medial PFC. To test this hypothesis, corticosterone (100 nM) was applied to slices containing the medial PFC and spontaneous mIPSCs and evoked IPSCs were analyzed. mIPSCs were recorded in the presence of the Na^+ channel blocker TTX (0.5 μM). Since the neuroendocrine and biochemical studies reported above indicate that the effects of glucocorticoids on endocannabinoid signaling are measureable 1 hr following the onset of stress, we performed electrophysiological recordings a minimum of 1 hr following incubation with corticosterone. Preincubation of the slices with corticosterone (100 nM, 20 min) significantly decreased the frequency of mIPSCs, and this effect was blocked by AM251 ($F_{(2,22)} = 4.69$, $p < 0.05$; Fig 2.5D). Corticosterone application had no significant effect on the mean amplitude of IPSCs ($F_{(2,22)} = 0.48$, $p > 0.05$; Fig 2.5E) or the cumulative probability distribution of the amplitude ($p > 0.05$; Fig. 2.5F). To further delineate the mechanism of corticosterone-mediated inhibition of IPSCs, we examined the PPR (paired-pulse interval = 100 ms) in control and corticosterone-treated slices. In control slices, the PPR, calculated as IPSC2/IPSC1, was significantly below one, indicative of a paired-pulse depression of IPSCs (Wilcox and Dichter, 1994). In corticosterone-treated slices, the PPR was significantly increased ($F_{(2,23)} = 4.03$, $p < 0.05$; Fig 2.5G), which is consistent with an effect of corticosterone to inhibit presynaptic GABA release. The effect of corticosterone on PPR was abolished by AM251.

Figure 2.5. Corticosterone depresses GABA-mediated currents in layer V principal neurons of the medial PFC via recruitment of endocannabinoid signaling.



A–C. mIPSCs recorded in layer V pyramidal neurons in medial PFC slices from ICR mice encompassing the prelimbic cortex that were treated with vehicle (control), or corticosterone (100 nM, 20 min) alone, or in combination with AM251 (2 μM). **D.** Corticosterone treatment decreased the mean frequency of mIPSCs, and this effect was blocked by AM251 (n = 8–9; *p < 0.05). **E and F.** Corticosterone treatment had no significant effect on the mean amplitude of mIPSCs (**E**) or the cumulative amplitude distributions of mIPSCs (**F**). **G.** Corticosterone treatment (100 nM, 20 min) increased the paired-pulse ratio in layer V pyramidal neurons of the medial PFC, and this effect was blocked by AM251 (2 μM; n = 8–10, *p < 0.05).

2.4. Discussion

Termination of HPA axis activation following exposure to stress is essential to limit the duration of glucocorticoid secretion and prevent the deleterious effects of persistently elevated levels of glucocorticoid hormones on cardiovascular, immune, metabolic and neural systems. Our data demonstrate that stress, via activation of glucocorticoid receptors, increases 2-AG levels within the medial PFC, and that endocannabinoid signaling is required for the appropriate termination of glucocorticoid secretion following cessation of stress. Immunohistochemical data indicate that CB_1 receptors are expressed on GABAergic terminals in layer V of the prelimbic region of the medial PFC, particularly on axons that synapse onto the soma of principal neurons. Functional studies support a role for CB_1 receptors to inhibit GABA release within this same neuronal population. Finally, our data demonstrate that incubation of slices from the medial PFC with corticosterone results in enhanced endocannabinoid-mediated inhibition of GABA release onto principal neurons. Collectively, these data support the hypothesis that glucocorticoid hormones released following stress exposure activate endocannabinoid/CB_1 receptor signaling within the medial PFC, inhibiting GABA release onto layer V pyramidal neurons in the prelimbic cortex. Consistent with this model, pharmacological disruption of $GABA_A$ receptor signaling within the medial PFC also decreases stress-induced activation of the HPA axis (Weinberg et al., 2010). Collectively, these data indicate that prefrontocortical endocannabinoid signaling links glucocorticoids and neuronal activation within the medial PFC and contributes to the long negative feedback loop responsible for terminating HPA axis activity following cessation of stress.

The present data are consistent with the established role of the medial PFC, and particularly the prelimbic region of the medial PFC, in the regulation of the HPA axis and termination of the stress response. Lesion studies have demonstrated have demonstrated that selective ablation of the prelimbic region of the PFC does not alter the magnitude, but rather the duration, of corticosterone secretion following exposure to a psychogenic stressor (Diorio et al., 1993; Radley et al., 2006a; Radley et al., 2009). In the present study, both genetic deletion of the CB_1 receptor, and local antagonism of CB_1 receptor signaling within the medial PFC prolonged the elevation in stress-induced levels of circulating corticosterone. These data confirm an important role for the prelimbic region of the medial PFC in the regulation of HPA axis activity during the recovery period following stress induction, and further indicate that activation of CB_1 receptor signaling within this brain region is required for normative decline in circulating corticosterone following stress. Our histological and electrophysiological experiments demonstrate the presence of CB_1 receptors on GABAergic terminals impinging upon pyramidal neurons within layer V of the prelimbic region of the medial PFC. These findings support the hypothesis that activation of CB_1 receptor signaling in this brain region will result in disinhibition of excitatory projections from the prelimbic medial PFC to other brain regions.

The circuit by which the efferent projection neurons from the prelimbic region of the PFC inhibit the HPA axis involves activation of inhibitory GABAergic neurons within subregions of the BNST (Spencer et al., 2005; Radley et al., 2009) or the peri-PVN region (Herman et al., 2005). Activation of each of these inhibitory circuits dampens neuronal activation of the CRH-secreting cells of the PVN (Herman et al.,

2005). Layer V of the prelimbic cortex is the primary site for projection neurons that extend to subcortical limbic structures (Gabbott et al., 2005), such as the BNST; thus, the identification of endocannabinoid-mediated regulation of neuronal excitability within layer V neurons in the prelimbic region of the medial PFC provides a neurochemical and functional mechanism that compliments the previously established neuroanatomical networks involved in prefrontocortical regulation of HPA axis activity.

Recent data indicate that excitatory afferents arising from the ventral subiculum activate the same inhibitory relays within the BNST as are activated by excitatory afferents originating from the medial PFC (Radley and Sawchenko, 2011). These data suggest that both hippocampal and medial PFC projections are involved in the long glucocorticoid-mediated negative feedback loop via inputs into the BNST. In the current study, disruption of endocannabinoid signaling within the medial PFC attenuated, but did not completely prevent, the return of circulating glucocorticoids to baseline concentrations. It is possible that the projections from the ventral subiculum to the BNST, which would not be affected by CB_1 receptor blockade in the medial PFC, are responsible for the ultimate return of circulating corticosterone concentrations to baseline.

The activation of these efferent projections from the medial PFC has been shown to function as a prominent pathway in glucocorticoid-mediated negative feedback. Local activation of glucocorticoid receptors within the medial PFC accelerates the decline in circulating levels of corticosterone following exposure to stress (Diorio et al., 1993) and downregulation of glucocorticoid receptors within the medial PFC following chronic stress or in aging is associated with impaired glucocorticoid feedback (Mizoguchi et al.,

2003; Mizoguchi et al., 2009). Unlike the local glucocorticoid effects in the PVN that rapidly decrease activation of CRH neurosecretory cells governing HPA axis output and promote fast feedback inhibition on the HPA axis (Di et al., 2003; Evanson et al., 2010), disruption of the medial PFC to PVN circuit prolongs the recovery to normal circulating glucocorticoids levels following stress exposure (Diorio et al., 1993; Radley et al., 2006a; Radley et al., 2009). As such, these data demonstrate that glucocorticoid-mediated negative feedback possesses both short-loop (locally within the PVN) and long-loop (distally within the medial PFC and ventral subiculum) components. Moreover, there is evidence that endocannabinoid signaling contributes to both of these phases of glucocorticoid feedback. The current data create an argument for a role of prefrontal cortical endocannabinoid signaling in the long-loop phase of glucocorticoid feedback. Additionally, it has recently been reported that local antagonism of the CB_1 receptor within the PVN impairs fast-feedback inhibition of HPA axis activity by glucocorticoids (Evanson et al., 2010). In the present study, we report that mice globally deficient in CB_1 receptors exhibit a larger peak in corticosterone secretion following stress, which is consistent with these mice lacking fast-feedback inhibition due to the absence of CB_1 receptor signaling within the PVN.

Taking these data together, we propose the following model for the integration of endocannabinoid signaling into the temporal phases of glucocorticoid feedback. Glucocorticoid hormones are released into the circulation in response to stress. In the PVN, these glucocorticoids evoke a rapid induction of endocannabinoid release through a non-genomic pathway, which results in a rapid suppression of glutamatergic inputs to CRH neurosecretory cells and decreases the excitatory drive to the HPA axis (Di et al.,

2003; Evanson et al., 2010). In the medial PFC, glucocorticoids produce a time-delayed increase in 2-AG, which, via CB_1 receptor activation, suppresses GABAergic inputs to principal neurons. This suppression of GABAergic inputs to principal neurons could act to increase the outflow of these projection neurons to inhibitory relays within the BNST, and thus contribute to the long loop of glucocorticoid negative feedback. In sum, our model proposes a temporally and structurally specific role of endocannabinoid signaling in distinct phases of glucocorticoid feedback.

The mechanism by which glucocorticoids regulate endocannabinoid signaling was not determined in the current study, but appears to be distinct from the process that occurs within the PVN. In the PVN, glucocorticoid regulation of endocannabinoid signaling is not blocked by an antagonist of the nuclear glucocorticoid receptor and is driven by glucocorticoid-induced G-protein signaling (Di et al., 2003). Furthermore, administration of glucocorticoids in the absence of stress can rapidly (~10 min) increase endocannabinoid content within the hypothalamus but not the medial PFC (Hill et al., 2010a). Thus, we hypothesize that the actions of glucocorticoids on endocannabinoid content within the medial PFC require coincident increases in neuronal activation, which occur following exposure to stress to produce a detectable increase in 2-AG content using bulk tissue measurements.

Termination of HPA axis activation following exposure to stress is an essential process for maintaining optimal health in the face of persistent stress. The data presented herein suggest an important role of the endocannabinoid system within the medial PFC in the termination of HPA axis activity following exposure to stress. The induction of 2-AG mobilization within the medial PFC following exposure to stress provides a mechanism

of coincidence detection that can fine tune the excitability of pyramidal neurons within the prelimbic region of the medial PFC and contribute to termination of HPA axis following cessation of stress exposure. These data contribute to our general understanding of the mechanisms subserving glucocorticoid-mediated negative feedback and stress recovery. Furthermore, they provide a mechanism that could underlie modulation by glucocorticoids of neuronal sensitivity in extrahypothalamic structures that contribute to feedback and recovery.

3. Prefrontocortical Anandamide Signaling Coordinates Coping Responses to Stress Through a Serotonergic Pathway[2]

3.1. Introduction

The endocannabinoid system has recently surfaced as a promising therapeutic candidate for the treatment of emotional disorders where stress is a contributing factor, and converging evidence has revealed a complex bidirectional relationship between endocannabinoid signaling and the neural stress circuit (Hill and McEwen, 2010; Riebe and Wotjak, 2011). Interactions with this circuit occur via stress-induced regulation of the endocannabinoid ligands AEA and 2-AG (Hill and McEwen, 2010) (Patel and Hillard, 2008). These neuroactive lipid signaling molecules activate presynaptic CB_1 receptors, which results in the inhibition of excitatory, inhibitory, and monoaminergic neurotransmitter release throughout stress-responsive corticolimbic brain regions including the PFC, amygdala, hippocampus, and hypothalamus (Freund et al., 2003). Following CB_1 receptor activation, these endocannabinoids are then metabolized by their respective degradative enzymes; FAAH metabolizes AEA while MGL is responsible for the degradation of 2-AG (Ahn et al., 2008). Alterations within any of the components of the endocannabinoid system can have profound consequences for proper stress responses (Finn, 2010; Hill and McEwen, 2010; Riebe and Wotjak, 2011).

Given the extensive presence of endocannabinoid signaling within corticolimbic circuits and its sensitivity to regulation by stress, it is not surprising that endocannabinoid

[2]A version of this chapter has been accepted for publication in *European Neuropsychopharmacology*. McLaughlin RJ, Hill MN, Bambico FR, Stuhr KL, Gobbi G, Hillard CJ, Gorzalka BB. Prefrontal cortical anandamide signaling coordinates coping responses to stress through a serotonergic pathway. Accepted January 12, 2012.

signaling is involved in the regulation of emotional behavior. Accordingly, facilitation of endocannabinoid signaling through the inhibition of AEA hydrolysis by FAAH has been shown to promote active coping responses to stress and reduce anxiety-like responses to aversive environmental stimuli (Gobbi et al., 2005; Patel and Hillard, 2006; Bortolato et al., 2007; Moreira et al., 2008; Haller et al., 2009; reviewed in Hill et al., 2009a; Zanettini et al., 2011). One putative mechanism subserving the ability of FAAH inhibition to modulate stress coping behavior is that stress exposure rapidly activates FAAH, which downregulates AEA signaling in corticolimbic structures such as the amygdala (Hill et al., 2009a). As such, this rapid loss in AEA/CB_1 signaling in response to stress could be a contributing factor to stress-induced alterations in emotional behavior. It is plausible that this endogenous mechanism may also dictate responses to stress within the PFC, a structure known to participate in stress coping strategies and emotional behavior, although this hypothesis has yet to be empirically validated.

Accordingly, to investigate whether stress-induced regulation of prefrontocortical endocannabinoid signaling contributes to changes in emotional behavior, we coupled biochemical analyses of endocannabinoid signaling following exposure to swim stress with local pharmacological manipulation of AEA hydrolysis. Since recent studies have demonstrated that the ventromedial subregion of the PFC is particularly essential for the modulation of emotional behavior by cannabinoid signaling (Bambico et al., 2007; Rubino et al., 2008b), our focus was on this brain region. Moreover, given the established role of 5-HT signaling in promoting active coping responses to swim stress (Cryan et al., 2005), we also sought to determine whether the ability of prefrontocortical

endocannabinoid signaling to modulate coping behaviors in the FST is dependent on 5-HT neurotransmission.

3.2. Materials and Methods

3.2.1. Subjects

Male Sprague Dawley rats (Charles River Breeding, Montreal, Canada; 300-350 g) were pair-housed unless they had been implanted with cannula, in which case they were individually housed in a room maintained at a temperature of 21±1°C with ad libitum food and water access. All experimental testing was conducted in accordance with the guidelines of the Canadian Council of Animal Care and was approved by the Animal Care Committee of the University of British Columbia.

3.2.2. Biochemical Studies

The medial PFC was rapidly harvested as described in Chapter 2 at four time points: no stress (Day 1 Basal), immediately following the initial 15 min swim stress exposure (Day 1 Stress), 24 hr following the first swim exposure (Day 2 Basal) and immediately following the 5 min swim stress exposure (Day 2 Stress). The medial PFC was dissected as a tissue block that was anatomically defined as the area dorsal to the anterior olfactory nucleus, ventral to the motor cortex and medial to the corpus callosum and claustrum formation. Tissue sections were frozen in liquid nitrogen within 5 min of decapitation and stored at -80 ºC until analysis. All cohorts for biochemical analyses consisted of 7-8 rats per group. For endocannabinoid analysis, lipids were extracted from tissue and contents of AEA and 2-AG were determined using isotope-dilution, liquid chromatography/mass spectrometry as described in Section 2.2.2.2.

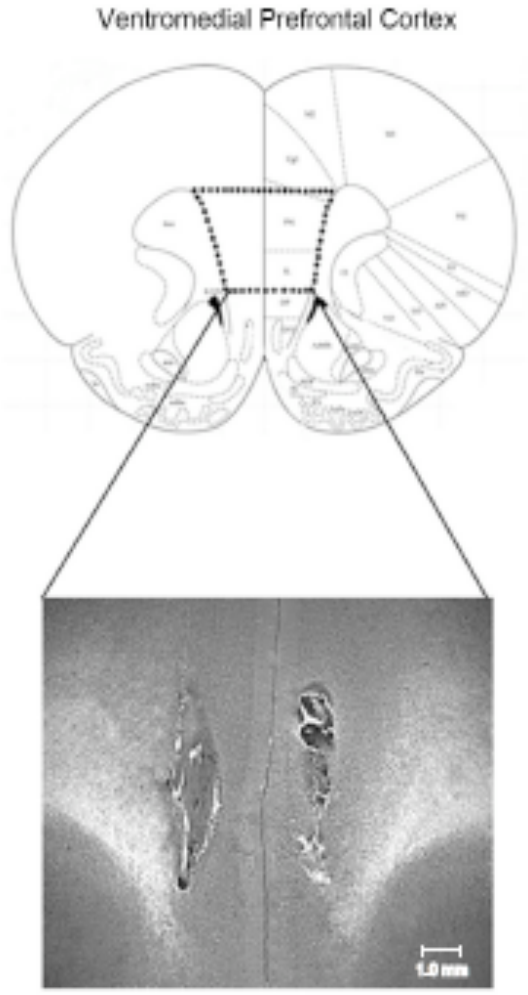
3.2.3. Behavioral Studies

Cylindrical glass containers (diameter 35 cm and height 45 cm) filled to 30 cm with water at a temperature of 24±1°C were used for all swim stress exposures. For both biochemical and behavioral studies, two swim sessions were employed. The first session was of 15 min duration and the second session of 5 min duration was performed 24 hr thereafter. Consistent with the analysis of stress coping behaviors in the FST (Porsolt et al., 2001), the first 15-min session was treated as an exposure session, while the second 5-min session was treated as the test session. Stress coping behaviors were analyzed during the 5-min session on day 2. In each study, animals were subjected to forced swim stress 1 hr after intra-PFC drug administration. Both active (swimming and struggling) and passive coping responses (immobility) were recorded and scored by trained observers blinded to treatment conditions. Immobility was defined as when the rat was stationary and only made the minimal movements necessary to stay afloat; swimming was defined as when the rat was actively moving at least two limbs to induce active motion around the swim chamber; struggling was defined as when the rat was thrashing with its forepaws above the water surface and was in a vertical position (Detke et al., 1995). The duration of each of these behaviors during the test session was scored.

In order to analyze the effects of intra-PFC inhibition of FAAH on coping behaviors in the FST, cannula were implanted bilaterally into the ventromedial PFC (coordinates from bregma: AP = +3.0mm, ML = ±0.7mm, DV = -3.4mm from dura mater). Four steel screws and dental acrylic were used to permanently affix the guide cannula to the skull. Behavioral testing began 7–10 days after the implantation surgery.

The FAAH inhibitor URB597 (Cayman Chemical; Ann Arbour, MI, USA), or its vehicle (1:9 DMSO: 0.9 % saline; injection volume of 200 nl for all infusions), was infused at a dose of 0.01 μg bilaterally into the ventromedial PFC 60 min prior to day 1 swim exposure, 60 min prior to day 2 swim exposure, or 60 min prior to both sessions. This dose was based on that demonstrated by Rubino and colleagues to increase AEA content and influence anxiety-like behavior (Rubino et al., 2008b). To determine whether the behavioral effect of intra-ventromedial PFC administration is a CB_1 receptor-dependent phenomenon, a separate group of rats received a cocktail of URB597 and the CB_1 receptor antagonist AM251 60 min prior to day 1 and day 2 swim exposure (0.01 μg URB597: 0.28 ng AM251). Another cohort of rats was used to determine whether 5-HT activity might mediate the effects of intra-ventromedial PFC URB597 administration. Vehicle or p-chlorophenylalanine (pCPA; 350 mg/kg,), a selective inhibitor of 5-HT synthesis, was administered via intraperitoneal (IP) injection 72 and 48 hr before URB597 administration and subsequent swim exposure. All cohorts for behavioral testing consisted of 7-8 rats per group. Following behavioral testing, placements were verified employing the stereotaxic atlas of Paxinos and Watson (1998). Histological analysis revealed that approximately 90% of cannula placements were in boundaries of the region of interest (see Fig. 3.1 for a representative photomicrograph illustrating bilateral cannula placement). Subjects with cannula outside of the desired subregion were excluded from subsequent analyses.

Figure 3.1. Representative illustrations depicting the boundaries for tissue extraction and cannula placements targeting the ventromedial PFC.



Coronal brain slice (3.0 mm anterior to bregma) depicting the ventromedial PFC tissue section extracted for biochemical studies, consisting of the prelimbic and infralimbic cortices (above). Representative bilateral cannula placement targeting the ventromedial PFC (below). All placements were confirmed according to the rat brain atlas of Paxinos and Watson (1998).

3.2.4. In Vivo Electrophysiology Studies

In *vivo* extracellular single-unit recordings of putative dorsal raphe 5-HT neurons were performed, as previously described (Bambico et al., 2007), to examine whether the firing rate of this subpopulation of neurons is affected by bilateral intra-ventromedial PFC administration of 0.01 μg URB597. Rats were anaesthetized with chloral hydrate (400 mg/kg, IP) and mounted in a stereotaxic frame. Extracellular single-unit recordings were performed using single-barreled glass micropipettes pulled from 2 mm Stoelting capillary glass on a Narashige (Tokyo, Japan) PE-21 pipette puller and preloaded with fiberglass strands to promote capillary filling with 2% Pontamine Sky Blue dye in sodium acetate (0.5 M, pH 7.5). The micropipette tips were broken down to diameters of 1–3 μm. Electrode impedances ranged from 2 to 4 MΩ. A burr hole was drilled on the cranial midline subtending regions above the entire rostrocaudal medial extent of the dorsal raphe, a region considered richest in 5-HT neurons (Descarries et al., 1982). The electrode was lowered 0.5–1.0 mm posterior to the interaural line on the midline and 2.5–3.5 mm from the dura mater, just beneath the Sylvian aqueduct. The first putative 5-HT neurons were encountered immediately within this region, and 5-HT neuron-containing coordinates stretch from 5.0 to 6.5 mm ventral to the dura mater. Single-unit 5-HT activity was recorded as discriminated action potentials amplified by a Tennelec (Oakridge, TN) TB3 MDA3 amplifier, postamplified and filtered by a Realistic 10 band frequency equalizer, digitalized by a CED1401 interface system (Cambridge Electronic Design, Cambridge, UK), processed on-line, and analyzed off-line by Spike2 software version 5.05 for Windows PC (Microsoft, Seattle, WA). Under physiological conditions, 5-HT neurons exhibit characteristic electrophysiological properties distinguishable from

non-5-HT neurons. These 5-HT neurons exhibit a slow (0.1–4 Hz) and a prominently regular firing rate (coefficient of variation ranges from 0.12 to 0.87), a broad biphasic (positive–negative) or triphasic waveform (0.8–3.5 ms; 1.4 ms first positive and negative deflections) (Baraban and Aghajanian, 1980; Allers and Sharp, 2003; Bambico et al., 2007). When the regularity of firing was apparently altered, putative 5-HT neurons were distinguished based on firing rate, spike shape, and duration, which are reliable markers for 5-HT neurons. Cannula aimed at the ventromedial PFC were also bilaterally implanted as described above. Once a stably firing 5-HT neuron was found and 1–3 min of baseline activity was established, microinfusion of URB597 or vehicle was administered directly into the ventromedial PFC and changes in the neuronal discharge pattern of dorsal raphe 5-HT neurons were monitored over the following 120 min. At the end of each experiment, the recording site was marked by iontophoretic ejection (5–10 μA, negative current for 10 min) of Pontamine Sky Blue for histological verification.

3.2.5. Statistics

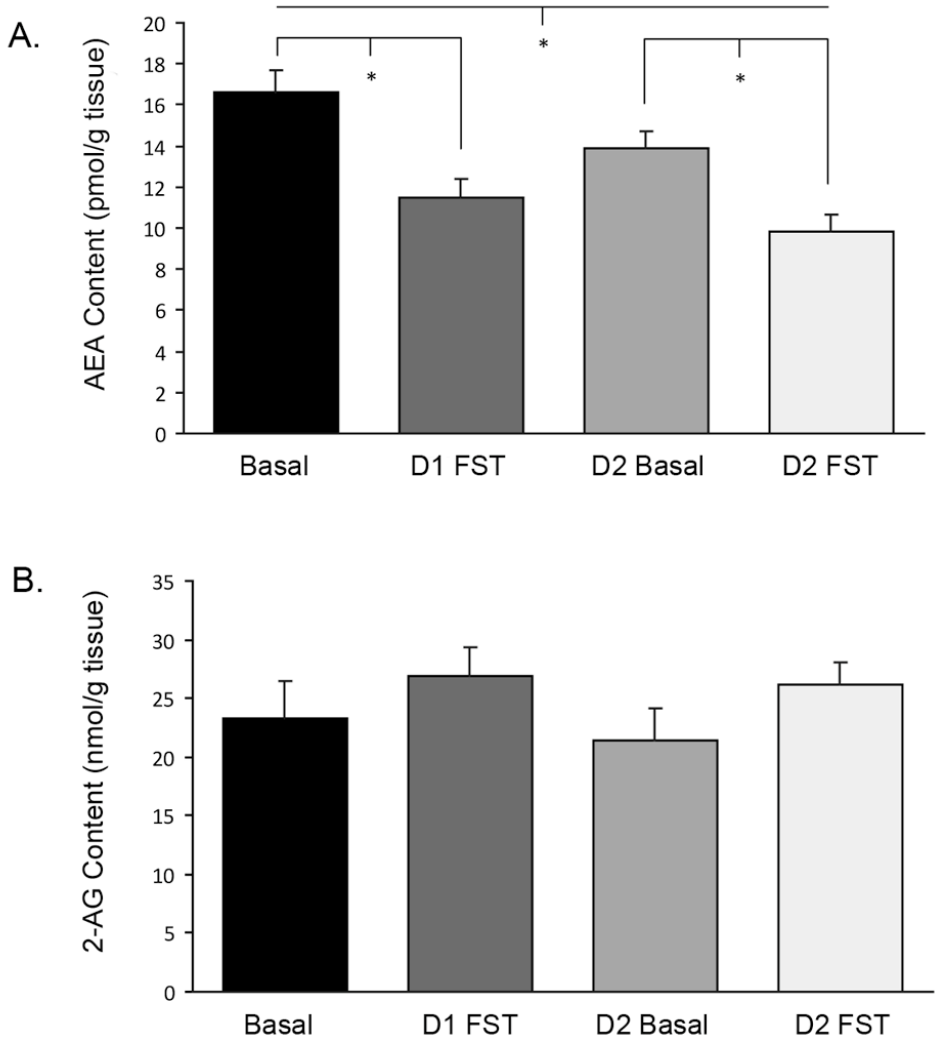
A one-way ANOVA was used to analyze the effects of swim stress exposure on PFC tissue content of AEA and 2-AG as well as the effects of intra-ventromedial PFC drug administration on behavioral parameters during swim sessions. An independent t-test was used to compare firing rates of 5-HT neurons following intra-ventromedial PFC URB597 or vehicle injections. All post-hoc analyses were performed using Dunnett's tests. All statistical significance levels were set at 0.05 and data are presented as means +/- standard error of the mean (SEM).

3.3. Results

3.3.1. Forced Swim Exposure Rapidly Suppresses Fatty Acid Ethanolamide Content in the Medial PFC

There was a significant effect of swim stress exposure on AEA content within the medial PFC [$F_{(3, 30)} = 11.23$, $p < 0.001$], with post-hoc analysis revealing that relative to day 1 basal, AEA content was significantly reduced following both day 1 and day 2 stress (both p's < 0.001). There was no difference between basal day 1 and basal day 2 AEA content, although exposure to the 5 min swim stress session on day 2 reduced AEA content relative to day 2 basal levels ($p < 0.02$; Fig. 3.2A). Consistent with these changes in AEA content, there was also significant effects of swim stress exposure on the tissue contents of both palmitoylethanolamide (PEA) [$F_{(3, 30)} = 3.58$, $p < 0.03$; Table 3.1] and oleoylethanolamide (OEA) [$F_{(3, 30)} = 4.17$, $p < 0.02$; Table 3.1] within the medial PFC, such that both PEA and OEA were significantly reduced on both day 1 and day 2 of swim stress exposure, relative to basal values (all p's < 0.05). These data indicate that stress increased metabolism of all fatty acid ethanolamides, consistent with a rapid increase in FAAH activity. There was no effect of swim stress on 2-AG content at any time point [$F_{(3, 30)} = 0.90$, $p > 0.05$; Fig. 3.2B].

Figure 3.2. Forced swim stress rapidly suppresses AEA content in the medial PFC.



A. Forced swim stress produced a rapid decline in medial PFC AEA content immediately following both day 1 (D1 FST) and day 2 (D2 FST) test sessions, relative to stress-naïve rats (Basal). Moreover, AEA content was also significantly decreased following forced swim exposure on day 2 (D2 FST) compared to animals sacrificed just prior to the day 2 test session (D2 Basal). **B.** Forced swim stress did not significantly alter 2-AG levels in the medial PFC at any time point relative to stress-naïve rats. Values are expressed as mean tissue levels ± SEM (n = 7-8 / treatment condition). * denotes significant differences at $p < 0.05$.

Table 3.1. Effect of forced swim stress on tissue content of fatty acid ethanolamides in the medial PFC.

	Day 1 Basal	**Day 1 FST**	**Day 2 Basal**	**Day 2 FST**
PEA Content (pmol/g)	129.2 +/- 10.6	102.5+/- 4.9*	111.9 +/- 6.6	100.8 +/- 3.0*
OEA Content (pmol/g)	60.1 +/- 5.8	42.2 +/- 2.4*	57.8 +/- 5.8	44.8 +/- 0.8*

Forced swim stress evoked a rapid decline in the tissue content of both palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) within the medial PFC on both the first and second days of exposure to swim stress. Data are presented as mean values +/- SEM (n = 7-8 / condition). * denotes significant differences relative to animals measured under basal conditions (D1 basal).

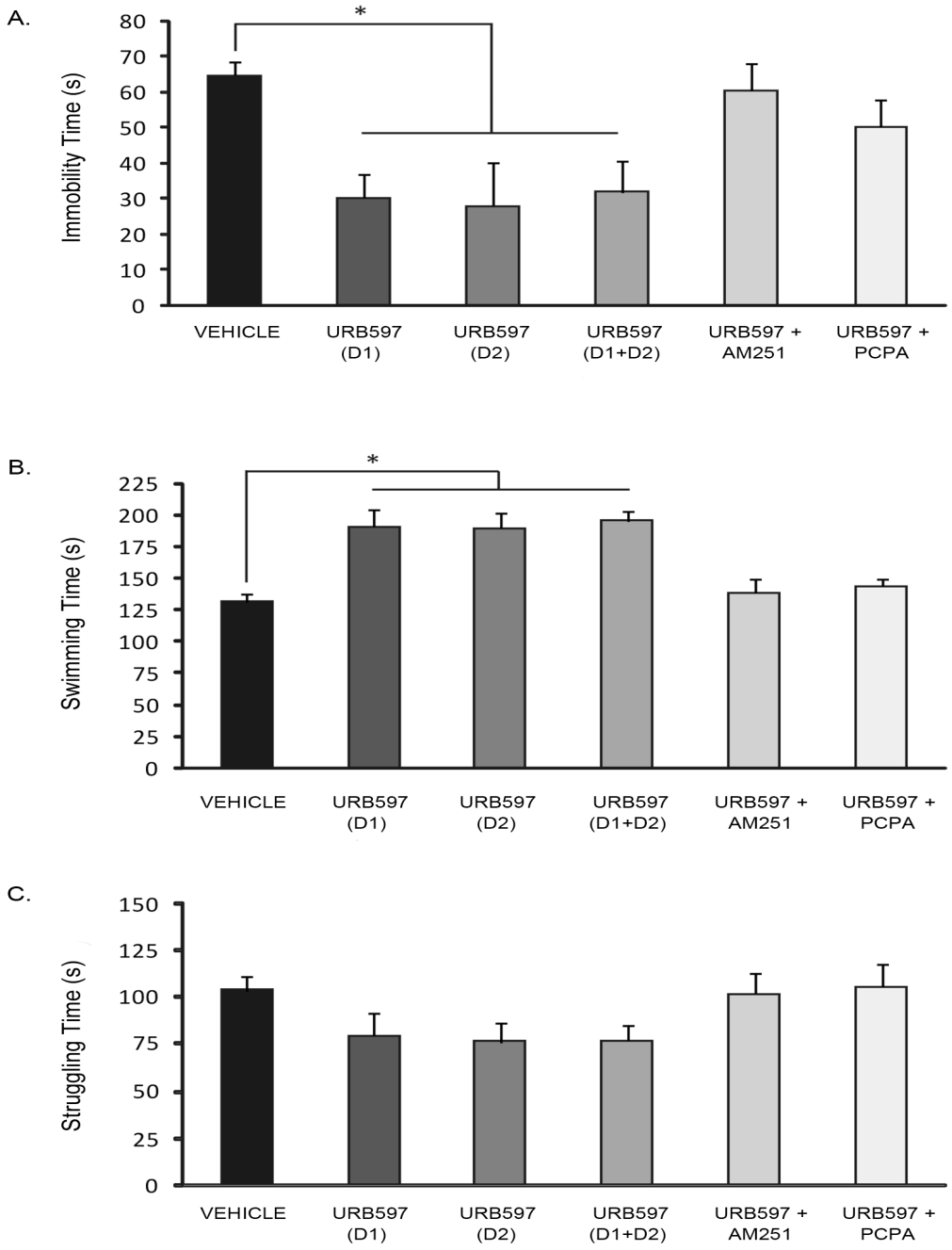
3.3.2. Inhibition of FAAH Within the Ventromedial PFC Prevents the Adoption of Passive Coping Responses Via a CB_1 Receptor-Dependent and 5-HT-Mediated Mechanism

To determine whether the stress-induced decrease in AEA content contributed to coping strategies during swim exposure, we examined the effect of intra-ventromedial PFC administration of the FAAH inhibitor URB597 on passive (immobility) and active (swimming and struggling) coping behaviors. Two additional cohorts of rats were also included to determine whether the behavioral effects of intra-ventromedial PFC URB597 administration were CB_1 receptor-dependent and/or 5-HT-mediated. A one-way ANOVA revealed that there was no significant difference between the control groups in each of these studies with respect to immobility [$F_{(2,16)} = 0.875$, $p > 0.05$], swimming [$F_{(2,16)} = 0.344$, $p > 0.05$], or struggling [$F_{(2,16)} = 0.085$, $p > 0.05$]. Thus, for all further behavioral analyses, these control groups were pooled into a single value and compared to all other treatment conditions.

A one-way ANOVA revealed a significant main effect of treatment on immobility [$F_{(5,43)} = 6.02$, $p < 0.001$]. Post-hoc analyses further showed that rats receiving intra-ventromedial PFC injections of URB597 exhibited significantly reduced immobility levels compared to vehicle-treated rats, regardless of whether they received the infusion prior to day 1, prior to day 2, or prior to both days (all p's < 0.005). Rats receiving the URB597/AM251 cocktail did not differ from vehicle-treated rats, nor did rats pre-treated with the 5-HT depletor pCPA (both p's > 0.05; Fig. 3.3A). A one-way ANOVA also revealed a main effect of treatment on swimming behavior [$F_{(5,43)} = 10.59$, $p < 0.001$]. Post-hoc analyses demonstrated that intra-ventromedial PFC administration

of URB597 significantly increased swimming, regardless of whether the infusion was given prior to day 1, prior to day 2, or prior to both days (all p's < 0.001). Accordingly, rats receiving the URB597/AM251 cocktail did not differ from vehicle-treated rats, nor did rats pre-treated with pCPA (both p's > 0.05; Fig. 3.3B). When struggling behavior was analyzed, a one-way ANOVA revealed no main effect of intra-ventromedial PFC treatment on this coping strategy [$F_{(5,43)} = 1.79$, $p > 0.05$; Fig. 3.3C]. Thus, intra-ventromedial PFC administration of URB597 induces a CB_1-receptor-dependent reduction in passive coping strategies (i.e., immobility) with a concomitant increase in active, escape-directed responses (i.e., swimming), which is dependent on the integrity of the 5-HT system.

Figure 3.3. Local medial PFC FAAH inhibition promotes active coping strategies in the FST in a CB_1 receptor-dependent and 5-HT-mediated mechanism.

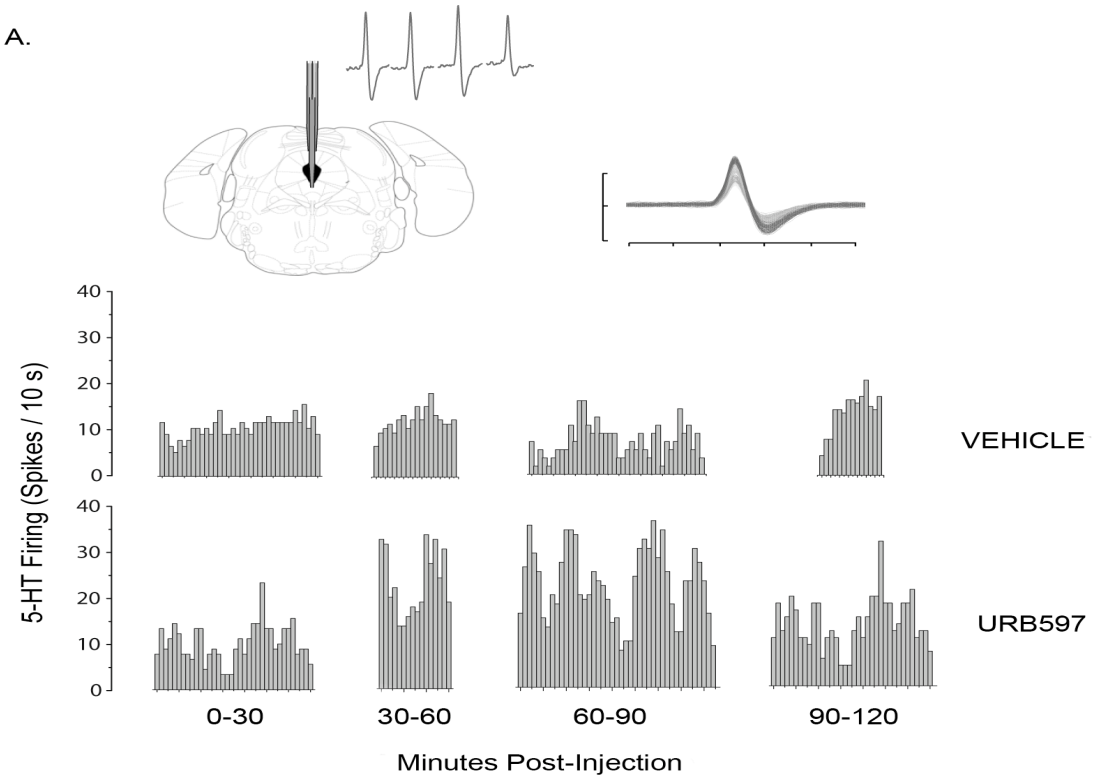


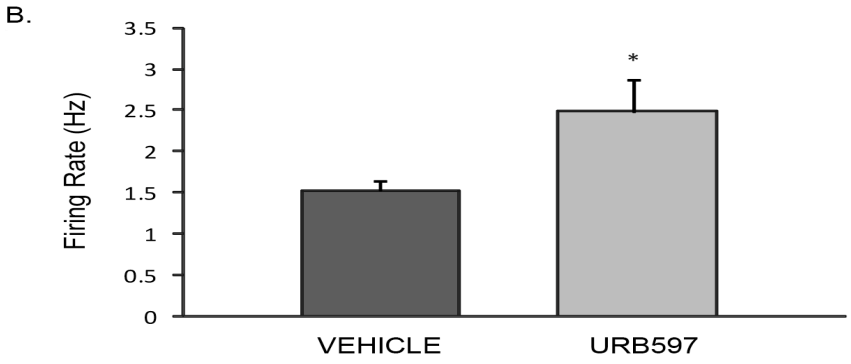
A and B. Local administration of the FAAH inhibitor URB597 (0.01 μg) into the ventromedial PFC significantly reduced immobility time (**A**) and increased swimming time (**B**) in the FST. These effects were prevented by concurrent administration of the CB_1 receptor antagonist AM251 (0.28 ng) or the 5-HT depletor pCPA. **C.** Local URB597 administration did not significantly alter struggling time in the FST. Data are presented as mean time (s) +/- SEM (n = 6-7 / treatment condition). * denotes significant differences from vehicle at $p < 0.05$.

3.3.3. Local Inhibition of FAAH Within the Ventromedial PFC Enhances the Firing Rate of 5-HT Neurons in the Dorsal Raphe

Because the behavioral effects of intra-ventromedial PFC URB597 administration were dependent on 5-HT signaling, we next determined if local inhibition of FAAH within the ventromedial PFC would alter the firing activity of 5-HT neurons in the dorsal raphe. For in *vivo* electrophysiological recordings, a total of 79 5-HT neurons were recorded from the dorsal raphe (vehicle, n = 41; URB597, n = 38; 4-5 rats/group). Figure 3.4 shows a representative integrated firing rate histogram of dorsal raphe 5-HT neurons recorded from rats receiving an intra-ventromedial PFC infusion of URB597 or vehicle, as well as a typical waveform of a recorded 5-HT neuron. An independent t-test revealed that rats receiving an intra-ventromedial PFC injection of URB597 displayed a significantly elevated 5-HT firing rate relative to vehicle-infused rats [$t_{(77)} = 2.41$, $p < 0.025$; Fig. 3.4]. These data are in agreement with the behavioral studies above, suggesting that inhibition of AEA metabolism within the ventromedial PFC coordinates behavioral coping responses to stress by modulating 5-HT neuronal output from the dorsal raphe.

Figure 3.4. Intra-medial-PFC administration of URB597 (0.01 μg) enhanced the firing rate of dorsal raphe 5-HT neurons in *vivo*.





A. Representative integrated firing rate histograms of 5-HT neuronal activity recorded from a rat that received an intra-ventromedial PFC infusion of vehicle (top) or URB597 (bottom). Abscissa represents time after infusion. **Upper left inset:** region of electrode contact within the dorsal raphe nucleus. **Upper right inset:** typical waveform characteristic of presumed 5-HT neurons encountered during electrophysiological recordings. Ordinate scale unit = 1 mV; abscissa scale unit = 1 ms. **B.** Local microinjection of URB597 into the medial PFC significantly enhanced the firing rate of dorsal raphe 5-HT neurons relative to vehicle-treated rats (vehicle, n = 41 neurons; URB597, n = 38 neurons). Data are presented as mean firing rate (Hz) +/- SEM (n = 4-5 rats / condition). * denotes significant differences at $p < 0.05$.

3.4. Discussion

The present study demonstrated that exposure to swim stress rapidly suppressed AEA content within the medial PFC. In parallel, facilitation of AEA signaling within the ventromedial PFC, through local FAAH inhibition, resulted in an increase in active behavioral coping responses to swim stress through a CB_1 receptor-dependent pathway. This increase in active coping responses following FAAH inhibition appeared to be mediated by an increase in 5-HT activity, as local FAAH inhibition within the medial PFC increased dorsal raphe neuron firing and depletion of 5-HT prevented the effects of intra-PFC FAAH inhibition on active coping responses to stress. Collectively, these data demonstrate that prefrontal cortical AEA signaling may function to couple stress induction to changes in emotional behavior.

The role of AEA within the ventromedial PFC in the coupling of stress induction to behavior parallels recent findings that stress exposure produces a rapid FAAH-mediated reduction in AEA signaling within the basolateral amygdala, which promotes activation of the HPA axis (Hill et al., 2009b). In accordance with the present results, local inhibition of FAAH within the BLA attenuated stress-induced activation of the HPA axis (Hill et al., 2009b). Taken together, these data suggest that stress induces a widespread decrease in corticolimbic AEA signaling which contributes to changes in both neuroendocrine secretion and behavioral coping strategies.

The current evidence also suggests that changes in serotonergic transmission account for the ability of AEA signaling within the ventromedial PFC to regulate stress coping behavior. In support of this hypothesis, the increase in active coping behaviors following FAAH inhibition is restricted to an increase in swimming behavior, but not

struggling, which is the pattern observed for a 5-HT-mediated behavioral response (Cryan et al., 2005). Consistent with this, depletion of 5-HT prevented the ability of local FAAH inhibition in the medial PFC to promote active coping responses, indicating that 5-HT signaling is required for this response. Furthermore, local inhibition of FAAH within the medial PFC increased the firing rate of serotonergic neurons in the dorsal raphe. This finding is consistent with previous studies demonstrating that local administration of a CB_1 receptor agonist into the medial PFC increases limbic 5-HT transmission and firing activity of dorsal raphe 5-HT neurons (Bambico et al., 2007), and that mice deficient in FAAH exhibit an increase in the firing activity of dorsal raphe neurons as well as corticolimbic 5-HT release (Bambico et al., 2010a; Cassano et al., 2011). Further, trans-section of prefrontal cortical afferents or depletion of 5-HT can mitigate the ability of local CB_1 receptor activation to promote active coping behaviors (Bambico et al., 2007). Collectively, these data indicate that facilitation of CB_1 receptor signaling within the medial PFC increases the excitability and output of prefrontal cortical projection neurons, ultimately resulting in augmented dorsal raphe neuronal firing and limbic 5-HT release. This facilitation of 5-HT neurotransmission provides a mechanism of action by which CB_1 receptor activation promotes active coping responses to stress; however, it should be noted that the 5-HT receptor subtypes involved in these behavioral changes have yet to be identified. The current data extends these findings by demonstrating that local AEA signaling within the ventromedial PFC regulates the activation of the dorsal raphe by prefrontal cortical outputs, and that this AEA signal is both sensitive to stress exposure and modulates behavioral coping responses to stress.

We have recently demonstrated that CB_1 receptors are predominantly localized to GABAergic terminals impinging upon pyramidal output neurons within layer V of the prelimbic region of the medial PFC (see Chapter 2), which is the origin of most prefrontal cortical projections to the dorsal raphe (Celada et al., 2002). As such, the mechanism by which AEA/CB_1 receptor signaling within the medial PFC increases the activity of projection neurons is likely driven by an inhibition of GABA release onto projection neurons. Based on these data, we propose the working model that AEA/CB_1 receptor signaling in the ventromedial PFC tonically regulates 5-HT firing in the dorsal raphe. Exposure to an aversive stimulus would elicit a FAAH-mediated reduction in AEA content, resulting in a disinhibition of GABAergic release onto pyramidal neurons in the medial PFC, thereby decreasing ventromedial PFC-mediated activation of dorsal raphe projections. Preventing the stress-induced decline in AEA signaling (via local administration of a FAAH inhibitor) could increase local activation of CB_1 receptors on GABAergic neurons in the medial PFC, thereby reducing GABA-mediated inhibition of pyramidal neurons and allowing for enhanced dorsal raphe 5-HT transmission. As mentioned previously, this increase in 5-HT transmission enables active coping strategies to stressful stimuli (Kirby et al., 2007).

It should be noted that the ability of AEA facilitation in the ventromedial PFC to promote excitability of dorsal raphe 5-HT firing might also be due to other mechanisms. For instance, not only do CB_1 receptors exist on GABAergic interneurons in the ventromedial PFC, they are also present to a lesser degree on glutamatergic pyramidal neurons in this region (Fortin and Levine, 2007). Medial PFC pyramidal output is known to exert both excitatory and inhibitory effects on dorsal raphe 5-HT activity via activation

of GABAergic/5-HT_{1A} receptors and AMPA/NMDA receptors, respectively (Hajos et al., 1998). Thus, it is possible that facilitation of AEA/CB_1 receptor signaling on glutamatergic ventromedial PFC pyramidal neurons acts to directly inhibit excitatory inputs to the dorsal raphe that synapse onto inhibitory GABAergic and/or 5-HT_{1A} neurons in this region, thereby promoting disinhibition of 5-HT firing locally within the dorsal raphe and enabling active stress coping responses. However, arguing against this theory is evidence suggesting that electrical stimulation of medial PFC projection neurons represents an effective antidepressant strategy and promotes proactive stress coping responses in humans and rodents, respectively. For instance, deep brain stimulation of the subgenual PFC in humans (which is functionally homologous to the ventromedial PFC in rodents) has emerged clinically as a promising novel therapeutic strategy for treatment-resistant cases of major depression (Price and Drevets, 2010). Likewise, deep brain stimulation of the rodent medial PFC promotes anxiolysis as well as a robust antidepressant-like response in the FST in a 5-HT-dependent manner (Hamani et al., 2010b). Moreover, electrical stimulation of the medial (but not lateral) PFC elicits substantial increases in limbic 5-HT output that likely contributes to the rapidly induced antidepressant effects of deep brain stimulation and electroconvulsive therapy (Juckel et al., 1999). Collectively, these data argue that the antidepressant profile obtained from intra-ventromedial PFC administration of URB597 is likely due to CB_1 receptor-mediated disinhibition of ventromedial PFC pyramidal neurons, thereby allowing for increased dorsal raphe 5-HT output to corticolimbic brain regions that are functionally implicated in stress coping and emotionality.

The endocannabinoid system is now a prominent target for the development of novel antidepressants, particularly in the form of FAAH inhibition rather than direct CB_1 receptor agonism (Gobbi et al., 2005; Hill et al., 2009a). The present data demonstrate that facilitation of endocannabinoid signaling within the ventromedial PFC is likely an important neural substrate for the ability of endocannabinoids to modulate stress-regulated emotional behaviors. In line with this, a recent report has demonstrated that FAAH inhibition within the ventromedial PFC produces anxiolytic effects, while local lentivirus-mediated overexpression of FAAH exerts the opposite behavioral effect (Rubino et al., 2008b). These data suggest that stress-induced regulation of AEA signaling within the ventromedial PFC may be important for the coordination and regulation of multiple facets of emotional behavior in response to stress. Together, this body of evidence suggests that AEA/CB_1 receptor signaling in the ventromedial PFC is tuned by environmental stimuli and through its ability to regulate 5-HT neurotransmission, could serve as both an important regulator of emotional responding and a determining factor in the nature of the coping response engaged in response to stressful stimuli.

4. Up-regulation of Cannabinoid CB_1 Receptor Binding in the Ventromedial Prefrontal Cortex is Adaptive Following Chronic Unpredictable Stress[3]

4.1. Introduction

The endocannabinoid system has recently emerged as a key component in the etiology of major depression and may represent a novel therapeutic candidate for its treatment (Hill and Gorzalka, 2009a). This system is comprised of a presynaptically located receptor (CB_1) and two endogenous ligands, AEA and 2-AG, which are synthesized on-demand and serve to modulate excitatory, inhibitory, and monoaminergic neurotransmission in brain regions involved in the regulation of emotionality and stress (Freund et al., 2003). Preclinical studies employing genetic deletion or chronic pharmacological antagonism of the CB_1 receptor have revealed a behavioral and neuroendocrine profile that closely resembles the phenotype of major depression in humans (Hill and Gorzalka, 2005a). Likewise, rats exposed to CUS, a valid and reliable animal model of depression (Willner, 2005), exhibit reduced CB_1 receptor binding and expression in subcortical limbic structures such as the hippocampus, hypothalamus, and ventral striatum (Hill et al., 2005; Hill et al., 2008b; Reich et al., 2009).

While exposure to CUS and the development of a depressive phenotype are associated with reductions in endocannabinoid signaling in most brain regions, a different pattern has emerged in the PFC. For instance, CUS exposure induces a robust

[3]A version of this chapter has been submitted for publication and is currently under review. McLaughlin RJ, Hill MN, Dang S, Hillard CJ, Gorzalka BB. Up-regulation of cannabinoid CB1 receptor binding in the ventromedial prefrontal cortex is adaptive following chronic unpredictable stress exposure. Submitted Dec. 18, 2011.

up-regulation of CB_1 receptor mRNA and binding in whole PFC tissue samples (Hillard et al., 2006; Bortolato et al., 2007; Hill et al., 2008b). Furthermore, this increase in CB_1 receptor binding in the PFC is normalized following chronic treatment with imipramine (Hill et al., 2008b) or URB597, an inhibitor of AEA degradation (Bortolato et al., 2007). Similarly, an increase in CB_1 receptor density and functionality in the PFC has been reported in the OBX animal model of depression (Rodriguez-Gaztelumendi et al., 2009). These changes were linked to alterations in anxiety-like behavior in the open field test; and both the increase in CB_1 receptor density and increased anxiety behavior were reversed following chronic fluoxetine treatment (Rodriguez-Gaztelumendi et al., 2009). These preclinical data are in agreement with post-mortem reports demonstrating that CB_1 receptor protein expression, binding site density, and signal transduction are all increased in the dorsolateral PFC of depressed, and alcoholic, suicide victims (Hungund et al., 2004; Vinod et al., 2005). Collectively, these data lend support to the notion that changes in CB_1 receptor activity within the PFC contribute to the development of major depression. However, it is currently not known whether this up-regulation of CB_1 receptor activity in the PFC represents a compensatory adaptive response initiated to dampen the behavioral symptoms of depression induced by chronic stress, or alternatively, a driving factor that contributes to the development of these changes.

The PFC is a structurally and functionally heterogeneous brain region that mediates a wide range of cognitive and emotional processes that are necessary to plan, control, and direct behavioral and neuroendocrine responses according to shifting environmental demands. Dorsomedial subregions of the PFC (in particular the prelimbic cortex) have been shown to suppress the neuroendocrine response to acute stress, while

the more ventromedial regions of the PFC (namely the infralimbic cortex) exert an opposing effect, promoting neuroendocrine activation (Radley et al., 2006a). Accordingly, human patients with bilateral ventromedial PFC lesions exhibit substantially lower rates of depression compared to patients with non-frontal lesions, whereas those with bilateral dorsolateral PFC lesions report greater severity of depressive symptoms (Koenigs et al., 2008). However, preclinical studies to date examining CB_1 receptor parameters have been exclusively conducted using whole PFC tissue samples and therefore, the effect of CUS on CB_1 receptor binding in dorsal versus ventral subregions of the PFC has yet to be empirically evaluated.

The first objective of the present study was to examine whether CUS differentially alters CB_1 receptor binding parameters in dorsal versus ventral components of the medial PFC in an effort to determine the precise localization of the CUS-induced increase in CB_1 receptor binding. Our second objective was to use the FST as a behavioral endpoint to examine the functional relevance of CUS-induced alterations in CB_1 receptor functionality within the PFC. Specifically, we sought to determine whether local pharmacological blockade of CB_1 receptor activity following CUS exposure would dampen, or alternatively exacerbate, despair-like passive responding in the FST.

4.2. Material and Methods

4.2.1. Subjects

Seventy-day-old male Sprague-Dawley rats (300 g; Charles River, Montreal, Canada) were used for the present study. All rats were housed in groups of three in standard maternity bins lined with contact bedding for the duration of the study, except following surgical procedures. In this case, rats implanted with cannula were individually

housed for 72 hr post-surgery to allow for proper healing and recovery, after which they were re-introduced to their original cage mates and group-housed for the remainder of the study. Colony rooms were maintained at 21 ºC, and on a 12-hr reverse light/dark cycle, with lights off at 0700hr. All rats were given free access to Purina Rat Chow and tap water. Rats were weighed on the morning prior to the initiation of the study and every week thereafter to allow for comparison between control and CUS-exposed animals. All experimental testing was conducted in accordance with the guidelines of the Canadian Council of Animal Care and was approved by the Animal Care Committee of the University of British Columbia.

4.2.2. Chronic Unpredictable Stress

Separate groups of rats were counter-balanced according to weight and randomly assigned to either CUS or control conditions prior to beginning the study (n=6/group for binding studies, n=7/group for microinjection studies). The CUS paradigm employed has been repeatedly used in our laboratory for both behavioral and biochemical analyses (Gorzalka et al., 1998; Hill et al., 2005; Hill et al., 2008b) and is adapted from the original chronic mild stress paradigm (Willner et al., 1987). This CUS paradigm consists of 2-3 stressors per day from the following list: 1 hr tube restraint; 1 hr exposure to social crowding with white noise/stroboscopic illumination; 5 min forced swim exposure; 18 hr food and/or water deprivation; 3 hr cage rotation to alter dominance hierarchies; and 18 hr social isolation in damp bedding. For studies where the FST was used as a behavioral endpoint, forced swim exposure in the CUS paradigm was replaced with 1 hr 30° cage tilt. All stressors were randomized and separated by a period of at least 2 hr. Rats

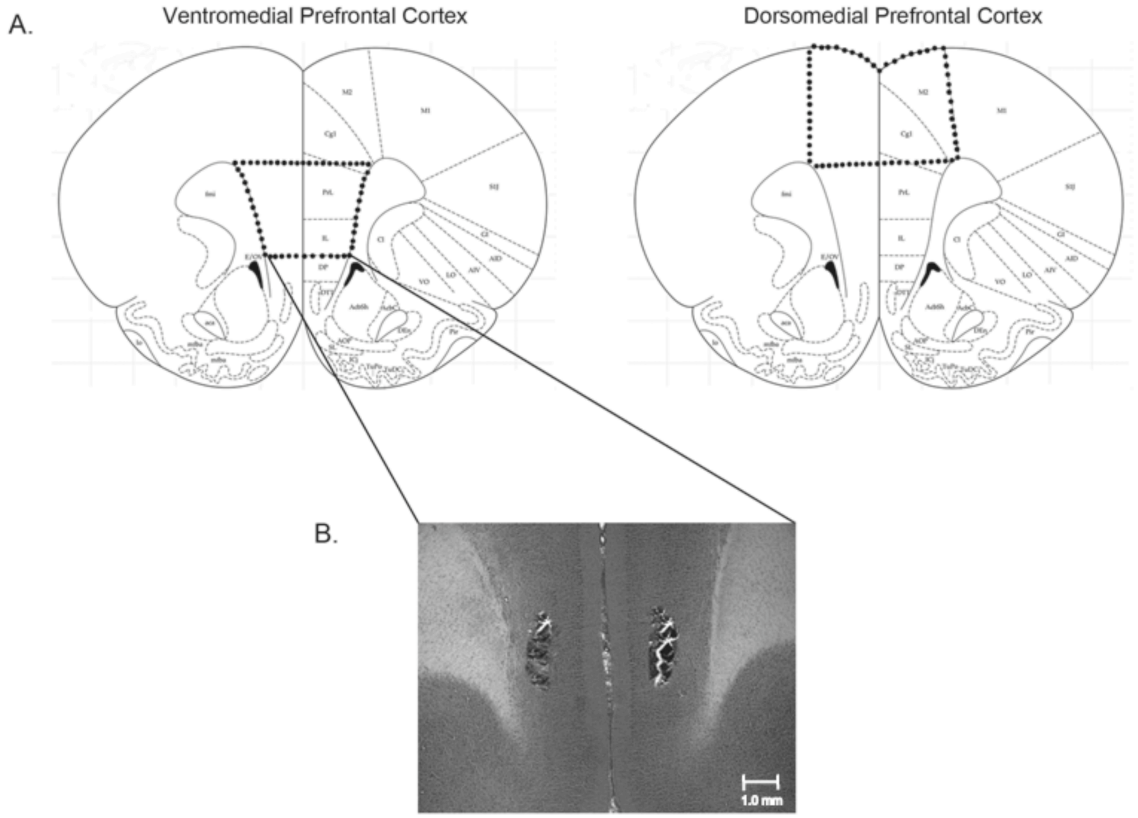
assigned to the control condition were handled three times per week for the duration of the study.

4.2.3. Radioligand Receptor Binding Studies

4.2.3.1. Membrane Preparation

On the morning after the final day of CUS (following 18 hr social isolation), rats used for CB_1 receptor binding analyses were decapitated and their brains were rapidly removed. The dorsomedial PFC (motor and anterior cingulate cortices) and ventromedial PFC (prelimbic and infralimbic cortices) were dissected, flash-frozen in liquid nitrogen, and stored at -80°C until analysis (see Fig. 4.1A for a representative diagram of the dissected brain regions). Brain sections were homogenized in 10 volumes of 0.32 M sucrose containing 3 mM HEPES (pH 7.5) and 1 mM EDTA. Homogenates were centrifuged at 18 000 x g for 20 min and the resulting pellet, which contains membranes, was re-suspended in 1-2 ml TME buffer (50 mM Tris HCl, pH 7.4; 1 mM EDTA and 3 mM $MgCl_2$). Protein concentrations were determined by the Bradford method (Bio-Rad, Hercules, CA, USA).

Figure 4.1. Representation of dissection boundaries and cannula placements within the medial PFC.



A. Coronal brain slice (3.0 mm anterior to bregma) depicting the boundaries for dissection for dorsomedial and ventromedial PFC tissue extractions. **B.** Representative photomicrograph showing a bilateral cannula placement for rats receiving microinfusions into the ventromedial region of the PFC. Placements were confirmed according to the atlas of Paxinos and Watson (1998).

4.2.3.2. CB_1 Receptor Radioligand Binding Assay

CB_1 receptor radioligand binding was performed using a Multiscreen Filtration System with Durapore 1.2-μM 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 0.1, 0.25, 0.5, 1.0 or 2.5 nM of [^{3}H] CP55940, a cannabinoid CB_1 receptor agonist. Ten μM THC was used to determine non-specific binding. The maximal CB_1 receptor binding site density (B_{max}) and affinity of [^{3}H] CP55940 for the CB_1 receptor (K_D) were determined by nonlinear curve fitting to the single site binding equation using GraphPad Prism (San Diego, CA, USA).

4.2.4. Microinjection Studies

4.2.4.1. Surgical Cannula Implantation

For behavioral studies examining the functional relevance of CUS-induced alterations in CB_1 receptor binding within the PFC, separate cohorts of animals were randomly assigned to one of four distinct groups (n=7/group): 1) CUS-VEH; 2) CUS-AM251; 3) CON-VEH; 4) CON-AM251. These animals were implanted with bilateral cannula aimed at the ventromedial subregion of the PFC prior to initiation of CUS, which occurred approximately 10-13 days post-surgery. Briefly, rats were anesthetized with a cocktail of 100 mg/kg of ketamine hydrochloride and 7 mg/kg xylazine, and implanted with 23 gauge stainless-steel guide cannula into the ventromedial PFC according to the following coordinates (AP = + 3.0 mm; ML = +/- 0.7 mm; DV = -3.4 mm; Paxinos and Watson, 1998). Four steel screws and dental acrylic were used to permanently affix the guide cannula to the skull and stainless steel stylets (30-gauge) were inserted into the

guide cannula until the time of infusion. Baytril antibiotic (0.5 ml) was added to water bottles for 72 hr following surgery, and animals were given a total of 10-12 days to allow for proper recovery and re-acclimatization with original cage mates before testing began.

4.2.4.2. Forced Swim Test

All behavioral testing occurred during the middle third of the animals' dark cycle on the days immediately following cessation of CUS exposure. Glass cylindrical containers (diameter 35 cm and height 45 cm) were filled to 30 cm, and water temperature was maintained at a constant 24±1°C. Consistent with the modified method of testing in the FST, animals were subjected to two swim sessions (Porsolt et al., 2001; Cryan et al., 2002). The first swim session was a 15-min pre-exposure session, followed by a 5-min test session 24 hr later. During the test session, the duration of immobility, swimming, and struggling was videotaped and later scored by trained assistants blinded to experimental conditions (see Section 3.2.3 for a description of scoring criteria for each behavioral component).

The FST was initially developed as a preclinical test to model behavioral despair, such that more time spent in a state of immobility reflected a greater level of behavioral despair and an increased reliance on passive coping strategies (Porsolt et al., 1977; Porsolt et al., 1978). Consistent with this reasoning, the occurrence of active, escape directed behaviors such as swimming and struggling is believed to represent an active coping strategy, as treatment with virtually all conventional antidepressants reduces the expression of immobility, and increases swimming and/or struggling behaviors (Borsini and Meli, 1988; Detke et al., 1995). Accordingly, CUS exposure reliably promotes passive coping strategies at the expense of escape-directed behaviors, and thus, the FST

has also been commonly used as a behavioral endpoint in assessing depressive-like responding following CUS exposure (Liu et al., 2009; Hellemans et al., 2010; Larsen et al., 2010).

4.2.4.3. Drug Administration

The CB_1 receptor antagonist AM251 (Tocris Cookson Ltd., Bristol, UK) or vehicle (0.9% saline) was administered at a dose of 0.28 ng (0.2 µl/side) directly into the ventromedial PFC through 30-gauge injection cannula via a microsyringe pump (Sage Instruments Model 341, Freedom, California, USA) connected to 10 µl Hamilton syringes by polyethylene (PE-20) tubing. This dose was chosen in accordance with recent studies demonstrating behavioral and neuroendocrine effects following intracranial microinjection of AM251 at this dose (Campolongo et al., 2009; Hill et al., 2011). Rats received injections of AM251 or vehicle 30 min prior to both forced swim sessions and were placed back into their home cages until testing began.

4.2.4.4. Histology

Following behavioral testing, tissue was sliced and stained with cresyl violet, and cannula placements were verified according to the stereotaxic atlas of Paxinos and Watson (1998). Histological analysis revealed that approximately 95% of cannula placements were in boundaries of the region of interest (see Fig. 4.1B for a representative photomicrograph). Subjects with cannula outside of the desired subregion were excluded from subsequent analyses.

4.2.5. Statistics

A mixed factorial ANOVA was used to examine differences in weight gain between control and CUS-exposed groups across the duration of CUS exposure. Post-hoc

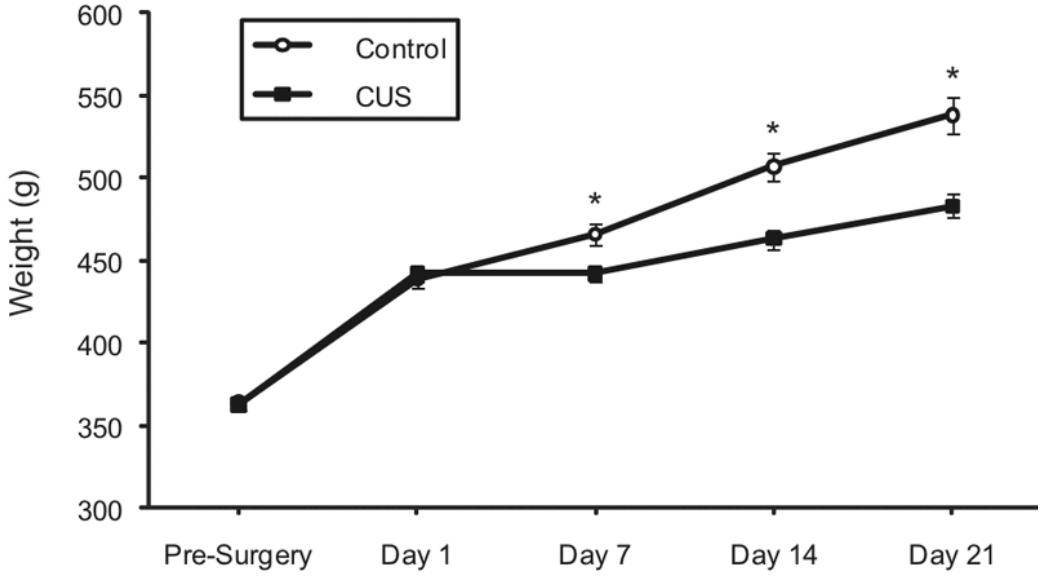
analyses were performed using Fisher's least significant difference test. A series of 2x2 between-subjects ANOVAs were used to compare the effects of CUS exposure on CB_1 receptor binding parameters in dorsal and ventral components of the medial PFC. Follow-up planned comparison t-tests were conducted to assess the region-specific effects of CUS exposure on CB_1 receptor binding parameters. A series of one-way ANOVAs were used to compare the effects of intra-ventromedial PFC saline or AM251 administration on immobility, swimming, and struggling in the FST following CUS exposure. Post-hoc analyses were performed using Fisher's least significant difference test.

4.3. Results

4.3.1. CUS Exposure Significantly Reduces Weight Gain

In order to examine the effect of 21-day CUS exposure on weight gain across the stress regimen, a 2x4 factorial ANOVA was conducted with group (CUS, control) as a between-groups factor and day of CUS (1, 7, 14, 21) as a within-group factor. The results demonstrated a main effect of group [$F_{(1, 28)} = 10.14$, $p < .005$], day [$F_{(3, 84)} = 240.92$, $p < .001$], and an interaction between the two [$F_{(3, 84)} = 40.45$, $p < .001$]. Follow-up analyses revealed that the weights of animals in the CUS and control groups did not differ on day 1 [$t_{(28)} = .41$, $p > .05$]; however, they did differ significantly on day 7 [$t_{(28)} = 2.76$, $p < .01$], day 14 [$t_{(28)} = 4.18$, $p < .001$], and day 21 [$t_{(28)} = 4.38$, $p < .01$]. This suggests that CUS-exposed animals gained significantly less weight compared to control animals over the 21-day stress regimen (Fig. 4.2).

Figure 4.2. 21-day chronic unpredictable stress (CUS) exposure significantly attenuated weight gain.

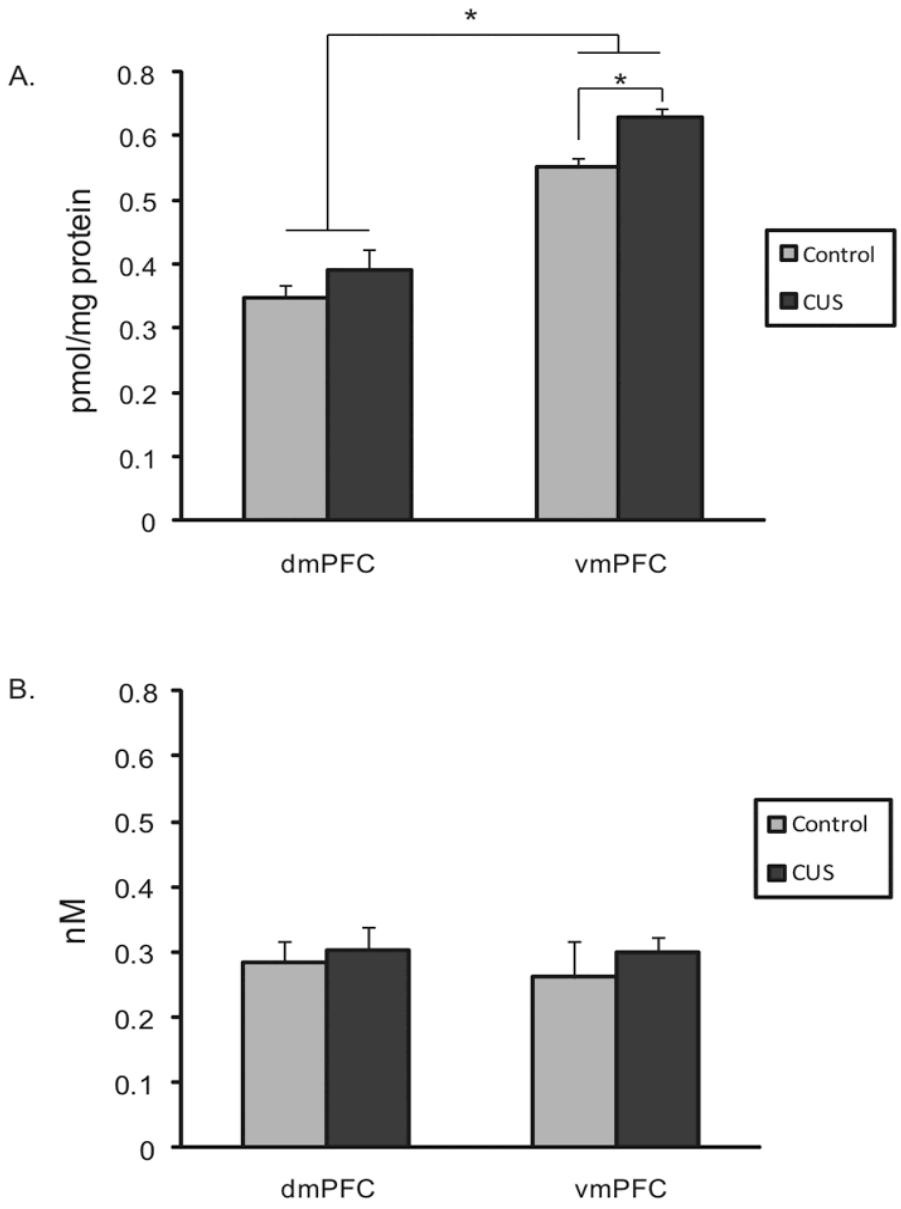


The weights of animals in the CUS and control groups were similar on day 1 of CUS exposure, but by day 7, and even more so by day 14 and day 21, animals in the CUS group had gained significantly less weight relative to animals in the control group. Values are expressed as mean weights (g) ± SEM. * denotes significant differences at $p < .05$.

4.3.2. CUS Exposure Increases CB_1 Receptor Binding in the Ventromedial PFC, but not Dorsomedial PFC

A 2x2 between-groups ANOVA was conducted to assess the effect of CUS exposure on the maximal binding site density (B_{max}) of CB_1 receptors in the dorsomedial and ventromedial subregions of the PFC. Results showed a main effect of stress [$F_{(1,19)} = 7.17$, $p < .05$] as well as a main effect of brain region [$F_{(1,19)} = 99.82$, $p < .001$], but no significant interaction [$F_{(1,19)} = 0.65$, $p > .05$]. Follow-up planned comparisons revealed a significant effect of CUS in the ventromedial PFC [$t_{(9)} = 3.85$, $p < .005$], but not in the dorsomedial PFC [$t_{(10)} = 1.09$, $p > .05$]. A 2x2 between-groups ANOVA was conducted to assess the effect of CUS exposure on the binding affinity (K_D) of [^{3}H] CP55940 for the CB_1 receptor in the dorsomedial and ventromedial subregions of the PFC. There was no significant main effect of stress [$F_{(1,19)} = 0.65$, $p > .05$] or brain region [$F_{(1,19)} = 0.14$, $p > .05$] and no significant interaction between these two variables [$F_{(1,19)} = 0.066$, $p > .05$]. These results suggest that the B_{max} (but not the K_D) of CB_1 receptors is preferentially increased in the ventromedial PFC (but not dorsomedial PFC) of CUS-exposed animals compared to those in the control group (Fig. 4.3).

Figure 4.3. 21-day chronic unpredictable stress (CUS) exposure significantly increased the maximal binding site density of CB_1 receptors in the ventromedial PFC.



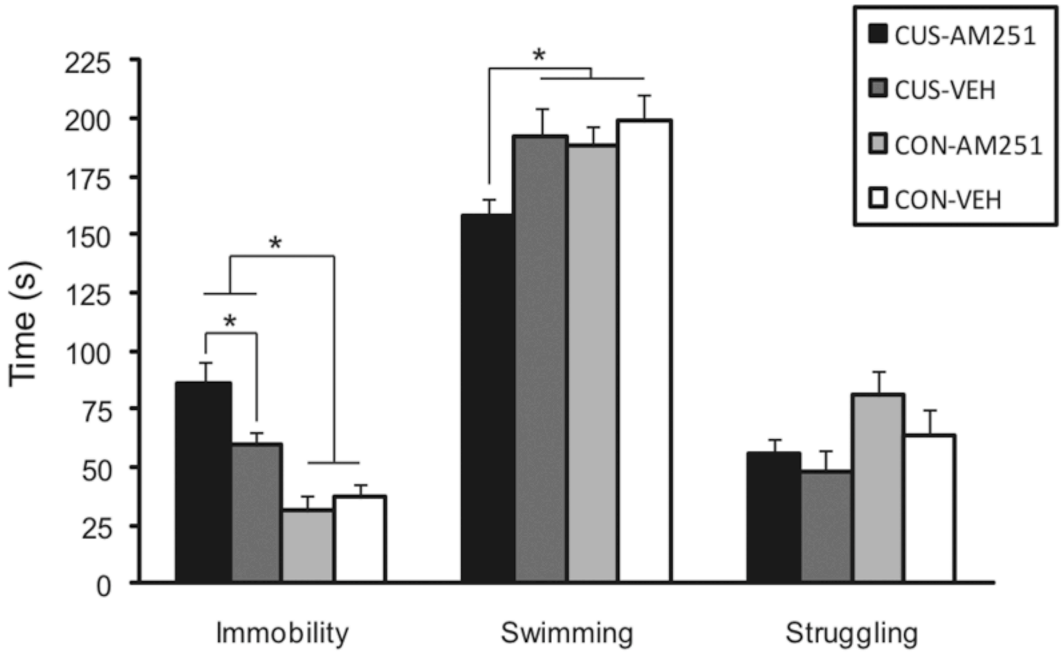
A. The maximal binding site (B_{max}; measured in pmol/mg protein) of the CB_1 receptor was substantially higher in the ventromedial PFC (vmPFC) compared to the dorsomedial PFC (dmPFC). Comparisons between control and CUS-exposed animals revealed that the B_{max} of the CB_1 receptor in the vmPFC was significantly higher in CUS-exposed animals compared to control animals, with no differences in B_{max} in the dmPFC. **B.** There was no significant difference in the binding affinity (K_D; measured in nM) between control and CUS-exposed animals in either the vmPFC or dmPFC. Values expressed as means ± SEM. * denotes significant differences at $p < .05$.

4.3.3. Local CB_1 Receptor Blockade Within the Ventromedial PFC Following CUS Exposure Further Exacerbates Despair-Like Responding in the FST

Because the CUS-induced increase in CB_1 receptor binding was restricted mainly to the ventromedial PFC, we next examined whether pharmacologically blocking this population of CB_1 receptors following CUS exposure would alter behavioral responses in the FST. Four separate groups were compared (CON-VEH; CON-AM251; CUS-VEH; CUS-AM251), and a one-way ANOVA revealed a significant effect of group on immobility [$F_{(3,24)} = 14.42$, $p < .001$]. Post-hoc analyses revealed that animals in the CON-VEH group spent significantly less time in an immobile posture compared to those in the CUS-VEH ($p = .002$) and CUS-AM251 ($p < .001$) groups, but not relative to those in the CON-AM251 group ($p = .53$), suggesting that CUS-exposed animals showed higher levels of immobility. Moreover, animals in the CUS-AM251 group showed significantly greater levels of immobility relative to those in the CUS-VEH group ($p = .03$). Thus, CUS-induced behavioral despair was further augmented by intra-ventromedial PFC CB_1 receptor blockade (Fig. 4.4).

A one-way ANOVA also revealed a significant effect of group on swimming behavior in the FST [$F_{(3,24)} = 3.28$, $p < .05$]. Follow-up analyses showed that although animals in the CON-VEH and CUS-VEH groups did not significantly differ ($p = .43$), those in the CUS-AM251 group did demonstrate a significant reduction in swimming compared to those in the CUS-VEH ($p = .04$), CON-VEH ($p = .01$), and CON-AM251 ($p = .04$) groups. There was no significant effect of group on struggling behavior [$F_{(3,24)} = 2.21$, $p > .05$; Fig. 4.4].

Figure 4.4. The effect of local ventromedial PFC administration of the CB_1 receptor antagonist AM251 on FST behaviors in control and CUS-exposed animals.



Four separate groups were compared: CON-VEH; CON-AM251; CUS-VEH; CUS-AM251. Animals exposed to chronic unpredictable stress (CUS) showed significantly higher levels of immobility compared to animals in the control (CON) groups, suggestive of enhanced behavioral despair in CUS-exposed animals. Moreover, animals in the CUS group treated with AM251 (0.28 ng/side) prior to forced swim exposure (CUS-AM251) showed significantly higher levels of immobility relative to animals in the CUS group receiving vehicle (VEH) infusions prior to swim exposure (CUS-VEH). Thus, CUS-induced behavioral despair was further augmented by intra-ventromedial PFC CB_1 receptor blockade. Animals in the CUS-AM251 group also showed significantly lower levels of swimming compared to animals in all other treatment groups, suggesting a reduced reliance on escape-directed proactive coping strategies in these animals. There were no significant differences between groups with respect to struggling behavior in the forced swim test. Data are presented as mean time (s) +/- SEM. * denotes significant differences at $p < 0.05$.

4.4. Discussion

The present study sought to determine the precise localization and functional relevance of alterations in PFC CB_1 receptor binding site density in rats subjected to 21 days of CUS. The results described herein reveal that the B_{max} for CB_1 receptor agonist binding is substantially higher in the ventromedial PFC compared to the dorsomedial PFC, and moreover, it is this population of CB_1 receptors that experiences an increase in maximal binding under CUS conditions. In light of this finding, we next used the FST as a behavioral endpoint to examine the functional contribution of this CUS-induced increase in CB_1 receptor binding in the ventromedial PFC. Animals in both CUS-exposed groups (CUS-VEH and CUS-AM251) showed greater levels of immobility compared to control animals, which is reflective of enhanced behavioral despair. Furthermore, animals in the CUS group pretreated with intra-ventromedial PFC infusions of AM251 prior to swim stress displayed the highest levels of immobility of all, even significantly more than those in the CUS group receiving vehicle infusions. Accordingly, those in the CUS-AM251 group also spent significantly less time swimming compared to those in all other treatment groups, which is suggestive of a reduced reliance on active, escape-directed coping responses in this cohort of animals. Together, these findings argue that the increase in the density of CB_1 receptor agonist binding sites documented in the ventromedial PFC of CUS-exposed animals is an adaptive response, as local antagonism of this population of CB_1 receptors further facilitated despair-like responses while reducing proactive coping strategies in the FST.

The CUS-induced increase in CB_1 receptor binding predominantly within the ventromedial PFC is intriguing, given that this region is a key determinant of depression-

like behavior and antidepressant responses both in clinical observations and preclinical studies. The rodent ventromedial PFC is functionally homologous to the subgenual region of the PFC in humans (Ongur et al., 2003), and notably, cellular and neuroimaging studies have revealed a reduction in immediate early gene expression and a substantial loss in gray matter in the subgenual PFC of depressed individuals (Drevets et al., 1997; Covington et al., 2010). Moreover, a variety of clinical interventions including pharmacological antidepressants, electroconvulsive shock treatment, and deep brain stimulation have all been associated with changes in subgenual PFC activity coinciding with symptom improvement (Mayberg et al., 2000; Drevets et al., 2002; Holthoff et al., 2004; Mayberg et al., 2005; Lozano et al., 2008). Accordingly, stress-susceptible rodents exposed to chronic social defeat stress exhibit similar reductions in immediate early gene activity in the ventromedial PFC, while optogenetic stimulation of this population of neurons produced antidepressant-like effects in these animals (Covington et al., 2010). Furthermore, deep brain stimulation of the rodent ventromedial PFC promotes a robust antidepressant-like response in the FST that is dependent on the integrity of the 5-HT neurotransmitter system (Hamani et al., 2010b). In agreement with this last report, local activation of CB_1 receptors within the ventromedial PFC has been shown to elicit a similar antidepressant-like response in the FST. This is accomplished via transynaptic activation of 5-HT outputs from the dorsal raphe (Bambico et al., 2007). Given that activation of CB_1 receptors within the ventromedial PFC exerts potent antidepressant-like responses similar to optogenetic and deep brain stimulation, it is not surprising that local pharmacological blockade of this population of CB_1 receptors further exacerbated

despair-like responding (i.e., immobility) and reduced escape-directed behaviors (i.e., swimming) in the FST.

Although local CB_1 receptor blockade within the ventromedial PFC effectively exacerbated despair-like responding in CUS-exposed animals, such an effect was not observed in control animals receiving intra-PFC administration of AM251. In previous studies, genetic deletion or global pharmacological CB_1 receptor blockade in mice has been shown to increase immobility in the FST (Steiner et al., 2008c). The lack of effect reported herein is likely due to the precise population of CB_1 receptors targeted. Evidently, local antagonism of CB_1 receptors in the ventromedial PFC is not sufficient to induce despair-like responding under normal conditions, which is consistent with previous findings (Bambico et al., 2007). The effects observed following global pharmacological blockade are likely attributed to cumulative actions of CB_1 receptor antagonism at many brain targets. Alternately, these differences may be due to species-specific factors, as previous studies were similarly unable to demonstrate an increase in immobility following global CB_1 receptor blockade in rats (Gobbi et al., 2005; Hill and Gorzalka, 2005b; Adamczyk et al., 2008).

The precise neurobiological mechanisms that are driving the CUS-induced increase in ventromedial PFC CB_1 binding are currently unknown. However, our laboratory has previously shown that a decrease in prefrontal AEA content also accompanies the increase in CB_1 receptor binding (Hill et al., 2008b), while local pharmacological facilitation of AEA/CB_1 receptor signaling elicits anxiety-like responding in the elevated plus maze (Rubino et al., 2008b). Thus, it is reasonable to speculate that the decrease in AEA content indirectly stimulates the compensatory up-

regulation of CB_1 receptor binding in the ventromedial PFC in an effort to maximize the diminishing AEA/CB_1 receptor activity induced by CUS exposure. This hypothesis is supported by evidence that pharmacological inhibition of FAAH during CUS exposure prevents the increase in CB_1 receptor mRNA expression that occurs within the PFC (Bortolato et al., 2007). Alternatively, it is possible that 2-AG could also be decreased in the ventromedial PFC following CUS exposure and that this change could be driving the increase in CB_1 receptor binding, especially given the high efficacy of 2-AG at CB_1 receptors (Sugiura et al., 1999). Following this logic, chronic inhibition of FAAH may be providing beneficial effects and preventing increased PFC CB_1 receptor expression by increasing AEA nonspecifically, thereby overcoming low amounts of 2-AG. However, these hypotheses remain speculative and will require further validation.

The amplified immobility response observed in CUS-exposed animals following intra-ventromedial PFC CB_1 receptor blockade was also accompanied by a reduction in swimming in the FST. Alterations in swimming behavior are traditionally thought to be mediated by changes in 5-HT transmission (Cryan et al., 2005), and consistent with this, the antidepressant-like effect of intra-ventromedial PFC CB_1 receptor activation occurs via interactions with this system (Bambico et al., 2007). Thus, it is possible that the increase in CB_1 receptor binding observed in the present study may be acting to facilitate 5-HT output from midbrain monoaminergic nuclei. Alternatively, the increase in endocannabinoid activity may be acting to regulate 5-HT_{2A} receptor activity (Gorzalka et al., 2005). However, more research will be needed in order to support these hypotheses.

CB_1 receptors are unique in that they are present on both glutamatergic and GABAergic neurons within the PFC, and as such, are ideally positioned to modulate both

excitatory and inhibitory projections (Freund et al., 2003). Although these data do not offer insight into which population of CB_1 receptors are primarily affected by CUS, in Chapter 2 of this dissertation we have demonstrated that CB_1 receptors are predominantly localized on GABAergic terminals impinging upon pyramidal output neurons within layer V of the ventromedial PFC, which is the origin of most prefrontal cortical projections to the dorsal raphe (Celada et al., 2002). Given that both CB_1 receptor mRNA and binding are increased within the PFC following CUS (current data; Hillard et al., 2006; Bortolato et al., 2007; Hill et al., 2008b), this would suggest that the increase is occurring within a local neuronal population, which is consistent with an increase of CB_1 receptor activity on local GABAergic terminals. It is possible that this increase in CB_1 receptor binding serves to limit local inhibitory tone provided to afferent projection neurons and to maintain their activity following exposure to chronic stress. Consistent with this hypothesis, mice lacking CB_1 receptors specifically on cortical glutamatergic neurons have been shown to exhibit decreased immobility in the FST (Steiner et al., 2008b), which would suggest that an increase in CB_1 receptors on glutamatergic terminals in the cortex would actually promote passive coping responses to stress, not constrain them.

Based on these, and previously published data, we propose the working model that CB_1 receptor signaling in the ventromedial PFC tonically regulates the output of midbrain serotonergic nuclei and may act via 5-HT_{2A} receptor activity. Exposure to CUS elicits a progressive reduction in AEA content, resulting in a disinhibition of GABAergic release onto pyramidal neurons in the medial PFC, thereby decreasing ventromedial PFC-mediated activation of dorsal raphe projections. As a result of this reduction in

AEA, CB_1 receptor binding becomes functionally enhanced in the ventromedial PFC in an attempt to maintain excitatory output to the dorsal raphe, thereby facilitating proactive stress coping. As mentioned above, an increase in 5-HT transmission is known to promote the adoption of proactive coping strategies to stressful stimuli (Kirby et al., 2007). If this is the case, therapeutic treatment with pharmacological agents that act to prevent the stress-induced decline in AEA signaling would be expected to increase local activation of CB_1 receptors on GABAergic neurons in the medial PFC, thereby reducing GABA-mediated inhibition of pyramidal neurons and allowing for enhanced dorsal raphe 5-HT transmission and an ensuing antidepressant-like response. Indeed, mounting evidence supports the notion that stimulating ventromedial PFC projection neurons represents an effective antidepressant strategy in both clinical and preclinical studies (Johansen-Berg et al., 2008; Covington et al., 2010). Moreover, daily administration of the selective FAAH inhibitor URB597, has been shown to ameliorate the reduction in body weight gain and sucrose intake induced by CUS and block the CUS-induced increase in CB_1 receptor mRNA within the PFC (Bortolato et al., 2007). Accordingly, drugs that target the endocannabinoid system, particularly those that facilitate AEA signaling, have recently emerged as promising candidates for the treatment of neuropsychiatric disorders where stress is a contributing factor (Bambico et al., 2009; Hill et al., 2009a).

In conclusion, the findings from the present study are in line with previous research suggesting that increased CB_1 receptor binding in the ventromedial PFC protects against the detrimental effects of chronic stress and facilitates proactive coping responses, as local blockade of CB_1 receptor activity in this region significantly

exacerbated behavioral despair and decreased escape-directed behaviors in the FST following exposure to CUS. Future studies are needed to determine whether the local reduction in prefrontal AEA content is somehow driving the increase in CB_1 receptor binding, and whether pharmacologically maintaining AEA tone over the course of CUS exposure can prevent this increase in binding and produce a stress-resilient phenotype via interactions with the $5\text{-}HT_{2A}$ receptor or other components of the 5-HT system. Nevertheless, these data offer valuable insight into the functional relevance of alterations in prefrontocortical CB_1 receptor binding induced by CUS exposure, and point to this system as a potential target for novel treatment strategies for major depression in clinical populations.

5. General Discussion

5.1. Overview

The overarching goal of this collective body of research was to examine alterations in endocannabinoid signaling parameters in the medial PFC following exposure to different kinds of stress (acute restraint stress, forced swim stress, and CUS) and to determine the functional role of medial prefrontocortical endocannabinoid signaling in regulating neuroendocrine and behavioral responses to these regimens. The research described in Chapters 2 and 3 argues that in the medial PFC, fluctuations in the two primary endocannabinoid ligands 2-AG and AEA are fundamentally involved in glucocorticoid-mediated negative feedback processes and the expression of emotional behavior, respectively, albeit through dissociable mechanisms. In Chapter 4 it was revealed that the region-specific enhancement of CB_1 receptor binding in the ventromedial PFC following CUS exposure represents a compensatory adaptation that is engaged to dampen stress-induced disturbances in emotional behavior, namely behavioral despair.

The remaining sections of this dissertation will first summarize the main findings of this research and describe the apparent mechanism of action hypothesized to underlie these effects in light of current research in the field of endocannabinoids and stress. Next, a unifying theory will be proposed outlining the underlying cause and resulting effect of endocannabinoid signaling fluctuations in the medial PFC under conditions of acute and chronic stress. This will be followed by a discussion of the strengths and limitations of the research, an assessment of the broader implications and potential therapeutic applications of these findings, and finally, a description of promising future directions.

5.1.1. Acute Stress Recovery is Mediated by 2-AG Signaling in the Medial PFC

Dysfunction of the HPA axis and the extrahypothalamic circuitry that gates its activation and facilitates its recovery represents a primary instigating factor in the development of stress-related pathologies, most notably melancholic depression (Gold and Chrousos, 2002). Individuals suffering from melancholic depression exhibit basal hypersecretion of CRH and prolonged release of glucocorticoids under stressful conditions (Gold et al., 1988a, b; Holsboer, 2000), suggesting that the inhibitory feedback processes governing HPA axis recovery are compromised, rendering this system hyperactive and prone to allostatic overload (i.e., the cumulative wear-and-tear that results from excessive activation or inefficient autoregulation of a biological system; McEwen and Wingfield, 2003; McEwen, 2007). Therefore, understanding the mechanisms that govern effective termination of the HPA axis under stressful circumstances is of paramount importance.

One brain region that expresses a high density of glucocorticoid receptors and participates in the long negative feedback loop responsible for termination of HPA axis activity is the medial PFC (Sanchez et al., 2000; Bizon et al., 2001). However, the physiological mechanism by which glucocortioids increase the excitability of principal neurons in the medial PFC to promote termination of HPA axis activity had been largely unknown. A substantial body of evidence from our laboratory and others has indicated that the endocannabinoid system tightly regulates the neuroendocrine response to stress, while stress in turn mobilizes endocannabinoids to re-establish homeostatic balance when the threat at hand is no longer deemed stressful (Hill and McEwen, 2010). In recent years, we have examined the complex mechanisms by which endocannabinoids modulate

HPA axis activity and stress habituation processes, particularly within the amygdala (Hill et al., 2009b; Hill et al., 2010b). Until now however, the precise role of endocannabinoid signaling in the medial PFC with respect to modulation of the HPA axis had remained an unexplored domain.

The research conducted in Chapter 2 of this dissertation employed a multidisciplinary approach to persuasively demonstrate that the mechanism by which glucocorticoids increase the excitability of principal neurons in the medial PFC to terminate HPA axis activity is via recruitment of endocannabinoid signaling. First, we confirmed that CB_1 receptor knockout mice display impaired corticosterone recovery following acute exposure to 30-min restraint stress. Accordingly, local pharmacological blockade of CB_1 receptors in the medial PFC of rats was shown to produce similar deficits in corticosterone recovery at 60 min following stress onset, suggesting that CB_1 receptors specifically in the medial PFC participate in termination of the HPA axis. Next, we used mass spectrometry to measure content of the endocannabinoids AEA and 2-AG 60 min following the onset of restraint stress in accordance with the timeframe of corticosterone recovery deficits outlined in the studies above. We revealed that 2-AG (but not AEA) content was increased specifically within the medial PFC at this time point, and that this mobilization of 2-AG was absent in animals pre-treated with a glucocorticoid receptor antagonist. Therefore, a glucocorticoid-mediated enhancement of 2-AG signaling within the medial PFC was suspected to promote feedback inhibition of the HPA axis.

Next, we performed immunohistochemical analyses and in *vitro* electrophysiological recordings to elucidate the precise mechanism of action underlying

this effect. Immunofluorescence studies revealed dense CB_1 receptor expression throughout layers II/III and V of the prelimbic region of the medial PFC in both rats and mice, a region of the medial PFC particularly important for glucocorticoid-mediated regulation of the HPA axis and termination of stress-induced corticosterone secretion (Diorio et al., 1993; Radley et al., 2006a; Radley et al., 2009). Patch clamp recordings in layer V pyramidal neurons within the prelimbic region of the medial PFC demonstrated that blocking CB_1 receptors prevents the induction of DSI as well as the expression of LTDi following repetitive stimulation of synaptic afferents, which are prototypic forms of endocannabinoid-mediated short- and long-term synaptic plasticity at GABAergic synapses, respectively. Accordingly, CB_1 receptor activation depressed the amplitude of evoked inhibitory currents. Finally, we demonstrated that incubation of medial PFC slices with corticosterone for 60 min decreased GABA-mediated currents in layer V medial PFC pyramidal neurons, and moreover, these effects were abolished following CB_1 receptor blockade. These data collectively suggest that endocannabinoids act on CB_1 receptors that are expressed on GABAergic synapses impinging on principal neurons in layer V of the prelimbic region of the medial PFC, and that the mechanism by which glucocorticoids depress GABA-mediated currents in pyramidal output neurons of the medial PFC is via recruitment of 2-AG released from postsynaptic neurons.

In light of these data, the following mechanism is proposed. In response to acute stress, CRH neurosecretory cells in the parvocellular region of the PVN become activated, allowing for the ensuing release of ACTH from the anterior pituitary and glucocorticoids (i.e., corticosterone) from the adrenal glands. Corticosterone enters the brain, binding to canonical glucocorticoid receptors within the medial PFC.

Glucocorticoid receptor activation induces the synthesis and release of 2-AG from postsynaptic neurons, which travel back across the synapse to bind to presynaptically located CB_1 receptors that are densely expressed on GABAergic neurons in layer V of the prelimbic medial PFC. Activation of this population of CB_1 receptors depresses GABA-mediated inhibition of pyramidal neurons emanating from layer V, thereby resulting in a disinhibition (and consequent activation) of these excitatory principal output neurons. Anatomical tracing studies have elegantly demonstrated that these medial PFC principal output neurons synergize with outputs from the ventral hippocampus at a common inhibitory relay site within the anterior BNST (Radley and Sawchenko, 2011), and that these circuits cooperate to dampen neuronal activation of CRH-secreting cells in the PVN, thereby leading to restoration of homeostasis.

5.1.2. Behavioral Despair is Mediated by AEA Signaling in the Medial PFC

It is widely known that exposure to severe or prolonged stress can have important ramifications for the emergence of emotional dysfunction. Although emotionality is a relatively abstract concept that isn't easy to objectively define in preclinical terms, the expression of behavioral despair-like responses under inescapable and/or highly stressful conditions effectively models a core component of negative mood states. In addition to its primary function as a screening test for novel antidepressant treatment strategies, the FST is also the most commonly used paradigm for assessing behavioral despair, mainly because responses in the FST can be dichotomized into two distinct coping strategies; active coping, which consists of escape-directed responses that can be quantified via measurements of swimming and struggling time, and passive coping, which is quantified by measuring time spent immobile and is akin to behavioral despair. Virtually all

antidepressants currently on the market selectively promote active coping responses in the FST (anxiolytics are ineffective in the FST for example) (Cryan et al., 2005), while regimens that engender depressive-like symptoms, such as the CUS and social defeat paradigms, increase the propensity to exhibit despair-like passive coping responses in this test (Rodriguez Echandia et al., 1988; Rygula et al., 2005; Hellemans et al., 2010). Therefore, understanding the neurobiological processes that elicit the expression of active vs. passive coping responses in the FST may provide valuable insight into the mechanisms that confer resilience and vulnerability to emotional dysfunction in clinical populations afflicted with stress-related pathologies.

In addition to the substantial role of the medial PFC in governing glucocorticoid-mediated inhibitory feedback processes (see Chapter 2), this brain region also fundamentally regulates the expression of emotional behavior. Reciprocal communication between the medial PFC and midbrain monoamine cell bodies such as the 5-HT-producing dorsal raphe have been argued to be a key determinant in whether a stressor is perceived as controllable (Maier and Watkins, 2010), while crosstalk between the medial PFC and the amygdala is known to encode the emotional salience of affective stimuli (Davidson, 2002; Quirk and Beer, 2006). Moreover, recent evidence has revealed that exogenous CB_1 receptor activation within the medial PFC mediates antidepressant-like active coping responses in the FST via a trans-synaptic enhancement of dorsal raphe 5-HT neurotransmssion (Bambico et al., 2007).

The series of studies described in Chapter 3 of this dissertation investigated the functional role of *endogenous* medial prefrontocortical cannabinoid signaling in the expression of coping responses in the FST using a combination of behavioral,

pharmacological, biochemical, and electrophysiological approaches. We first examined how FST exposure affects endocannabinoid ligand content in the medial PFC using mass spectrometry, and revealed that AEA (but not 2-AG) content experiences a rapid and robust decline immediately following the first FST exposure session. AEA content had partially (but not fully) restored when examined 24 hr later, but was subject to an even greater decline following a second FST exposure session, and this was accompanied by the expression of behavioral despair. Thus, fluctuations in medial PFC AEA signaling were suspected to mediate the transition between active and passive coping strategies. To support this claim, we next demonstrated that local medial PFC inhibition of FAAH, the enzyme responsible for the degradation of AEA, reduced the expression of passive, despair-like coping responses (i.e., immobility) in the FST and consequently augmented the expression of a subset of active coping responses (i.e., swimming) that are known to be 5-HT-mediated (Cryan et al., 2005). The enhancement of swimming cannot be attributed to a general increase in locomotion, since previous reports have demonstrated that URB597 does not significantly affect basal locomotor activity (Adamczyk et al., 2008). This effect in the FST was blocked by co-administration of a CB_1 receptor antagonist, as well as by global pharmacological depletion of 5-HT precursors, suggesting that the ability of FAAH inhibition within the medial PFC to promote active coping strategies in the FST is both CB_1 receptor-dependent and 5-HT-mediated. Finally, using in *vivo* single unit extracellular recordings, we demonstrated that local inhibition of FAAH within the ventromedial PFC enhanced the firing rate of dorsal raphe 5-HT neurons on a time course that mirrors the behavioral effects in the FST. Together, these studies argue that AEA/CB_1 receptor activity in the ventromedial PFC mediates the

expression of active coping responses in the FST via an enhancement of dorsal raphe 5-HT neuronal firing, which is in line with the findings of Gobbi and colleagues (Bambico et al., 2007).

One conspicuous difference between the findings from Chapters 2 and 3 is that AEA and 2-AG appear to be differentially altered by forced swim stress and acute restraint, respectively. Whereas Chapter 2 revealed robust effects on 2-AG signaling in response to restraint stress with no change in AEA, Chapter 3 revealed forced swim-induced alterations in AEA without significantly affecting 2-AG. The likely reason for these discrepancies is the time frame in which these endocannabinoids were measured following stress exposure. In Chapter 2, endocannabinoid content was measured 60 min post-stress onset, while in Chapter 3 these measurements were taken 5 min after removal from the forced swim chamber. Thus it appears that in response to stress, the effect on AEA signaling is more immediate, and this response contributes to the behavioral responses that are so vital during the initial stages of stressor exposure. In contrast, the effect of stress on 2-AG signaling is more delayed and contributes to the modulation of synaptic inhibition that underlies neuroendocrine homeostatic recovery.

It is not surprising that AEA is the primary endocannabinoid responsible for these behavioral responses, as Parolaro and coworkers have similarly demonstrated that lentivirus-mediated local overexpression of FAAH in the medial PFC increases anxiety-like behavior, while local administration of a FAAH inhibitor or the metabolically stable AEA analogue methanandamide into this region elicits an anxiolytic profile at low doses (Rubino et al., 2008b). Thus, fluctuations in medial prefrontocortical AEA signaling evidently mediate the expression of distinct forms of emotional behavior (anxiety and

behavioral despair), providing further support for the notion that this system is generally implicated in the expression of behavioral responses to various forms of emotionally aversive stimuli.

The results from Chapter 2 revealed that CB_1 receptors in the medial PFC are predominantly localized to GABAergic neurons that govern the excitability pyramidal output neurons. Moreover, it is known that activation of medial PFC pyramidal output neurons increases the excitability of dorsal raphe 5-HT neurons. Therefore, the mechanism by which augmented AEA/CB_1 receptor signaling enhances dorsal raphe 5-HT firing is likely via enhanced inhibition of GABAergic synapses, thereby leading to disinhibition of ventromedial PFC pyramidal output neurons. Under basal conditions, tonic AEA/CB_1 receptor activity in the ventromedial PFC is high, likely in an effort to constrain GABAergic signaling at pyramidal output neurons. Exposure to swim stress dampens AEA content throughout the medial PFC, effectively strengthening GABAergic inhibition of pyramidal output neurons and decreasing medial PFC-mediated activation of dorsal raphe 5-HT projections. This diminished afferent input to dorsal raphe 5-HT neurons is accompanied by behaviors in the FST that resemble behavioral despair. By preventing the stress-induced decline in AEA signaling in the medial PFC via local administration of a FAAH inhibitor prior to the induction of stress, CB_1 receptor signaling at GABAergic synapses becomes enhanced, thereby dampening GABA-mediated inhibition of pyramidal neurons and allowing for enhanced dorsal raphe 5-HT transmission. This increase in 5-HT transmission increases postsynaptic concentrations of 5-HT in the limbic forebrain, culminating in a proactive behavioral coping response in the FST. Accordingly, increased excitability of dorsal raphe 5-HT neurons and synaptic

5-HT concentrations also serve as the biological endpoint of the selective 5-HT reuptake inhibitor (SSRI) class of antidepressants, while active coping responses in the FST are the primary indicator of potential antidepressant efficacy at the preclinical stage (Lucki, 1997). Thus, augmenting AEA signaling in the ventromedial PFC not only promotes the adoption of active coping strategies over despair-like responses, but may also elicit antidepressant-like effects, both behaviorally and neurophysiologically, perhaps without invoking the negative side effect profile of conventional antidepressants.

5.1.3. Increased PFC CB_1 Receptor Binding Protects Against CUS-Induced Behavioral Disturbances

Exposure to CUS is a valid and reliable model of melancholic depression that promotes hypersecretion of glucocorticoids accompanied by alterations in hedonic reactivity, emotional behavior, immunosuppression, reductions in body weight, decreased grooming behaviors, and a lack of habituation to stress (Willner, 2005). Our laboratory and others have recently shown that CB_1 receptor binding and mRNA in whole PFC tissue samples is selectively increased following CUS exposure (Hillard et al., 2006; Bortolato et al., 2007; Hill et al., 2008b), while post-mortem reports of depressed suicide victims have reported a similar phenomenon, albeit in the dorsolateral PFC (Hungund et al., 2004). However, the precise prefrontal subregion implicated and the functional relevance of these changes had not been previously explored. Chapter 4 of this dissertation first sought to examine whether CB_1 receptor binding parameters are differentially altered in dorsomedial vs. ventromedial PFC subregions in response to CUS. This study revealed that the increase in prefrontal CB_1 receptor binding is primarily

localized to the ventromedial region of the PFC, consisting of the prelimbic and infralimbic cortices.

Next, we examined whether local pharmacological blockade of CB_1 receptors in the ventromedial PFC following CUS exposure would significantly exacerbate the expression of behavioral despair-like responses in the FST. The intent of this study was to determine whether enhanced CB_1 receptor binding in the ventromedial PFC serves an adaptive compensatory purpose that is engaged to dampen the negative effects of CUS, or alternately, a detrimental consequence of CUS that contributes to the depressive-like phenotype. Results revealed that the propensity to engage in passive, despair-like coping responses following CUS exposure was augmented by local pharmacological blockade of ventromedial PFC CB_1 receptors prior to forced swim exposure, coupled to a reduction in swimming time that collectively indicates an exacerbated depressogenic response in this paradigm.

Our interpretation that enhanced CB_1 receptor signaling in the ventromedial PFC serves an adaptive function is corroborated by the fact that endocannabinoids promote biochemical signals resulting in a pro-survival fate while inducing a selective death in glia-derived tumor cells (Massi et al., 2008). Moreover, under neuropathological conditions, glial cells have been shown to release an increased amount of endocannabinoids and over-express CB_1 receptors in the PFC, which may constitute an endogenous defense mechanism that prevents additional cell damage (Massi et al., 2008). In agreement with this notion, CB_1 receptor knockout mice have been shown to exhibit HPA axis dysregulation along with exacerbated excitotoxic/neuroinflammatory responses in the PFC (Zoppi et al., 2011). Interestingly, daily treatment with a CB_1

receptor agonist is capable of preventing increases in pro-inflammatory molecules, lipid peroxidation, and decreased glutamate uptake that results from chronic stress (Zoppi et al., 2011). Given the negative impact of CUS exposure and the neuroprotective capacity of CB_1 receptors in the PFC, it is apparent that the increase in CB_1 receptor binding observed in the present study is indeed a compensatory response.

Although the mechanisms driving this CUS-induced increase in CB_1 receptor binding are currently unknown, we have previously demonstrated that AEA content is significantly reduced in the PFC in response to CUS exposure (Hill et al., 2008b). Therefore, the most parsimonious explanation is that CB_1 receptor binding in the ventromedial PFC becomes enhanced in an effort to maximize the binding opportunities in response to decreasing levels of AEA. In Chapter 3, we demonstrated that AEA content is similarly reduced in response to forced swim stress, and that this physiological change is fundamentally involved in the expression of behavioral despair. It is therefore possible that maintaining AEA tone in the ventromedial PFC could represent a viable therapeutic treatment strategy for combating pathological states that are characterized by HPA axis disturbances, behavioral despair, and excitotoxic/neuroinflammatory responses, such as melancholic depression. Indeed, pharmacological administration of antidepressants has been shown to prevent the increase in prefrontal CB_1 receptor binding induced by CUS (Hill et al., 2008b) and OBX (Rodriguez-Gaztelumendi et al., 2009), while chronic inhibition of FAAH is similarly effective at reversing reductions in body weight gain and sucrose consumption invoked by CUS exposure (Bortolato et al., 2007). Because animals exposed to CUS (and theoretically individuals afflicted with major depression) already express a greater maximal CB_1 receptor binding site density in

the PFC, chronic treatment with a FAAH inhibitor could substantially improve AEA signaling at primed CB_1 receptor synapses within this region. Such an intervention would be expected to reinstate AEA tone at GABAergic synapses that have been rendering pyramidal output neurons hypoactive due to excessive inhibitory input. As argued in Chapter 3, increased AEA tone in the ventromedial PFC translates into increased excitability of pyramidal output neurons that heavily innervate the 5-HT-producing dorsal raphe, thereby leading to increased synaptic 5-HT concentration and an ensuing antidepressant-like behavioral response. Although this model remains speculative, it is apparent that alterations in AEA/CB_1 receptor signaling specifically within the ventromedial PFC could underlie behavioral and neuroendocrine disturbances observed in rodents exposed to CUS, and possibly, humans afflicted with major depression.

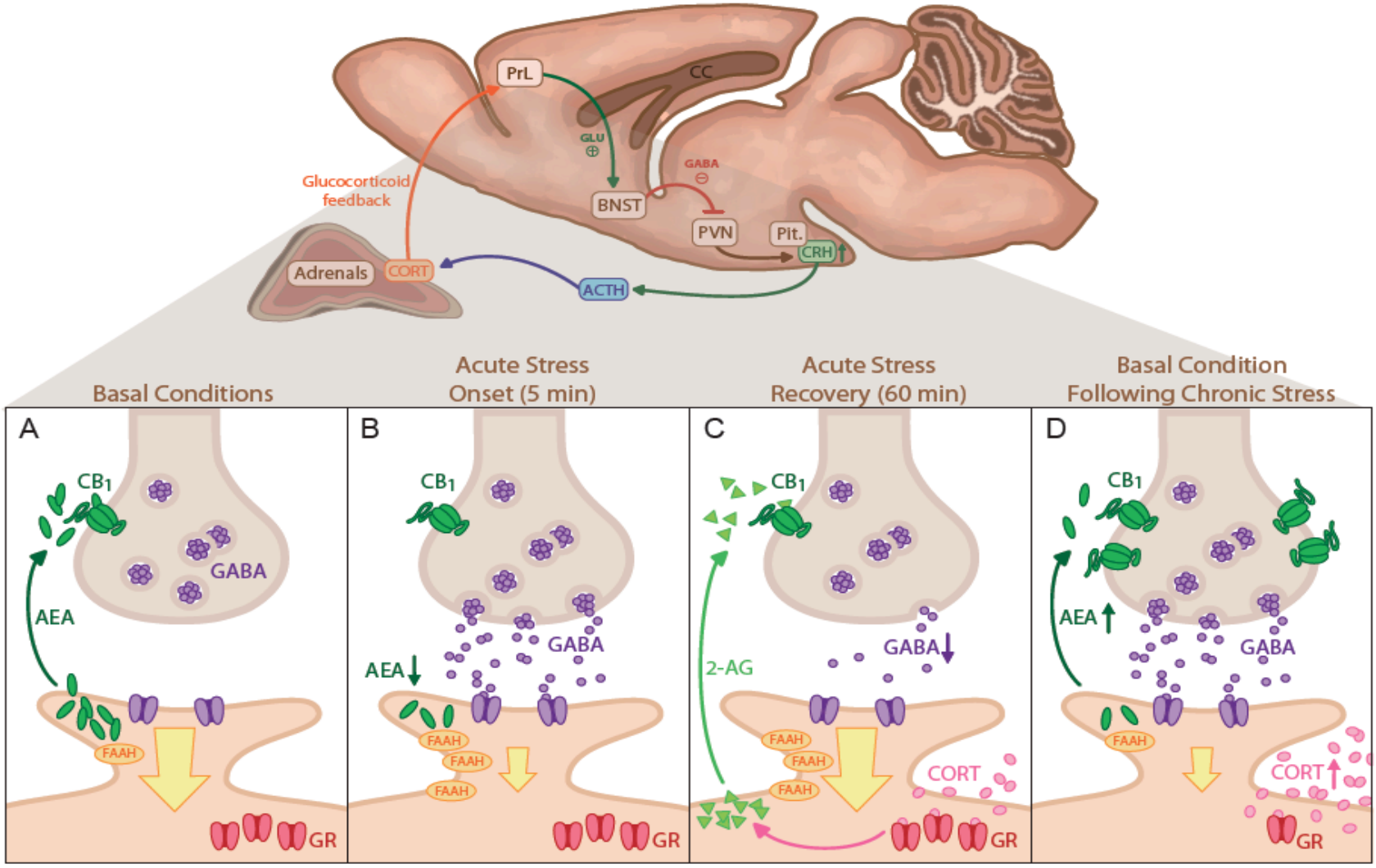
5.2. A Unifying Theory of Endocannabinoid Signaling in the Medial PFC Under Conditions of Acute and Chronic Stress

The body of research described in this dissertation has portrayed three distinct scenarios where different components of the endocannabinoid system (AEA, 2-AG, and CB_1) are altered in the medial PFC. In Chapter 3, it was revealed that as early as 5 min post-stress onset, AEA content is robustly decreased in the medial PFC, while preventing this phenomenon engages proactive stress coping strategies via interactions with the 5-HT system. In Chapter 2, we demonstrated that by 60 min post-stress onset, content of the other primary endocannabinoid, 2-AG, is enhanced in the medial PFC in a glucocorticoid-dependent manner, and that this mobilization of 2-AG participates in negative feedback processes that serve to terminate HPA axis activation and promote restoration of neuroendocrine homeostasis. In Chapter 4, we provided evidence that 21

days of CUS exposure, which is characterized by deficient HPA inhibitory feedback processes and produces a ubiquitous downregulation of AEA throughout the corticolimbic circuit (Hill et al., 2008), also increases the binding site density of CB_1 receptors in the ventromedial PFC. This increase in CB_1 receptor binding was suggested to counter the reduction in AEA signaling by maximizing the number of binding sites for AEA, thereby helping to preclude the adoption of maladaptive stress coping strategies that are a hallmark of CUS exposure.

Integrating these findings into the current state of knowledge, we propose the following model of prefrontocortical endocannabinoid signaling under conditions of acute and chronic stress (see Figure 5.1 for a schematic diagram of this model). Under basal conditions (panel A), AEA/CB_1 signaling at presynaptic GABA neurons in the medial PFC is tonically high, thus constraining GABA-mediated inhibition of medial PFC pyramidal neurons and allowing for normal pyramidal neuron output. Immediately following the onset of stress (panel B), there is an increase in postsynaptic FAAH, which rapidly degrades AEA and reduces AEA-mediated activation of CB_1 receptors. This allows for an increase in GABA release, resulting in a net inhibition of pyramidal neuron output. At 60 min post-stress onset, after the stressful encounter has ended (panel C),

Figure 5.1. A theoretical model outlining the role of endocannabinoid signaling in the medial PFC under conditions of acute and chronic stress.



Top Panel. Depiction of the neuroendocrine cascade resulting in activation and eventual termination of the HPA axis. CRH neurons in the parvocellular region of the hypothalamic PVN become activated, releasing CRH into the median eminence, where it is then transported to the anterior pituitary, stimulating the release of ACTH into the bloodstream. ACTH induces the release of glucocorticoids (cortisol in humans, corticosterone in rodents) from the adrenal cortex into general circulation. Glucocorticoids cross the blood-brain barrier and bind to glucocorticoid receptors located in the prelimbic (PrL) region of the medial PFC (as well as other regions; not shown). Activation of these inhibitory feedback neurons stimulates activation of GABAergic relay centers in the anterior BNST, which directly inhibit PVN activation, thereby promoting termination of the HPA axis and proper stress recovery. **Bottom Panel.** Theoretical model outlining the mechanism by which alterations in medial prefrontocortical endocannabinoid signaling modulate pyramidal neuron output under conditions of acute and chronic stress. **A.** Under basal conditions, AEA signaling is tonically high, suppressing GABA-mediated inhibition of pyramidal neurons. **B.** Immediately after stress onset, AEA experiences a FAAH-mediated decline, allowing for increased GABA transmission, reduced pyramidal output and an ensuing neuroendocrine and behavioral response. **C.** Following the cessation of stress, corticosterone binds to intracellular glucocorticoid receptors (GR), leading to the induction of 2-AG which binds to presynaptic CB_1 receptors. This reduces GABA-mediated inhibition of pyramidal output neurons that project to the BNST (see above panel), which directly inhibits PVN output, ultimately resulting in termination of the HPA axis and restoration of homeostasis. **D.** Under conditions of chronic stress, corticosterone is hypersecreted, glucocorticoid receptors are downregulated, and CB_1 receptor binding is increased. AEA is persistently reduced, resulting in greater GABA-mediated inhibition, thus suppressing normal pyramidal neuron output and rendering the medial PFC perpetually hypoactive.

there is an increase in corticosterone binding at intracellular glucocorticoid receptors, which induces the synthesis of 2-AG. The 2-AG then traverses the synapse and binds to presynaptic CB_1 receptors, which potently inhibits GABAergic currents. This disinhibition of medial PFC pyramidal output neurons allows for greater activation of HPA inhibitory feedback structures such as the anterior BNST, ultimately resulting in termination of HPA axis activity (Radley et al., 2006a; 2009; 2011). Note that AEA content remains decreased at this time point.

If the stressor is an isolated, non-traumatic incident, endocannabinoid content eventually regresses to baseline levels and neuroendocrine homeostasis is restored, but if stress is persistent and unpredictable, as in the CUS paradigm, this circuit is subject to allostatic overload, resulting in hyperactivation of the HPA axis and enhanced vulnerability to stress-related illnesses (panel D). In this scenario, corticosterone is hypersecreted, glucocorticoid receptors are downregulated in the postsynaptic neuron, and the binding density of presynaptic CB_1 receptors is increased. AEA is persistently reduced under these conditions, as it would be following acute exposure to stress, although in this case the reduction in AEA is likely attributed to a deficit in biosynthesis rather than an increase in FAAH-mediated metabolism (Hill et al., 2008). The decrease in AEA signaling results in greater GABA-mediated inhibition, thus suppressing normal pyramidal neuron output and rendering the medial PFC perpetually hypoactive in rats exposed to CUS and theoretically, humans with major depression. Although this model is a broad simplification that does not consider the effects of other brain regions, neurotransmitters, and neurosteroids in modulating responses to acute and chronic stress,

it is nevertheless apparent that endocannabinoid signaling in the medial PFC represents a key player in these responses.

5.3. Strengths and Limitations

A major strength of this collective body of work is the use of a multidisciplinary approach. We used a variety of techniques to examine how endocannabinoid signaling is altered at the neuroendocrine, biochemical, physiological, and behavioral level. This diverse set of complementary techniques, which included mass spectrometry, neurochemical receptor binding, immunohistochemistry, genetic disruption of the CB_1 receptor, in *vitro* and in *vivo* electrophysiology, and pharmacological manipulations targeting both systemic and local receptor populations, have all been implemented to provide strong support for our research hypotheses described above.

However, despite these strengths, there are a few limitations that deserve consideration before the proposed hypothetical model of prefrontocortical endocannabinoid signaling under conditions of acute and chronic stress can be fully accepted. First, it is worth mentioning that although the results of Chapter 2 do convincingly support our hypotheses, we did not actually measure neuronal activation in the medial PFC during acute stress recovery. In order to argue that endocannabinoid signaling in the medial PFC disinhibits principal output neurons to promote stress recovery, an immediate early gene marker such as c-*fos* should have been employed to provide additional evidence to support our theoretical model. Nevertheless, previous research in the field has repeatedly shown that acute stress induces robust c-*fos* activation and enhanced glucose mobilization in both the prelimbic and infralimbic subregions of the PFC (Duncan et al., 1993; Cullinan et al., 1995), and that this heightened activation

participates in inhibition and stimulation of the HPA axis, respectively (Diorio et al., 1993; Figueiredo et al., 2003; Radley et al., 2006a). Therefore, we can be reasonably confident that our proposed mechanism of action in Chapter 2 is accurate without conducting additional measures of neuronal activation.

Another limitation of this research is that only activity of CB_1 receptors located on GABAergic synapses was considered, while the role of CB_1 receptors expressed on prefrontocortical glutamatergic synapses were not discussed. Indeed, CB_1 receptors are expressed on both GABAergic and glutamatergic neurons in the medial PFC, although it should be noted that they are more heavily expressed on GABAergic neurons relative to glutamatergic neurons (Lafourcade et al., 2007; Wedzony and Chocyk, 2009; Hill et al., 2011b). Despite this discrepancy in CB_1 receptor localization, it should be noted that a previous study employing conditional knockout mice lacking CB_1 receptors specifically on cortical glutamatergic neurons revealed that this subpopulation is necessary for proper neuroendocrine and behavioral responses to stress (Steiner et al., 2008b). Because of their expression directly on principal output neurons, it is certainly plausible that activating these receptors could override the disinhibitory influence of CB_1 receptor activity at GABAergic synapses and result in a net inhibition of these principal output neurons. However, this may largely occur when CB_1 receptor occupancy on GABAergic neurons is saturated, as in cases where THC is administered at relatively high doses (Monory et al., 2007). Since the dose of CB_1 receptor agonists used in our studies was at the lower end of the spectrum (2 μM), it is unlikely that CB_1 receptor activation on glutamatergic synapses is influencing our results. However, this assumption will nonetheless require further validation.

Another important consideration is our interpretation of behaviors in the FST in Chapters 3 and 4. As is customary in the field, we interpreted immobility in the FST as an indication of behavioral despair. However, there is a long-standing controversy in the literature over whether immobility in the FST is actually analogous to behavioral despair. Some have argued that immobility is not necessarily a maladaptive coping failure, but instead reflects a successful coping strategy that employs energy conservation strategies (West, 1990). Thus, it has been suggested that immobility in the FST reflects an adaptation governed by learning and memory processes rather than an adoption of behavioral despair. While this may be a plausible interpretation, there is evidence to suggest otherwise. For instance, CUS (which induces a deficit in stress adaptation) increases immobility in the FST (Liu et al., 2009; Hellemans et al., 2010; Larsen et al., 2010; Chapter 4 of this dissertation) and this occurs in tandem with a constellation of depressive-like symptoms. Furthermore, chronic antidepressant administration is capable of reducing immobility upon exposure to a single forced swim session (Overstreet et al., 2004), which argues against the theory that immobility on day 2 reflects a learned behavioral response. Moreover, benzodiazepines, which negatively impact memory consolidation, are ineffective in the FST (Soubrie et al., 1976), while alpha-melanocye-stimulating hormone, which has been shown to enhance memory (Sandman and O'Halloran, 1986), actually reduces immobility time (Kastin et al. 1978). Therefore, we can be reasonably confident in our interpretation that immobility in the FST reflects a maladaptive coping strategy rather that a learned adaptive response.

It is also worthwhile to mention that although the CUS-exposed rats used in Chapter 4 showed greater levels of immobility compared to vehicle-treated rats, we did

not measure preferences for rewarding stimuli such as sucrose. Lack of motivation to pursue rewarding stimuli is a core symptom of major depression that is often used to validate the CUS paradigm (Willner, 2005). Thus, we cannot speak to whether these animals were actually anhedonic. However, the 21-day CUS protocol used in our laboratory has been used extensively in the past and has previously revealed a significant impairment in sexual motivation as demonstrated by an increased latency to engage in sexual activity with a receptive female rat (Hill et al., 2008).

Indeed, future studies employing different behavioral endpoints are necessary to assess the broader implications of CB_1 receptor up-regulation in the context of major depression. However, it should be noted that we chose to assess the functional impact of this receptor population specifically in the context of stress coping for several reasons. This study was specifically geared to addressing the adoption of proactive stress coping strategies, which are already known to be dependent on ventromedial PFC CB_1 receptor activation (Bambico et al., 2007). A study assessing sucrose preference is more likely to reveal CB_1 receptor-mediated alterations in reward processing centers such as the striatum or VTA. Consistent with this notion, a previous report has clearly demonstrated that activation of CB_1 receptors within subfields of the nucleus accumbens is involved in sucrose preference (Mahler et al., 2007). As such, examination of CB_1 receptor activation within the nucleus accumbens following chronic stress may be a more appropriate neural site to target if alterations in sucrose preference are investigated as the dependent variable following CUS exposure. Although previous work does indicate that global pharmacological blockade of CB_1 receptors further exacerbates chronic stress-induced reductions in sucrose consumption (Rademacher and Hillard, 2007), a large-scale

biochemical/behavioral analysis would be required to fully determine whether region-specific changes in CB_1 receptor signaling are functionally implicated in the adoption of anhedonic responses following CUS exposure.

Finally, as is often the case in the field of neuroscience, we chose to perform these studies in male subjects only. This is a particularly important caveat that prevents us from making more broad generalizations, especially considering that in the context of major depression, prevalence rates in females are roughly two to one relative to males (Kornstein, 1997; Kendler, 1998). Furthermore, a recent study has indicated that in direct contrast to the studies in male CUS-exposed rats, female rats exposed to CUS exhibit an up-regulation of CB_1 receptors in the dorsal hippocampus (Reich et al., 2009). Moreover, in non-stress control animals, males were observed to have higher levels of CB_1 receptors compared to females (Reich et al., 2009). These findings indicate that the endocannabinoid system is differentially altered in response to CUS in male and female rats. Therefore, it is especially important to further explore gender differences in the endocannabinoid system under conditions of acute and chronic stress.

5.4. General Applicability and Implications

In addition to describing the functional role of prefrontocortical endocannabinoid signaling in response to different stress regimens, these data also provide further evidence that targeting AEA in the brain could represent a viable pharmacotherapeutic strategy for treating major depression. Section 1.7.2 of this dissertation discussed recent evidence suggesting that pharmacological blockade of FAAH, the enzyme responsible for the degradation of AEA, produces potent anxiolytic and antidepressant-like effects in a wide variety of preclinical paradigms and strengthens extinction of emotionally

aversive memories (Kathuria et al., 2003; Gobbi et al., 2005; Patel and Hillard, 2006; Hill et al., 2007b; Naidu et al., 2007; Varvel et al., 2007; Moreira et al., 2008; Scherma et al., 2008). Indeed, the FAAH inhibitor URB597 shares many common features with chronic antidepressant treatments, including increased 5-HT and NE neurotransmission (Gobbi et al., 2005), enhanced tonic activity of postsynaptic hippocampal 5-HT_{1A} receptors (Bambico et al., 2010a), and increased hippocampal neurogenesis (Aguado et al., 2005). Moreover, URB597 administration promotes resilience to the anxiogenic effect of social defeat stress (Rossi et al., 2010) and reverses CUS-induced anhedonia (Bortolato et al., 2007). These beneficial effects have also been corroborated in mice lacking the FAAH gene, and generally speaking, these mice exhibit a phenotype that is in stark contrast to CB_1 receptor knockout mice, which closely mimic the symptom profile of individuals suffering from melancholic depression (Hill and Gorzalka, 2005a).

The use of cannabinoids in clinical practice has been fraught with controversy in the past. In addition to sharing the same mechanism of action with THC, the psychoactive constituent of marijuana, exogenous CB_1 receptor agonists are at risk for greater abuse potential and produce a constellation of negative side effects, especially at high doses. In addition to the compelling beneficial effects demonstrated in the preclinical studies listed above, FAAH inhibitors, which act to augment an existing endogenous pathway, evidently do not exhibit the addiction liability of direct CB_1 receptor agonists and also do not produce the negative side effects of conventional antidepressants (Bambico and Gobbi, 2008). Interestingly, primates that had been previously trained to self-administer THC, AEA, or cocaine did not self-administer URB597, suggesting that it is largely devoid of reinforcing properties and is thus

unlikely to exhibit abuse potential (Justinova et al., 2008). Moreover, URB597 self-administration also did not promote reinstatement of extinguished drug-seeking behavior that had been previously maintained by THC, AEA, or cocaine (Justinova et al., 2008). Therefore, not only do these compounds lack abuse potential in primates, but they also do not trigger relapse to other drugs of abuse, making them an especially intriguing candidate for therapeutic intervention, especially compared to direct CB_1 receptor agonists.

Although FAAH inhibitors and direct CB_1 receptor agonists inevitably work on the same receptor, there are a number of reasons why these compounds might be safer and more efficacious. For instance, FAAH inhibitors exhibit a different pharmacokinetic profile from CB_1 receptor agonists, inducing a slower, more progressive increase in 5-HT firing that is likely attributed to the on-demand nature of AEA synthesis (Bambico and Gobbi, 2008). Also, increasing doses of FAAH inhibitors do not produce the biphasic anxiogenic, depressogenic, and psychotomimetic effects that are commonly observed following high-dose CB_1 receptor agonist administration (Bambico and Gobbi, 2008), again because the biosynthesis of AEA likely serves as the rate-limiting step that prevents excessive activation. It should also be noted that FAAH and CB_1 receptors are not always co-localized, and as such, FAAH inhibitors may act on a slightly different population of neurons compared to direct CB_1 receptor agonists, which indiscriminately act on all CB_1 receptors regardless of whether FAAH is proximally expressed. These factors could ultimately determine the dissociable pharmacological profile of these two compounds and point to why inhibition of FAAH may represent the superior treatment strategy for individuals afflicted with major depression.

In addition to providing evidence for why FAAH inhibition may exert antidepressant potential, these data have also advocated the ventromedial PFC as an important site of action underlying these beneficial behavioral and neuroendocrine effects. An extensive body of literature has convincingly demonstrated that the rodent ventromedial PFC, which is analogous to the human subgenual PFC (Takagishi and Chiba, 1991; Ongur et al., 2003), is structurally and functionally altered following CUS exposure and in individuals suffering from major depression, respectively (see section 1.4). Moreover, various clinical interventions including antidepressant administration (Mayberg et al., 2000; Drevets et al., 2002; Holthoff et al., 2004), electroconvulsive shock treatment (Nobler et al., 2001), and deep brain stimulation (Mayberg et al., 2005) have all been associated with altered subgenual PFC activity that coincides with symptom improvement. This is not surprising given the hierarchical control the PFC exerts over executive, emotional, and neuroendocrine processes in the brain. With respect to deep brain stimulation, it is interesting to note that a similar antidepressant-like profile has been observed when the ventromedial PFC has been targeted in rodents, and moreover, this effect was shown to be dependent on the 5-HT system similar to the results obtained in Chapter 3 (Hamani et al., 2010a; Hamani et al., 2010b). Thus, it is possible that local inhibition of FAAH within the ventromedial PFC produces antidepressant-like effects via a similar mechanism to that observed following deep brain stimulation, which is quickly emerging as one of the most effective treatment strategies for major depression.

As a whole, this body of work not only extends our understanding of neural circuits in the brain that regulate responses to acute and chronic stress, but can also be

applied to more effectively treat stress-related disorders such as major depression. Only time will tell whether ongoing clinical trials with FAAH inhibitors in clinical populations will yield the success that the preclinical research predicts.

5.5. Future Directions

There are a number of possible future directions that deserve further exploration given the results obtained in this dissertation. As mentioned above, one potential caveat of our model is that it does not consider the functional impact of CB_1 receptors expressed on cortical glutamatergic neurons. Despite the fact that CB_1 receptors are more heavily expressed on GABAergic neurons as opposed to glutamatergic neurons in the PFC (Lafourcade et al., 2007; Wedzony and Chocyk, 2009; Hill et al., 2011b), multiple lines of evidence suggest that CB_1 receptors expressed on cortical glutamatergic neurons are involved in the behavioral effects induced by high-dose THC exposure (Monory et al., 2007), neuroprotection from excitotoxic seizures (Monory et al., 2006), and appropriate behavioral and neuroendocrine responses to stress (Steiner et al., 2008b). Therefore, a proper examination of the role of CB_1 receptor-expressing cortical glutamatergic neurons in behavioral and physiological responses to acute and chronic stress is needed before the model proposed above can be accepted. This could be achieved by conducting similar studies to those described herein using conditional knockout mice lacking CB_1 receptors on cortical glutamatergic or GABAergic neurons. Such a study would contribute considerable insight into the precise mechanisms underlying endocannabinoid-mediated actions in the medial PFC.

Although the research conducted in this dissertation focused exclusively on elucidating the role of endocannabinoids in the medial PFC in response to stress, it is

equally plausible that endocannabinoids in the hippocampus also contribute to these responses. Radley and coworkers have recently shown that extrinsic projections from the hippocampal ventral subiculum converge with projections from the prelimbic region of the medial PFC onto a common relay in the anterior BNST, and these inputs synergize to potently inhibit the HPA axis in an additive fashion (Radley and Sawchenko, 2011). Given the evidence reported herein suggesting that endocannabinoid signaling in the medial PFC mediates inhibition of the HPA axis via disinhibition of principal output neurons, as well as the studies demonstrating a similar 2-AG-mediated regulation of GABA release in the hippocampus (Gao et al., 2010; Pan et al., 2011; Wang et al., 2011), it is likely that glucocorticoid receptor activation also induces the synthesis of 2-AG to disinhibit projections from the ventral hippocampus. This theory is supported by a recent study showing that acute stress or corticosterone administration produces a delayed increase in hippocampal 2-AG content along with a corresponding enhancement of DSI in hippocampal slices, which is dependent on activation of glucocorticoid receptors (Wang et al., 2011). Therefore, future studies should be aimed at more closely examining the possible common pathways by which glucocorticoids mobilize 2-AG content in the medial PFC and hippocampus to regulate inhibitory feedback and restore neuroendocrine homeostasis.

Similarly, activation of CB_1 receptors in the dorsal hippocampus has also been shown to promote proactive behavioral coping strategies in the FST (McLaughlin et al., 2007); thus, it is possible that endocannabinoid signaling parameters are altered in the hippocampus and that this also contributes to the expression of passive and active coping strategies. Indeed, unpublished findings from our laboratory have revealed that similar to

the results obtained in the medial PFC, AEA signaling is also reduced in the hippocampus following forced swim stress. However, it should be noted that despite this intriguing evidence, previous attempts at locally inhibiting FAAH within the dorsal hippocampus have failed to produce antidepressant-like effects, although this may have been attributed to the high dose of URB597 administered or the precise subregion targeted (McLaughlin et al., 2007). There are distinct anatomical and functional differences between the dorsal and ventral hippocampus that deserve consideration in the context of stress and emotional behavior. The dorsal hippocampus performs primarily cognitive functions, while the ventral hippocampus coordinates actions that are relevant for stress, emotion, and affect (Fanselow and Dong, 2010). Accordingly, both subregions differ markedly in their anatomical connections. For example, the ventral hippocampus projects to regions of the PFC, whereas the dorsal hippocampus does not (Goldman-Rakic et al., 1984). Moreover, the ventral hippocampus is closely connected to the BNST, amygdala (Petrovich et al., 2001), and other sub-cortical structures associated with the HPA axis (Siegel and Tassoni, 1971; Krettek and Price, 1977a, b; Petrovich et al., 2001). Therefore, future studies should examine whether local inhibition of FAAH, particularly within the ventral region of the hippocampus, also engages proactive coping strategies in accordance with the data described in Chapter 3.

It is widely acknowledged that chronic stress is a primary instigating factor in the pathophysiological development of major depression. However, stress does not invariably lead to depression in all individuals; indeed, most individuals exposed to chronic stress exhibit resilience and are able to employ coping strategies to avoid such pathologies. Similarly, not all rodents subjected to models of depression display

depressive-like symptoms. Researchers are only now beginning to recognize the necessity for exploring the biological basis of individual differences in stress responsivity. Thus, it would be of considerable interest to compare two divergent, naturally occurring populations; those that are susceptible to the physiological and behavioral disturbances elicited by chronic stress regimens, and those that exhibit resilience to these stress-related deficits. Given the results from this dissertation and the fact that vulnerability to depression is preceded by dysfunction of the PFC and monoaminergic systems, it is suspected that alterations in AEA/CB_1 signaling in the ventromedial PFC dictate vulnerability and resilience to chronic stress. To execute this study, rats could be dichotomized according to their latency to defeat in the resident-intruder paradigm, an ethologically relevant model that recapitulates several aspects of melancholic depression (Wood et al., 2010). Then one could examine endocannabinoid content in the ventromedial PFC in these sub-populations, and locally manipulate CB_1 receptor signaling to examine whether this intervention can alter monoaminergic neurotransmission and behavioral coping strategies in these two divergent phenotypes. This research would substantially contribute to our understanding of the neurophysiological mechanisms that confer vulnerability and resilience to stress and offer additional insight into the therapeutic potential of prefrontocortical CB_1 activation.

In rodents subjected to chronic stress and humans suffering from melancholic depression, glucocorticoids are hypersecreted in an effort to limit excessive HPA axis activity, yet stress recovery is impaired in part because inhibitory feedback processes may be compromised. Perhaps this is due to a diminished ability of glucocorticoids to mobilize 2-AG in the medial PFC and/or hippocampus. Thus, an interesting extension of

the research described in Chapter 2 would be to determine the magnitude of the enhancement of 2-AG in the medial PFC and hippocampus of CUS-exposed animals treated with corticosterone. One might expect that CUS exposure dampens the ability of glucocorticoids to induce 2-AG synthesis in these two important inhibitory feedback structures, and that this contributes to overstimulation of the HPA axis and hypersecretion of glucocorticoids.

We have previously shown that AEA is significantly downregulated in the PFC following CUS exposure (Hill et al., 2008) and in Chapter 3 we established that acute inhibition of FAAH within the ventromedial PFC is sufficient to produce an increase in proactive stress coping behaviors. However, it remains to be seen whether FAAH inhibition within the ventromedial PFC is capable of preventing CUS-induced deficits. Therefore, another possible future direction could be to determine whether sustained suppression of FAAH with RNA silencing in the ventromedial PFC can prevent CUS-induced behavioral deficits. This is an especially intriguing study, as it could offer insight into whether local inhibition FAAH in the ventromedial PFC can increase resilience to behavioral and physiological disturbances observed in rats exposed to CUS, and possibly, humans afflicted with major depression.

Lastly, it is worth mentioning that there are striking similarities between the results described in Chapter 3 and recent studies employing deep brain stimulation of the ventromedial PFC in rodents. This intervention, which is quickly emerging as one of the more efficacious treatments for combating major depression in humans, has been shown to elicit antidepressant-like effects in the FST via a 5-HT-mediated mechanism (Hamani et al., 2010b), similar to our data using local inhibition of FAAH within the ventromedial

PFC. Moreover, these researchers have revealed that deep brain stimulation is also capable of reversing CUS-induced deficits in sucrose preference and hippocampal BDNF levels, but only in rats not receiving 5-HT-depleting lesions within the dorsal raphe (Hamani et al., 2012). The parallels between these studies and those described in the present body of work suggest that deep brain stimulation and facilitation of AEA/CB_1 receptor signaling in the ventromedial PFC may actually be activating a common pathway to elicit antidepressant-like effects (i.e., by increasing dorsal raphe 5-HT firing). It would be particularly interesting to examine this possibility further by measuring endocannabinoid levels in the ventromedial PFC in an effort to determine whether deep brain stimulation induces the synthesis of endocannabinoids to promote antidepressant-like responding. Evidently, there are a seemingly endless number of possible research paths that can be derived from the results obtained in this dissertation. These are just a few of the many possible avenues that are worth exploring in pursuit of fully comprehending the complex role of endocannabinoid signaling in the medial PFC under conditions of acute and chronic stress.

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