DYNAMIC CHANGES IN ADOLESCENT ENDOCANNABINOID SIGNALING IN MALE RATS: RELEVANCE TO STRESS RESPONSIVITY AND EMOTIONAL BEHAVIOUR

by

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Abstract

Adolescence is a period characterized by many distinct physical, behavioural and neural changes during the transition from child- to adulthood. In particular, adolescent neural changes often confer greater plasticity and flexibility, yet with this period of fluidity also comes the potential for heightened vulnerability to external perturbations such as stress exposure or recreational drug use. There is substantial evidence to suggest that factors such as adolescent stress exposure have longer lasting and sometimes more deleterious effects on an organism than stress exposure during adulthood. Moreover, the adolescent neuroendocrine response to stress exposure is different from that of adults, suggesting that further maturation of the adolescent hypothalamic-pituitary-adrenal (HPA) axis is required. The endocannabinoid system is a potential candidate underlying these age-dependent differences given that it is an important regulator of the adult hypothalamic-pituitary-adrenal (HPA) axis and neuronal development. Therefore, three studies were conducted in male rats to examine this possibility. The results of these experiments suggest that there is divergent age-dependent endocannabinoid tone between adolescents and adults (Chapter 2), age-dependent endocannabinoid regulation of HPA axis stress responsivity (Chapter 3) and a moderate organizational role of adolescent cannabinoid receptor type 1 (CB_1R) activation on adult stress responsivity and emotionality (Chapter 4). Taken together, the emerging picture suggests that the endocannabinoid system mediates interactions between HPA axis stress responsivity, emotionality and maturational stage. These findings may also be particularly relevant to our understanding of the development of affective disorders and the risks of adolescent cannabis consumption on emotional health and stress responsivity.

Preface

All of the work presented in this dissertation was conducted at the University of British Columbia (Point Grey campus), the Medical College of Wisconsin and the University of Calgary under the supervision of Dr. Boris B. Gorzalka. All animal research methodology was consistent with the protocols of the Canadian Council on Animal Care and were approved by the University of British Columbia Research Ethics Board (certificate #A13-0140 and #A11-0369) and the University of Calgary Research Ethics Board (certificate #AC11-0059).

Chapter 1. Figures 1.1 and 1.2, Tables 1.1 and 1.2 as well as portions of the introductory text are used with permission from: Lee TT & Gorzalka BB (2012). Timing is everything: evidence for a role of corticolimbic endocannabinoids in modulating hypothalamic-pituitary-adrenal axis activity across developmental periods. Neuroscience, 204, 17-30. I conceived the ideas in this review and wrote the manuscript. Additionally, a small portion of the introductory text (1.4 The developmental influence of gonadal hormones on the HPA axis) has been previously published: Goel N, Workman JL, Lee TT, Innala L, Viau V (2014). Sex differences in the HPA axis. Comprehensive Physiology, 4(3), 1121-1155. I wrote the sections of this review outlining the impact of gonadal hormones on the developing HPA axis an assisted in the editing of the manuscript as a whole.

Chapter 2. A version of this material has been previously published: Lee TT, Hill MN, Hillard, CJ & Gorzalka BB (2013). Temporal changes in N-acylethanolamine content and metabolism throughout the peri-adolescent period. Synapse, 67(1), 4-10. I conducted all the experiments and wrote the manuscript. I also performed additional studies and analyses of corticolimbic 2-AG content (unpublished).

Chapter 3. A version of Chapter 3 (Experiment 1) is currently under review for publication. I conducted all the experiments (with the assistance of JM Gray and MN Hill for mass spectrometry, some membrane preparation and hypothalamic MAGL enzyme activity assays), analyzed the data and wrote the manuscript.

A portion of this material has also been previously published (Experiment 2): Lee TT & Hill MN (2013). Age of stress exposure modulates the immediate and sustained effects of repeated stress on corticolimbic CB(1) receptor binding in male rats. Neuroscience, 249, 106-114. I conducted the experiment and wrote the manuscript with MN Hill.

I also performed additional analyses on ventral and dorsal hippocampal samples for MR and GR protein levels (unpublished).

Chapter 4. A version of chapter 4 is currently under review for publication. I conducted all the behavioural testing and analyses, HPA axis stress responsivity challenges and assisted in the mass spectrometry and CB_1 receptor binding assays, as well as wrote the manuscript.

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List of Abbreviations

µg – Microgram

µl – Microliter

µM – Micromolar

ºC – Degrees celsius

2-AG – 2-arachidonoylglycerol

5-HT – 5-hydroxytryptamine (serotonin)

ACTH – Adrenocorticotropic hormone

AEA – N-arachidonoylethanolamine

ANOVA – Analysis of variance

AVP – (Arginine) vasopressin

B_{max} - Maximal binding site density

BLA – Basolateral amygdala

BSA – Bovine serum albumin

Ca^{2+} - Calcium

CB - Cannabinoid

CBG – Cortisol binding globulin

CB_1R – Cannabinoid receptor type 1; CB1 receptor

CORT – Corticosterone

CRH – Corticotropin releasing hormone

DAGL – Diacylglycerol lipase

DMSO – Dimethyl sulfoxide

DSI – Depolarization-induced suppression of inhibition

DSE – Depolarization-induced suppression of excitation

eCB – Endocannabinoid

EDTA – Ethylene diamine tetraacetic acid

FAAH – Fatty acid amide hydrolase

GABA - γ-aminobutyric acid

GR – Glucocorticoid receptor

GTPγS – Guanosine 5'-O-[gamma-thio]triphosphate

HPA axis - Hypothalamic-pituitary-adrenal axis

Hr – Hour(s)

IP – Intraperitoneal

K_D – Dissociation constant (binding affinity of AM-251 to CB_1 receptors)

K_m – Binding affinity (substrate concentration at 1/2 the maximum reaction velocity)

kg – kilogram

MAGL – Monoacylglycerol lipase

MDEP – Maternal deprivation

mg – Milligram

$MgCl_2$ – Magnesium chloride

min – Minute(s)

mL – millilitre

MR – Mineralocorticoid receptor

mRNA – Messenger ribonucleic acid

NAE – N-acylethanolamine

NAPE - *N*-acyl phosphatidylethanolamine

NAPE-PLD – N-acyl phosphatidylethanolamine - specific phospholipase D

nL - nanoliter

nM – nanomolar

OEA - Oleoylethanolamine

PEA - Palmitoylethanolamine

PE - Phosphatidylethanolamine

PFC – Prefrontal cortex

pmol – Picomoles

PND – Post-natal day

PVN – Paraventricular nucleus of the hypothalamus

RNA – Ribonucleic acid

Sec – second

SEM – Standard error of the mean

THC – Δ-9-tetrahydrocannabinol

TME - Tris-HCl- MgCl2-Ethylenediaminetetraacetic acid

V_{max} – Maximum reaction velocity

VEH - vehicle

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To one of the strongest women I know, Laura Jay.

1. General Introduction

Adolescence is often referred to as a "perfect storm," given the numerous physical, neural and behavioural changes occurring simultaneously. Behaviourally, the adolescent is somewhat erratic with increased intensity of emotional states, oppositional attitudes, and increased risk taking behaviour coupled to a limited ability to engage in self-control in order to override these emotions and behaviours (Casey et al., 2011; Walker et al., 2004). These behaviours eventually cease and stabilize with the onset of young adulthood (Walker et al., 2004). However, in some cases behavioural or adjustment problems are precipitated during the onset of puberty and lead to psychiatric disorders that persist in adulthood (Kim-Cohen et al., 2003; Pine et al., 1998; Walker et al., 2004). In fact, over 75 percent of adults with a fear/anxiety-related disorder met diagnostic criteria as children or adolescents (Kim-Cohen et al., 2003). Similarly, estimates indicate that one in five adolescents have a mental illness that will persist into adulthood (Paus et al., 2008), and depression and anxiety disorders occur in as many as one in 10 adolescents (Costello et al., 2005; Kessler et al., 2005). Together, these findings support a sobering reality that the prevalence of mental illness, particularly emotional and anxiety disorders is specifically heightened in adolescence. Moreover, these bleak statistics highlight the importance of understanding the physiological and neural mechanisms underlying emotional development and stress regulation development (Pattwell et al., 2013).

To echo the sentiments of many developmental researchers, infants, children and adolescents are not simply miniature adults functioning with less developed cognitive, neural and behavioural processes. The demands required of the individual in each of these periods are also fundamentally different from those of adults, thus guiding these processes in very different ways. Infancy and childhood are characterized by heavy dependence on parental care for survival and learning of basic skills (e.g., suckling) and are coupled with significant neural changes that facilitate the ability to learn these associations and relationships. Building on top of

these acquisitions, adolescent development is a period of transition from parental dependence to independence, increased novelty and risk taking behaviour, establishment of more complex peer social relationships, as well as cognitive and behavioural processes involved in higher order processing indicative of the simultaneous and subtle neural reorganization of this age (Spear, 2000).

Interestingly, certain aspects of adolescent development appear non-linear and unique relative to young children and adults. Underlying these distinctive adolescent behavioural characteristics, numerous maturational processes such as synaptic pruning, reduction of grey matter, increased myelination, and strengthening of connectivity between neural structures, occur throughout the brain in a time-dependent and region-specific manner (Crews et al., 2007; Guerry and Hastings, 2011). For example, human imaging studies indicate that adolescent neurodevelopment involves maturation of motor and sensory cortices earlier than association areas that govern higher-order cognition and behaviour (Gogtay et al., 2004). It has been suggested that a transient developmental imbalance occurs between the structural and functional maturity of neural circuitry involved in emotional and reward-based behaviour and circuitry governing cognition and impulse control, giving rise to adolescent-specific characteristics such as heightened emotional reactivity and increased risk taking behaviour (Somerville et al., 2010). Moreover, it has been hypothesized that exacerbation of this transient structural and functional imbalance by various factors such as stress exposure, contributes risk for mental illness (Lee et al., 2014a). However, the exact mechanisms of action and conditions by which this kind of exacerbation can occur remains unclear.

The hypothalamic-pituitary-adrenal (HPA) axis is the body's major neuroendocrine response to stress exposure, with activity rising to promote processes (e.g., glucose mobilization) that meet the energetic demands associated with resolving the imminent perceived or real threat (e.g., running away from the threat). In the short term, the HPA axis stress response is adaptive;

however, studies examining the impact of chronic stress exposure indicate that prolonged glucocorticoid exposure is associated with a number of detrimental consequences ranging from cardiac disease to depression. Moreover, dysfunction of the adult HPA axis has been associated with emotional disorders such as depression and anxiety disorders such as post-traumatic stress disorder (Miller et al., 2007). Numerous studies indicate that maturation of the HPA axis also occurs over childhood/early life and adolescence before reaching the full functionality observed in adulthood. Moreover, processes devoted to pubertal maturation coincide with HPA axis maturation and sharp elevations in circulating gonadal hormones are known to modulate subsequent neural and HPA axis development. Deviations from the normative developmental trajectory induced by disruptions such as stress exposure appear to elicit profound long-term deficits or impairments to adult HPA axis stress reactivity and emotional behaviour, providing confirmation that early life and adolescence are windows of susceptibility to stress.

There are separate, but compelling lines of evidence supporting the idea that the endocannabinoid (eCB) system contributes regulation over both neurodevelopmental processes and adult HPA axis stress responsivity and emotional behaviour. Moreover, there are preliminary indications reviewed in the current chapter suggesting that corticolimbic eCB signaling is an important mediator of interactions between HPA axis stress responsivity and maturational stage (e.g., adolescence). Studies employing cannabinoid agonists suggest that early life and adolescence are periods of vulnerability that also coincide with distinct maturational alterations in the eCB system to stimulate age-dependent effects on HPA axis activity. These studies also indicate that cannabinoid exposure, whether by maternal use or during adolescence, in these periods of sensitivity can contribute to long-term dysregulation of the brain, emotionality and anxiety-like behaviour, reminiscent of the results of extended glucocorticoid exposure. Thus, further investigation of the mechanisms underlying eCB system mediation between development and stress-induced HPA axis functioning are important.

However, a significant gap exists in the adolescent literature investigating the regulation of stress responsivity by adolescent corticolimbic eCB signaling. Therefore, Chapter 1 will provide a review of: 1) functionality of the HPA axis during early life and adolescence, 2) how gonadal hormones modulate the developing HPA axis, 3) how the eCB system is an important regulator of HPA axis activity and neurodevelopment and lastly, 4) evidence indicating that corticolimbic eCB signaling acts as a mediator between developmental stage and HPA axis stress responsivity. Following this, the studies described in Chapter 2 and Experiment 1 in Chapter 3 were designed with the intent of providing a foundational understanding of normative adolescent corticolimbic signaling as a function of HPA axis activity (i.e., basal and stress-induced conditions). Furthermore, the studies in Chapters 3 (Experiment 2) and 4 are aimed at gaining an understanding on the long-term effects of stress exposure and adolescent CB_1R blockade on the development of the adult HPA axis, eCB system and emotional behaviour. Collectively, this body of work provides preliminary evidence indicating that corticolimbic eCB signaling contributes to adolescent neurodevelopmental processes and HPA axis stress regulation and may provide a neural substrate by which stress exposure can exert its detrimental effects.

1.1 Adolescence and Pubertal Maturation

One of the challenges in adolescent research is that the adolescent period remains ill-defined in terms of lacking distinct age boundaries. Defining an individual's maturational stage from infancy to adulthood remains somewhat difficult, particularly when the criteria accompanying each developmental category are not always agreed upon and individuals mature at different rates (McCormick et al., 2010). Furthermore, considering maturational stage in the context of species comparisons can further complicate one's understanding of developmental findings.

Many developmental rodent studies adhere to age ranges outlined in excellent reviews by Spear (2000; Figure 1.1), Eiland and Romeo (2013) as well as McCormick and colleagues (2010). In the rodent literature, "neonatal" typically refers to the first two weeks of life and "juvenile" refers to the period spanning from the age of weaning (generally post natal day (PND) 21) to early adolescence or pre-puberty (Eiland and Romeo, 2013). Rodent "adolescence" is conservatively regarded as PNDs 28-42, although this period has been reported by some authors to last at least until PND 55 in male rodents (Spear, 2000). Moreover, age of pubertal onset is often used as an indicator of adolescence (Spear, 2000). In the male rodent, pubertal onset generally occurs around PND 42 and and can be defined by balano-preputial separation in which the prepuce exhibits complete retraction from the head of the penis (Lewis et al., 2002). As in human females, pubertal onset occurs earlier in female rodents with vaginal opening in the rat appearing around PND 30 (Lewis et al., 2002). While it should be noted that these age ranges will vary by individual and strain of rodent, the current work adheres to the terminology described in previous work (i.e., McCormick et al., 2010; Spear, 2000) for consistency.

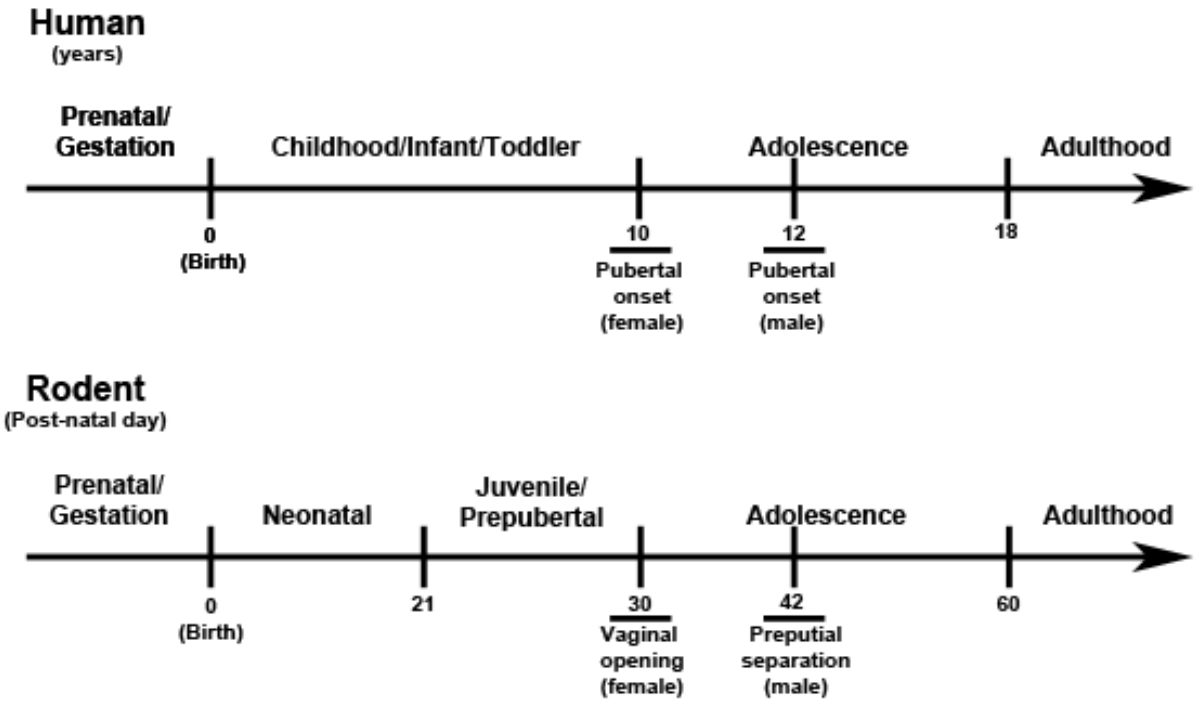

Figure 1.1 Approximate comparison between human and rodent development.

Puberty is a key developmental process that occurs during the adolescent period and includes behavioural and gonadal maturation that allows transition of an organism from a non-reproductive to reproductive state (Sisk and Foster, 2004). “Puberty” is often used interchangeably with the term “adolescence;” however, specialists use the term “puberty” to refer specifically to developmental processes of the hypothalamic-pituitary-gonadal axis that ensure gonadal maturation whereas “adolescence” may include pubertal processes as well as maturation of neural, cognitive, social and emotional processes in the transition from child to adult (Sisk and Zehr, 2005). One of the biological signatures of pubertal maturation is the dramatic rise in circulating gonadal hormones that are not only necessary for achieving reproductive maturation, but also produce secondary sex characteristics such as breast development or facial hair growth. The developing adolescent brain is also exquisitely sensitive to elevated levels of gonadal hormones, particularly in the remodeling of cortical and limbic circuits (Guerry and Hastings, 2011; Johnson et al., 2009; Juraska et al., 2013; Rice and Barone, 2000) that lead to adult cognition and behaviour. However, the complex interaction between the pubertal rise in gonadal hormones and timing of protracted maturational neural processes does vary by individual, making the adolescent brain a moving target for actions of these hormones (Sisk and Zehr, 2005). For example, age of pubertal onset varies among individuals and thus creates variability in the point at which gonadal hormones are exposed to the brain to modulate the developmental trajectory of neural and behavioural maturation (Sisk and Zehr, 2005).

Timing of pubertal onset is tied to an increased frequency of gonadotropin-releasing hormone (GnRH) pulse secretions generated by the median eminence of the hypothalamus. In order to support full gonadal and reproductive functioning, GnRH pulses must have relatively short interpulse intervals, but it is not currently known what mechanisms trigger an increase in GnRH pulse secretions from a quiescent state to initiate pubertal maturation (see review, Sisk and Zehr, 2005). The available literature suggests that these permissive signals vary by species

and sex, but predominantly relate to energy balance (Sisk and Zehr, 2005). Interestingly, there is also a transient rise in GnRH pulsatility to activate the hypothalamic-pituitary-gondal axis and stimulate gonadal steroid hormone circulation during late prenatal to early postnatal life, which contributes to sexual differentiation. Increasing pulses of GnRH into the circulation stimulate the pituitary gland to synthesize and release luteinizing hormone (LH) and follicle-stimulating hormone (FSH) to direct production of ova and spermatozoa as well as the gonadal steroid hormones from ovarian and testicular cells. Gonadal steroid hormones participate in follicle maturation and spermatogenesis and provide feedback on GnRH pulse secretion.

Due to the high degree of neural plasticity and maturation occurring during early life, researchers have long identified this period as a critical window in which the organism is particularly vulnerable to perturbations (e.g., maternal substance abuse), which can have long-lasting and even permanent consequences on the adult brain and behaviour (McEwen, 1992). More recent research acknowledges adolescence, also a period of high neural plasticity, as another distinct and critical stage of susceptibility to disturbances such as stress exposure (Andersen and Teicher, 2008; Casey et al., 2010; Eiland and Romeo, 2013; Lee et al., 2014a). In some ways, adolescence is similar to child development, characterized by substantial physical, emotional and behavioural alterations (Casey et al., 2010). However, the nature of adolescent development is distinct from that of childhood. Neural maturation in adolescence comes in the form of fine-tuning the brain via synaptic sprouting and pruning, myelination of nerve fibres, elimination of unnecessary connections and reorganization of neurotransmitter systems, all with the goal of facilitating neural efficiency and flexibility (Guerry and Hastings, 2011; Rice and Barone, 2000; Sisk and Zehr, 2005). Preclinical rodent studies have reported an overproduction of cortical axons and synapses during early adolescence, which are quickly pruned in late adolescence (Crews et al., 2007). Similarly, adolescent dendritic pruning occurs in the amygdala and nucleus accumbens, whereas growth of fibres between the amygdala and prefrontal cortex

(PFC) continues into early adulthood (see reviews, Brenhouse and Andersen, 2011; Casey et al., 2008). Thus, adolescence also confers greater neural plasticity and flexibility compared to adulthood, yet with this fluidity also comes the potential for heightened vulnerability to external disruptions (e.g., stress exposure) that further modulate normative brain and behavioural maturation.

1.2 The Neurobiology of Stress

Stress is considered a state of strain elicited by a real or perceived threat to homeostatic functioning. An organism exposed to a stressor activates two reflex arcs; the first is activation of the hypothalamic-sympathethic-adrenomedullary axis triggered by sympathetic neural efferents that stimulate release of adrenomedullary catecholamines (Tasker and Herman, 2011; Ulrich-Lai and Herman, 2009). The second reflex arc activated is the neuroendocrine reflex arc, which involves stimulation of the hypothalamic-pituitary-adrenal (HPA) axis by hypothalamic hormones (Tasker and Herman, 2011; Ulrich-Lai and Herman, 2009). It is the HPA axis that represents the major neuroendocrine axis responsible for the maintenance of homeostatic functioning in the face of stress exposure (Ulrich-Lai and Herman, 2009). Both reflex arcs converge on the adrenal glands, with the autonomic response stimulating rapid catecholamine release by the adrenal medulla and the neuroendocrine response resulting in enhanced glucocorticoid secretion by the adrenal cortex. It is believed the autonomic response contributes to the "fight-or-flight" response to promote survival whereas the neuroendocrine response promotes glucose mobilization from muscle and liver to enhance cardiac function, inhibit growth, reproductive and immune responses in an effort to divert all energy stores towards dealing with the threat at hand (Tasker and Herman, 2011; Ulrich-Lai and Herman, 2009).

The typical adult neuroendocrine HPA axis response to acute stress exposure involves hypothalamic activation of corticotropin releasing hormone (CRH) neurosecretory cells in the

paraventricular nucleus (PVN), with CRH stimulating anterior pituitary gland secretion of adrenocorticotropin releasing hormone (ACTH) into the circulatory system. Detection of ACTH in the blood stimulates the adrenal cortex to release glucocorticoids, primarily corticosterone (CORT) in rodents and cortisol in humans. Cessation of this neuroendocrine stress response occurs via negative feedback with glucocorticoids crossing the blood-brain barrier and binding to glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) found in the PVN and other extra-hypothalamic sites such as the amygdala, PFC and hippocampus.

MRs have a higher binding affinity (~10-fold) for corticosteroids than the more widely distributed GR (Rupprecht et al., 1993). As a result, MRs are readily occupied even under basal (non-stress) conditions (ter Heegde et al., in press). In contrast, GR's lower binding affinity results in high occupancy only under conditions in which CORT concentrations peak, such as stress exposure. Intracellular GR acts as a ligand-activated transcription factor to exert genomic long-acting changes in gene transcription (de Kloet et al., 1998; McEwen, 1973) to facilitate recovery and adaptation to stressors, while the non-genomic effects occur within minutes of glucocorticoid release and are thought to be mediated by activation of putative membrane-associated GRs to provide rapid negative feedback inhibition of the HPA axis and promote recovery by mobilizing energy resources (Orchinik et al., 1991; Tasker and Herman, 2011; Ulrich-Lai and Herman, 2009). Relatively recent work suggests that glucocorticoid binding to MR contributes to maintenance of limbic stress circuitry with the ability to set the threshold for HPA axis activation (De Kloet et al., 2007; Joëls et al., 2008). Studies on possible non-genomic effects indicate there is a lower affinity membrane bound MR involved in appraisal of the stressful stimulus and choosing the appropriate behavioural responses to cope with the stressor (Joëls et al., 2008); however the exact mechanisms underlying these cognitive processes remain to be determined. From what is known, MR and GR have complementary functions in the regulation of HPA axis stress responsivity and it is currently hypothesized that disturbances to

the normative ratio of MR:GR can result in HPA axis dysfunction and contribute to the development of psychiatric disease such as depression (De Kloet et al., 2007).

The brain also plays an important governing role in the regulation of the HPA axis based in part on the stressor type (Herman et al., 2003). Reactive stressors are stimuli that present a direct challenge to physical homeostatic functioning such as cold, blood loss, and infection (Ulrich-Lai and Herman, 2009). In contrast, anticipatory stressors refer to stimuli that are perceived as stressful, based on previous experience or innate species-specific preconceptions, rather than directly disrupting homeostasis (Herman et al., 2003; Ulrich-Lai and Herman, 2009).

It is important to note that the distinction between physiological and psychological stressors is experience-dependent. Thus, exposure to a reactive or physiological stressor in a specific context can be conditioned and result in a psychological or anticipatory response upon subsequent exposure to that same environment (Herman et al., 2003). Accordingly, the neural pathways differ in their responses to reactive versus anticipatory stressors. Exposure to a physiological stressor is recognized by peripheral sensory pathways that signal homeostatic disruption and travel up the spinal cord to activate the brainstem. An autonomic response to this input engages reflex arcs involving preganglionic sympathetic neurons in the intermediolateral cell column of the spinal cord and hindbrain regions such as the rostral ventrolateral medulla (Herman et al., 2003). Furthermore, activation of the HPA axis is engaged via ascending catecholaminergic pathways that are primarily noradrenergic and originate from the nuclei of the solitary tract and project to the parvocellular region of the PVN (Ulrich-Lai and Herman, 2009). However, the response to psychological stressors also relies on indirect inputs from forebrain structures that are responsible for determining whether a stimulus is threatening or non-threatening, such as the amygdala, hippocampus and PFC (Ulrich-Lai and Herman, 2009).

Indeed, corticolimbic structures of the forebrain assist in regulating psychological stress-induced HPA axis responses; the PFC, amygdala and hippocampus, provide input to the

hypothalamus mostly via the bed nucleus of the stria terminalis, (McEwen, 1992; McEwen, 2005; Romeo and McEwen, 2006). The medial PFC is subdivided into two regions with opposing contributions to the stress response: the prelimbic region contributes to inhibition and termination of HPA axis and autonomic responses to psychological stressors while the infralimbic region of the medial PFC is associated with initiating autonomic and HPA axis responses (Figueiredo et al., 2003; Radley et al., 2006). Stimulation of the hippocampus also has an inhibitory effect on HPA axis activity (e.g., decreases glucocorticoid secretion) and lesion studies have demonstrated that the hippocampus contributes to termination of the stress response (Ulrich-Lai and Herman, 2009). The amygdala is also functionally heterogeneous with numerous downstream targets that modulate the HPA axis and autonomic system. The central nucleus of the amygdala is activated by physiological stressors and the integration of autonomic components to psychological stressors whereas the medial and basolateral amygdala nuclei (BLA) are activated by psychological stressors and do not appear to have a role in regulating autonomic responses (Ulrich-Lai and Herman, 2009).

The brain plays an integral role in terminating the stress response by promoting feedback inhibition of the HPA axis and restoring homeostasis. Negative feedback regulation is critical to normative HPA axis functioning since it prevents hypothalamic and pituitary stress hormone depletion, allowing for the mounting of successive stress responses, and prevents prolonged exposure to high glucocorticoid levels (Sapolsky et al., 1984). Furthermore, under conditions of high circulating glucocorticoid levels such as those of chronic stress exposure, vasopressin (AVP), which potentiates the stimulatory effects of CRH on ACTH secretion, also contributes to maintaining corticotroph responsiveness (Aguilera and Rabadan-Diehl, 2000). While it is believed that in the short term, activation of the neuroendocrine stress response is adaptive and beneficial, chronically elevated levels of glucocorticoid hormones, whether resulting from impaired negative feedback or chronic stress exposure, can lead to many long term harmful

consequences (Chrousos, 2009). Indeed, chronic stress exposure can lead to pathological disorders ranging from diabetes and cardiac failure to depression and anxiety disorders. (McEwen, 2007). In addition to HPA axis negative feedback mechanisms to combat these detrimental conditions from developing, an adaptive form of HPA axis plasticity emerges in response to repeated exposure to the same stressor, termed the habituation response. In this case, the organism learns that the stressor is no longer a threat to survival with repeated exposure, resulting in a decrease in activation of neural structures in the corticolimbic circuit as well as an inhibition of glucocorticoid synthesis and release (Jaferi and Bhatnagar, 2006).

1.3 HPA Axis Development

The HPA axis undergoes maturational processes prior to becoming fully functional in adulthood. The rat neonate has been reported to experience a "Stress Hyporesponsive Period" (SHRP) whereby the HPA axis appears to exhibit comparatively low basal CORT levels (PND 4-14) and stress exposure results in little or no increase in those CORT levels (Schoenfeld et al., 1980). Previous research indicates that the SHRP is specific to hyporesponsiveness of adrenal gland output rather than hypothalamic or pituitary function (Dent et al., 2000). Furthermore, it appears that humans also experience a functionally similar SHRP that emerges in infancy and may extend throughout childhood given that it is difficult to induce elevations in cortisol levels, although basal concentrations are comparable to those in adults (Lupien et al., 2009). It is unknown whether the mechanisms contributing to this stress hyporesponsiveness are similar to those of the neonatal rat (Gunnar and Donzella, 2002) and it has been suggested that the human and rat neonate SHRP are likely related to neonate-maternal interactions.

However, there is relatively recent support for a reconceptualization of the SHRP. CORT responses to stress appear functional at birth (Widmaier, 1990) which is supported by findings that the human neonatal HPA axis is responsive to pain (Stang et al., 1988). Moreover, rodent

pups have been reported to launch a 300-400% increase in CORT levels in response to maternal separation or hypothermia (see review, Molet et al., 2014). These findings indicate that the neonatal HPA axis is in fact, functional yet is more finely tuned to stressors that are relevant to early life (Molet et al., 2014). Specifically, maternal sensory stimulation during nursing and grooming maintain relatively low CORT levels in pups (Stanton and Levine, 1990; Sullivan and Holman, 2011). This has led to the development of a relatively new, more naturalistic animal model of early life stress in which pregnant dams are provided with limited bedding materials that prevent construction of a satisfactory nest, thus serving as a maternal stressor. This stress is associated with fragmented rather than less maternal care and produces parameters indicative of early life stress in those pups (i.e., elevated CORT levels, higher adrenal weights, reduced body weight; Molet et al., 2014). Furthermore, this model provokes cognitive and emotional deficits in weanling rats that persist into adulthood, coupled to a loss of hippocampal synapses and dendritic morphology (Brunson et al., 2005) and an upregulation of hippocampal CRH mRNA expression (Ivy et al., 2010). In contrast, consistent and predictable maternal care results in a phenotype that is more resilient to stress exposure and depressive-like behaviours and is associated with reduced hypothalamic CRH mRNA expression (Baram et al., 2012; Fenoglio et al., 2005).

Maternal deprivation paradigms within the first few weeks of life have been most commonly used as rodent models of early life stress and are shown to have permanent, adverse consequences, such as reduced hippocampal MR expression, on adult HPA axis stress responsivity and plasticity in the corticolimbic stress circuit (Vazquez, 1998). Moreover, maternal deprivation contributes to depression- and anxiety-like symptoms through temporary disruption of typical dam-pup interactions (Holmes et al., 2005; Levine, 2000; Meaney, 2001). This paradigm has also been used to investigate the impact of early life stress on subsequent HPA axis functioning, neural maturation and behavioural models of depression and anxiety.

Length of exposure and duration of separation session vary by laboratory (1-24 hr for 1-14 days), with longer exposures of maternal separation stress generally upregulating adult stress-induced HPA axis sensitivity (Holmes et al., 2005) as well as initiating structural and functional neural degeneration in the medial PFC and hippocampus (McEwen, 2005). The effects of exposure to this paradigm are similar to those of peri-adolescent chronic variable stress exposure on adult HPA axis function and hippocampal morphology (Isgor et al., 2004). On the other hand, the negative programming effects of brief exposure to maternal deprivation have been shown to be reversible with environmental stimulation, such as handling, to decrease this HPA axis hypersensitivity and influence emotional and anxiety-related behaviours in adulthood (Anisman et al., 1998; Francis et al., 2002; Holmes et al., 2005). These experiments provide an interesting model for "stress inoculation-induced" resilience and the mechanisms of this adaptability appear promising, but require further study (Lyons et al., 2010).

Following the SHRP, the HPA axis possesses the ability to launch a neuroendocrine response to stress and basal glucocorticoid levels comparable to those of adults (Pignatelli et al., 2006; Romeo, 2010b; Romeo et al., 2006; Romeo and McEwen, 2006). However, numerous studies demonstrate divergent stress-induced HPA axis functioning between prepubertal and adult rodents. Acute restraint stress (Doremus-Fitzwater et al., 2009; Romeo et al., 2004a; Romeo et al., 2004b), intermittent exposure to foot shock (Goldman et al., 1973) or ether vapours (Vázquez and Akil, 1993), elicit comparable basal and peak CORT levels in adolescent and adult rats. However, roughly twice as much time is required to return to basal levels relative to that of adults with the same treatment. This effect is observed in both sexes exposed to acute restraint, with females exhibiting higher basal and stress-induced increases in CORT levels relative to males (Doremus-Fitzwater et al., 2009; Romeo, 2010a; Romeo, 2010b).

Foundational work by Vazquez and colleagues (see review, Vazquez, 1998) indicate that chronic intermittent stress exposure in both the adult and juvenile rodent (PND 25) yield

elevated CORT levels; however, the way in which this is achieved is vastly different. Whereas adults increase the secretion of ACTH and reduce negative feedback at the pituitary gland to stimulate elevations in CORT, the developing rat increases proopiomelanocortin (precursor of ACTH) processing to stimulate only a nominal increase in ACTH; however, the combination of reduced responsiveness to negative feedback and a more sensitive adrenal gland renders the prepubertal animal capable of responding to even small amounts of ACTH, thus increasing CORT levels. At the same time, this combination also results in longer exposure to circulating glucocorticoids while the HPA axis itself is undergoing maturation (Vazquez, 1998). Using acute restraint stress, Romeo and colleagues (2014) complemented the work of Vazquez by demonstrating that adrenal expression of the ACTH receptor, melanocortin 2 receptor, and the expression of melanocortin receptor accessory protein, which chaperones this receptor to the cell surface is greater in prepubertal rats relative to adults. Moreover, exogenous ACTH administration resulted in higher CORT levels at lower doses of ACTH in prepubertal animals 60 min following injection, which indicates that the prepubertal protracted HPA axis stress response is at least partly due to greater adrenal sensitivity to ACTH and also suggests that prolonged exposure to ACTH, itself, leads to greater CORT responsivity (Romeo et al., 2014). However, potential central mechanisms subserving age-dependent differences in negative feedback remain to be determined.

Further evidence of age-dependent stress reactivity has been observed using a repeated restraint stress paradigm (seven consecutive days of 30 min restraint) in which adult rats exhibit habituated (lowered) peak CORT levels whereas adolescent rats fail to achieve this (i.e., peak CORT levels during the final restraint session, yet display accelerated recovery to basal levels (Doremus-Fitzwater et al., 2009; Romeo et al., 2004a; Romeo et al., 2004b). These hormonal observations were associated with region-specific patterns of neuronal activation in corticolimbic circuitry and increased activation of CRH neurons in the PVN of prepubertal, but not adult rats

(Romeo et al., 2006). However, somewhat surprisingly, these age-dependent effects were not dependent on differences in hippocampal glucocorticoid mRNA or protein expression (Dziedzic et al., 2014; Romeo et al., 2008). Using another model, chronic variable stress exposure during early-, mid-, and late-adolescence periods as well as adulthood revealed age-dependent effects on HPA axis function and emotionality. Whereas the rats exposed to chronic variable stress in late adolescence exhibit elevated basal CORT and oxytocin levels, only the adult chronic variable stress group displayed an increase in stress-coping behaviour in the forced swim test (Jankord et al., 2011). When an ecologically relevant resident-intruder model of social stress is employed, basal and stress-induced CORT levels in adolescent (PND 28) rats do not differ from those of adults, although proactive behaviours in the defensive burying and forced swim test are increased in the adolescents (Bingham et al., 2011). These data suggest a general sensitivity to adolescent stress-induced HPA axis function relative to that observed in the adult rat.

Stress experience during development has also been shown to have long term, even permanent, negative effects on subsequent stress reactivity and corticolimbic neurocircuitry. Rats housed in social isolation (PND 30-50) were shown to exhibit long-term sex- and region-dependent consequences. There was an increased stress response (i.e., elevated CORT and hypothalamic AVP mRNA) to acute and repeated restraint as well as increased hippocampal brain-derived neurotrophic factor (BDNF; promotes neuroplasticity) mRNA in females exposed to adolescent social isolation whereas males in the same condition exhibited a reduced CORT response to acute restraint, reduced orexin (an arousal and attention promoting neuropeptide) mRNA, and reduced anxiety-like behaviour in the elevated plus maze (Weintraub et al., 2010). Adolescent chronic variable stress exposure also decreases hippocampal volume, downregulates basal GR expression, yields a protracted CORT response to a 15 min session in the open arm of an elevated plus maze, as well as deficits in spatial learning in the Morris water maze (Isgor et

al., 2004). Together, the literature indicates that adolescents are more sensitive to stress exposure both in the immediate and long-term, compared to adults.

1.4 Developmental Influence of Gonadal Hormones on the HPA Axis

As discussed earlier, pubertal maturation involves a significant rise in gonadal hormones (primarily testosterone in males and estradiol in females), which not only function to ensure reproductive maturation, but also influence the developing brain and HPA axis. In fact, sex differences in the rodent HPA axis begin to emerge relatively early in life. These differences are likely related to the release of gonadal hormones at pre- and postnatal developmental stages. In male rodents, a prenatal surge of testosterone occurs in the fetus on days 18-19 of gestation and a second surge occurs 2-4 hours after birth (Corbier et al., 1978; MacLusky and Naftolin, 1981; McGivern et al., 1993). Both periods of hormone exposure are considered critical for the masculinization and defeminization of the male brain (Arnold and Gorski, 1984; Feder et al., 1966; MacLusky and Naftolin, 1981). This hormone exposure is also known to have organizational effects that are important for sex-specific development of the HPA axis. There is little to no gonadal hormone secretion during juvenile stages; however, with the onset of puberty, gonadal organs mature and begin to release adult levels of hormone. As such, HPA axis activity varies as a function of age and sex, both under basal and stress conditions (Viau et al., 2005), shifting to meet fluctuating demands of the developing organism's environment (e.g. Levine, 1994; Sullivan and Holman, 2011).

There is evidence that naturally high levels of testosterone at birth provide key organizing effects on the developing male brain and HPA axis to modulate the adult neuroendocrine stress response. This is demonstrated by elevated ACTH and CORT levels induced by an ethanol injection in adult male rats gonadectomized at birth (Ogilvie and Rivier, 1996). Furthermore, adult male rats that were gonadectomized at birth display higher ACTH and

CORT levels (McCormick et al., 1998) as well as Fos activation in the medial parvocellular region of the PVN that were coupled to reductions in androgen receptors and AVP-positive cells in the bed nucleus of the stria terminalis and medial nucleus of the amygdala in response to restraint stress (Bingham and Viau, 2008). Neonatal, but not adult, testosterone replacement appears to normalize these effects (Bingham and Viau, 2008; McCormick et al., 1998). To further support this idea, a single injection of testosterone propionate in females within 24 hr of birth has been reported to result in permanent, masculinized HPA axis activity (Seale et al., 2005a). However, other studies have reported that a single injection of testosterone in newly born female pups fails to elicit the masculinized adult HPA axis stress responsivity as described above (Goel and Bale, 2008; Ogilvie and Rivier, 1996).

The aromatization of testosterone to estradiol has also been shown to be critical for the organizing effects of neonatal testosterone on HPA axis responsivity. Male rat neonates implanted with an aromatase inhibitor capsule within the first 12 hours of life (removed at weaning) exhibit elevations in adult CORT responses to acute restraint stress and Fos expression in the PVN (Bingham et al., 2012) at a magnitude that parallels adult females. Similarly, Seale and colleagues (Seale et al., 2005b) found that males pre- and postnatally treated with an anti-androgen (flutamide) or aromatase inhibitor (1,4,6-androstatriene-3,17-dione) secreted higher basal and stress-induced (noise or LPS) adult CORT levels than controls. Both groups of male rats also had higher AVP and CRH mRNA in the PVN, and lower GR mRNA in the PVN following the cessation of the stressors (Seale et al., 2005b). Lastly, female rats treated with estradiol from birth to post-natal day 7 exhibited permanent "de-feminization" of HPA axis activity with greater levels of CRH and AVP mRNA in the PVN and reduced GR mRNA in the hippocampus than adult females in diestrus (Patchev et al., 1995).

Pubertal onset is considered a key developmental event signaling maturation from a non-reproductive to a functional reproductive state. The HPA axis undergoes maturation during early

adolescence demonstrated by adrenarche (i.e., increased production and secretion of adrenal steroids). This process precedes increasing pulses of gonadotropin releasing hormone (GnRH), that stimulate luteinizing and follicle-stimulating hormone release from the pituitary gland to contribute to increases in sex steroid production from the gonads. The sharp and rapid elevations in gonadal hormones facilitate reproductive maturation, physical growth, and development of secondary sex characteristics in the adolescent. The rising gonadal hormone levels also facilitate organizational and activational effects on the developing adolescent brain. These elevated levels of gonadal hormones also exert influence on the HPA axis, likely contributing to the emergence of sex differences in HPA axis functionality, though the mechanisms by which these sex steroids influence the brain to regulate the HPA axis remain to be determined (Viau, 2002).

From what is known, increasing cortisol reactivity to stressors occurs in parallel with pubertal onset in human females beginning in gonadarche (i.e., the early stages of puberty) 1-2 years earlier than males (Marshall and Tanner, 1969; Marshall and Tanner, 1970). Interestingly, stress-induced increases in cortisol are known to decrease as males progress through puberty (Di Luigi et al., 2006). As such, differences in age of pubertal onset may also contribute to differing developmental trajectories in HPA axis development, ultimately facilitating sex differences in HPA stress responsivity. This is consistent with neuroanatomical maturational processes occurring in adolescents. Cortical refinement is initiated earlier in females, sex differences in brain volume emerge, and existing sex differences in limbic volumes during childhood/early life are further amplified during adolescent development (see review, Ordaz and Luna, 2012).

While it is generally accepted that sex differences in HPA axis activity begin to emerge with pubertal onset, there is a considerable gap in our understanding of the role of gonadal hormones on adolescent development of HPA axis activity. The existing literature suggests that pubertal maturation, which is associated with rising levels of androgens, estradiol and other estrogens, sets in motion maturational processes that contribute to the development of adult HPA

axis stress responsivity. This has been demonstrated in an elegant series of studies demonstrating that when exposed to acute restraint stress, gonadectomized prepubertal males with testosterone replacement still exhibit a protracted CORT response to acute restraint stress exposure (Romeo et al., 2004a). These results indicate that the effects of testosterone on HPA axis activity are functional only after pubertal maturation given that HPA axis activity to acute restraint was not altered in prepubertal males given testosterone replacement (Romeo et al., 2004a). Similarly, prepubertal females treated with estradiol and exposed to acute restraint 5 days later display reduced ACTH and CORT responses to restraint; however adult females with the same estradiol treatment displayed increased CORT levels to the same stressor, again indicating that the effects of estradiol on female HPA axis stress responsivity is functional only after pubertal maturation occurs (Evuarherhe et al., 2009). Collectively, the results of these studies indicate that pubertal maturation is a key developmental process contributing to HPA axis development.

In sum, numerous studies indicate that testosterone exposure shortly after birth is an important factor in developing the male pattern of stress responsivity, and the mechanisms likely involve activation of androgen and estrogen receptors. Furthermore, studies on the organizational impact of gonadal hormones and maturation of the HPA axis across various developmental stages indicate that there are several critical periods for sexually-dichotomous processes. Given the complexity of activational and organizational influences, the numerous brain regions involved, and the ever-changing nature of sex differences across development, a greater understanding of the role of sex steroids and their underlying mechanisms in the development of the HPA axis is warranted.

1.5 The Endocannabinoid System

Cannabis consumption has been documented over centuries of human civilization, commonly used for its therapeutic, mood-enhancing and stress-relieving properties. The main

psychoactive component of cannabis, **Δ**-9-tetrahydrocannabinol (THC), was first discovered and characterized in 1964 (Gaoni and Mechoulam, 1964) and has since given rise to an entire field of research devoted to understanding a myriad of cognitive, behavioural, emotional and physiological processes under the regulation of the endogenous cannabinoid system. The endocannabinoid (eCB; Figure 1.2) system interacts with THC to exert its physiological and behavioural effects and it includes two inhibitory G-protein coupled receptors, the CB_1 and CB_2 receptors. The CB_1 receptor (CB_1R) couples to intracellular $G_{i/o}$ proteins while the CB_2 receptor only couples to G_i proteins (Howlett et al., 2002). CB_1Rs are widely expressed in the brain while CB_2 receptors are predominantly found in peripheral tissues. CB_2 receptors have been detected in the central nervous system relatively recently (Onaivi et al., 2006; Van Sickle et al., 2005), although the majority of these reports localize their presence in microglia rather than neurons (e.g. Cabral et al., 2008).

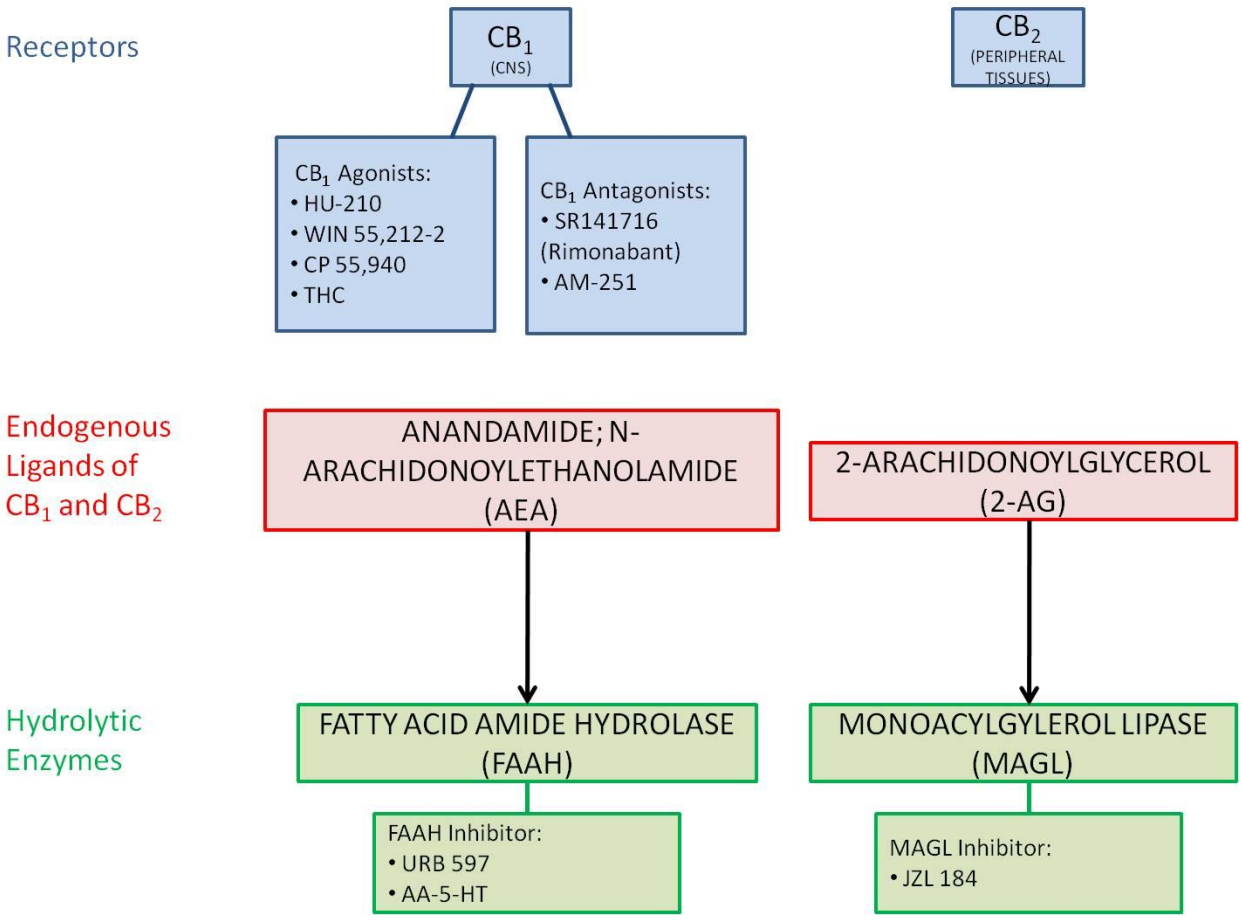

Figure 1.2. Major components of the endocannabinoid system and their respective agonists/antagonists/inhibitors: receptors (blue), endogenous ligands (red), and degradative enzymes (green)

CB_1Rs are found presynaptically on a number of neuronal populations including glutamate, gamma-aminobutyric acid (GABA), monoamines and catecholamines (Haring et al., 2012). Moreover, they are widely expressed throughout the brain, with high densities in the hippocampus and neocortex, moderate densities within the amygdala and a relatively lower distribution in the hypothalamus (Herkenham et al., 1991). The wide distribution of CB_1Rs across different neuronal subtypes and structures of the brain make them ideally situated to exert regulation over a variety of cognitive, behavioural and physiological processes including HPA axis stress responsivity and emotionality.

The eCB system also possesses two major endogenous ligands, N-arachidonoylethanolamine (AEA; anandamide) and 2-arachidonoylglycerol (2-AG) which are synthesized "on demand" and act as retrograde messengers to regulate the release of other neurotransmitters (Freund et al., 2003; see review Jutras-Aswad et al., 2009) and contribute to both short- and long-term synaptic plasticity (Mackie, 2006). Both AEA and 2-AG are synthesized post-synaptically by activity-dependent cleavage of phospholipid head groups. The synthesis of 2-AG is achieved by the conversion of phosphatidylinositol by phospholipase C into diacylglycerol, then converted to 2-AG by diacylglycerol lipase (DAGL; Hillard, 2000; Sugiura et al., 2002). The biosynthesis of AEA is less clear, although it begins with the conversion of phospholipid precursors to N-acyl phosphatidylethanolamine (NAPE) via a calcium-dependent transacylase enzyme; however, there are three known distinct and independent pathways of AEA production from NAPE involving enzymes such as phospholipase A2, C and D (Ahn et al., 2008). AEA and 2-AG are synthesized following post-synaptic membrane depolarization and are released into the synapse, travelling retrogradely to activate pre-synaptic CB_1Rs. Activation of these pre-synaptic CB_1Rs hyperpolarizes the membrane and results in reducing post-synaptic currents thereby reducing subsequent neurotransmitter release (Di Marzo, 2011). Both ligands are subject to rapid intracellular degradation primarily by hydrolytic enzymes, fatty acid amide

hydrolase (FAAH) for AEA and monoacylglycerol lipase (MAGL) for 2-AG (Di Marzo, 2011). Hydrolysis of AEA results in arachidonic acid and ethanolamine whereas hydrolysis of 2-AG yields arachidonic acid and glycerol (Ahn et al., 2008).

It is not entirely clear why there are two ligands for CB_1Rs; however, distinct biosynthetic and degradative pathways as well as pharmacokinetic differences between AEA and 2-AG facilitate differential patterns of signaling that could be contributing to the intricacies of regulation over a variety of complex processes with multiple targets, such as HPA axis stress responsivity and emotional behaviour. AEA is readily bound to the CB_1R, yet induces somewhat poor intracellular signal transduction (i.e. partial agonist properties); in contrast, 2-AG has a relatively low binding affinity to the CB_1R, but produces a robust intracellular response (Hillard et al., 1995). Moreover, FAAH is mostly found post-synaptically, whereas MAGL is mostly colocalized with CB_1Rs pre-synaptically (Haring et al., 2012). It is currently believed that these differences in signaling properties implicates 2-AG as representing a phasic signal (stimulus-induced) in response to sustained depolarization and is involved in several forms of activity-dependent synaptic plasticity, particularly two models of retrograde neurotransmission known as depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation which is discussed below (DSE; Blankman and Cravatt, 2013). In contrast, AEA appears to represent a tonic signal that gates and maintains steady state conditions (Hill and Tasker, 2012). In support of this, blockade of neuronal firing in hippocampal slice preparations is found to facilitate AEA uptake and degradation, thereby reducing AEA tone, and resulting in a reduction of CB_1R-mediated suppression of GABA release (Kim and Alger, 2010). Moreover, *in vivo* studies of FAAH disruption suggest AEA pathways control select behavioural processes such as anxiety behaviour (Bluett et al., 2014); although, simultaneous increases in both AEA and 2-AG via inhibition of MAGL and FAAH are known to produce some synergistic CB_1R-

mediated effects, indicating cross-talk between the two pathways to regulate behaviour (Blankman and Cravatt, 2013).

DSI and DSE are two related forms of short-term synaptic plasticity which act to transiently silence GABAergic and glutamatergic transmission, respectively (Diana and Marty, 2004). DSI/DSE are evoked by elevated intracellular calcium levels, induced by the activation of voltage-gated calcium channels through depolarization of a post-synaptic cell for 1-10 seconds (Diana and Marty, 2004; Pitler and Alger, 1992). The elevation in intracellular calcium levels is believed to trigger a cascade of intermediate steps, which ultimately lead to a transient inhibition of pre-synaptic GABA or glutamate release. Shortly after the discovery of this phenomenon, it was determined that eCBs mediate DSI/DSE, with the post-synaptic rise in calcium leading to the synthesis and release of eCBs that traverse in retrograde fashion and consequently activate pre-synaptic CB_1Rs, then acting to transiently silence neurotransmitter release (Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001). Thus, DSI/DSE can be used to provide real-time information about eCB actions at pre-synaptic CB_1Rs (Regehr et al., 2009).

1.6 Endocannabinoid Regulation of Neurodevelopment

Based on *in vitro* and *in vivo* studies employing pharmacological manipulation of eCB signaling as well as human and rodent descriptive work characterizing eCB system activity from late gestation to adulthood (see Table 1.1), involvement of the eCB system in neurodevelopment appears reliant on developmental stage (Borcel et al., 2004). On a cellular level, previous work has established that eCB signaling plays a multifaceted role in structural and functional neurodevelopment (see review Harkany et al., 2008; Maccarrone et al., 2014), regulating proliferation of neural progenitors and cell lineage commitment (Mulder et al., 2008), immature neuronal migration and axonal path finding (Berghuis et al., 2005; Berghuis et al., 2007; Harkany et al., 2008; Mulder et al., 2008), as well as initiation of synaptic communication of neural networks in perinatal rat tissue (Berghuis et al., 2007; Bernard et al., 2005).

Table 1.1 Neonatal and adolescent corticolimbic endocannabinoid system development in the rat

eCB Marker	Sex/Strain	Age	Brain Region	Developmental Trajectory	Reference
CB_1R binding	Male and Female, Wistar	PND 10-70	Limbic System	↑ from PND 10 to PND 30-40, then ↓ to PND 70	(de Fonseca et al., 1993)
CB_1R binding	Male and Female, Wistar	PND 7-21, 60	Striatum, Hippocampus, Hypothalamus	↑ from PND 7 to PND 60 (no adolescent time points included)	(Belue et al., 1995)
CB_1R mRNA	Sex unknown, Wistar	Gestation day 21, PND 1, 5, ~8 weeks old	BLA, Hippocampus, Ventromedial and PVN	↑ from gestational day 21 to PND 1, then ↓ PND 5 and ~8 weeks (no adolescent time points included)	(Berrendero et al., 1999)
CB_1R mRNA	Male, Sprague-Dawley	PND 25, 40, 70	PFC	↓ from PND 25 to 70	(Heng et al., 2011)
AEA, 2-AG	Sex unknown, Wistar	Gestation day 21, PND 1, 5, ~8 weeks old	Whole Brain	AEA ↑ from gestational day 21 to ~ 8 weeks; 2-AG ↑ from gestational 21 to PND 1, then ↓ PND 5 and ~8 weeks (no adolescent time points included)	(Berrendero et al., 1999)
AEA	Female Sprague-Dawley	PNDs 5, 15, 25, ~30, 250	Hypothalamus	AEA peaks immediately before pubertal onset (vaginal opening) ~ PND 30, then decreases to adult levels	(Wenger et al., 2002)
AEA, 2-AG, CB_1R	Male, Long Evans	PND 29, 38, 50	PFC	AEA ↑ from PND 29 to 50; 2-AG ↓ from PND 29 to 38, then ↑ to PND 50; CB_1R ↓ from PND 29 to 50	(Ellgren et al., 2008)
AEA, 2-AG, CB_1R binding, FAAH/MAGL activity	Female, Long Evans	PND 46, 60, 75	PFC	AEA ↑ from PND 46-60, then ↓ by PND 75; 2-AG is lowest ~PND 60; CB_1R binding ↑ from PND 46-60, then ↓ by PND 75; no differences in FAAH and MAGL activity	(Rubino et al., 2014)

A functional eCB system is present in the rat central nervous system as early as gestational days 11-14 as indicated by the presence of CB_1 receptors (Berrendero et al., 1999; de Fonseca et al., 1993), AEA and 2-AG (Berrendero et al., 1999; Harkany et al., 2008). Furthermore, 2-AG plays a role in ensuring appropriate neonatal suckling and is detected in the neonatal brain (Berrendero et al., 1999) and maternal milk (Fride et al., 2001). CB_1 receptors have been detected in human fetal tissue as early as gestational week 9 (Zurolo et al., 2010). These receptors were shown to be functional in the rat neonatal and human fetal brain given that application of the cannabinoid agonist, WIN55,212-2, stimulated [^{35}S]GTPγS binding (Berrendero et al., 1999; Mato et al., 2003). Moreover, examination of specific neural structures revealed that high CB_1R expression is limited to the amygdala and hippocampus, with low distributions in striatum, thalamus and cerebral cortex during early to mid-gestation in the human fetus (gestational weeks 17-22) and rat neonate (de Fonseca et al., 1993), which is in contrast to reports of higher and wider density of these ubiquitous receptors in the adult rat (Herkenham et al., 1991) and human brain (Jutras-Aswad et al., 2009). This unequal distribution of CB_1 receptor expression throughout the immature brain suggests a region-dependent role in ensuring normative neurodevelopment (Campolongo et al., 2011; Fride et al., 2005; Harkany et al., 2008; Jutras-Aswad et al., 2009; Marco et al., 2009a; Schneider, 2008).

In addition to region-dependent CB_1R expression levels in stress-relevant neurocircuitry, sex-dependent temporal specificity has also been reported. Limbic, striatal and midbrain structures exhibited low concentrations of CB_1R expression at PND 10, gradually peaking in mid-adolescence at PND 30-40, then decreasing to reach adult levels of expression by PND 70 in male and female rats (de Fonseca et al., 1993). Sex differences were most apparent in the midbrain with a higher density of CB_1Rs in females at PND 10, but this effect was inverted after 5 days, at which time males had a consistently higher CB_1R density than females (de Fonseca et al., 1993). In contrast, CB_1R binding from birth to PND 60 in both male and female rat brains

has also been reported to increase gradually to adult levels (Belue et al., 1995). In another study examining eCB ligand content in rats on gestational day 21, PND 1, PND 5 and adult (> 8 weeks; sex not reported), divergent, age-dependent concentration patterns have been described. Whereas AEA appeared to exhibit a steady increase to PND 5, then reach even higher levels in adulthood, 2-AG appeared to peak at PND 1 and then decreased to PND 5 concentrations which were comparable to those found in adults (Berrendero et al., 1999; Fride, 2004). However, maturation might not have been complete in the eight week old animals in this study as other evidence suggests that adulthood is not reached until about 10 weeks of age in rats (McCormick et al., 2010; Schneider, 2008).

The comparative lack of data on the adolescent eCB system represents a fundamental gap in our existing knowledge of eCB system development and its role in neural maturation. What is known, however, is that adolescent CB_1R expression in the rat appears to be highest with the onset of adolescence (PND 25-29), then a general linear decline occurs to adult levels within the PFC (Ellgren et al., 2008; Heng et al., 2011). This is in agreement with the findings of Rodriguez and colleagues (1993) who reported declining CB_1R expression in limbic, striatal and cortical structures beginning in mid-adolescence. More specific examination has revealed differential rates at which these declines occur between limbic/associative and sensorimotor cortical regions; declines in limbic/associative regions occurred gradually throughout adolescence whereas major changes in sensorimotor regions are not exhibited until mid- to late- adolescence (Heng et al., 2011). Functionality of these receptors, as measured by DSE, also followed the same developmental pattern (Heng et al., 2011). Investigation of the maturational trajectory of eCB ligands in the PFC also revealed interesting but divergent results. In this region, AEA exhibits a gradual and progressive increase (i.e., PND 29-50) to adult levels while 2-AG levels were highest in early adolescence (PND 29), decreasing in mid-adolescence (PND 38) before increasing again in late adolescence (PND 50; Ellgren et al., 2008). In other reports, PFC AEA

concentration is high, 2-AG levels are low and no differences in FAAH and MAGL activity are detected during the late adolescent period of female rats (Rubino et al., 2014). Moreover, hypothalamic AEA content is observed to increase immediately preceding vaginal opening (as a physical marker of pubertal onset; Wenger et al., 2002). Despite the paucity of research on adolescent corticolimbic eCB signaling development, these reports indicate that eCB ligand concentrations fluctuate throughout adolescence and the age- and region-dependent observations suggest a regulatory role for eCB signaling in neural maturation, though further characterization within this developmental period is certainly warranted.

1.7 Endocannabinoid System Regulation of the HPA Axis in Adulthood

The eCB system has been shown to regulate the HPA axis in the maintenance of both basal and stress-induced responses (e.g. Finn, 2010; Hill et al., 2010b; Hill et al., 2010c). eCB signaling exerts inhibition on HPA axis activity, contributing to the maintenance of low glucocorticoid levels during basal conditions and functioning to restrict HPA axis activity in situations of acute stress (Gorzalka and Hill, 2009). In support of this, genetic studies have revealed that CB_1R deficient mice have increased basal levels of CRH mRNA in the PVN, and elevated ACTH and CORT secretion (Finn, 2010; Steiner et al., 2008). This is further supported by pharmacological studies demonstrating that administration of the CB_1R antagonist, Rimonabant (SR141716), results in elevated circulating CORT levels in rodents (Patel et al., 2004; Steiner et al., 2008). *In vitro* evidence also indicates that eCB signaling negatively regulates the HPA axis given that glucocorticoid application stimulates synthesis and release of AEA and 2-AG in the PVN. This, in turn, activates CB_1Rs on presynaptic glutamatergic terminals to suppress excitatory inputs to post-synaptic CRH neurons in the PVN, thus contributing to termination of the stress-induced HPA axis response (Di et al., 2003; Malcher-Lopes et al., 2006).

As discussed earlier, it has been hypothesized that based on their separate biosynthetic/catabolic pathways and pharmacodynamic properties, AEA contributes to a "tonic-like" mechanism whereas 2-AG promotes a "burst-like" mechanism in CB_1R activation (Ahn et al., 2008; Gorzalka et al., 2008; Hill et al., 2010b). The differing roles of 2-AG and AEA are evident in the regulation of the HPA axis. On a functional level, acute stress decreases tonic AEA levels by increasing FAAH-mediated hydrolysis in the corticolimbic system (see review Hill and McEwen, 2009; Hill et al., 2010b), supporting the "gatekeeper" hypothesis which posits that the presence of AEA contributes to maintaining basal levels of glucocorticoids in an organism, while removal of this AEA tone facilitates activation of the HPA axis and an increase in the frequency of emotional and anxiety-like behaviours (Hill and McEwen, 2009; Patel et al., 2004). In contrast, stress exposure generally elicits a delayed increase in corticolimbic 2-AG (Hill et al., 2011b; Hill and Tasker, 2012; Wang et al., 2011). For example, this is supported by findings that acute stress exposure causes a glucocorticoid-dependent increase in hippocampal 2-AG content through the genomic actions of GRs to contribute to feedback inhibition of the HPA axis, thus indicating a role in promoting termination of the stress response (Wang et al., 2011).

There is compelling genetic, pharmacological and electrophysiological evidence indicating that eCBs mediate fast feedback inhibition actions within the PVN to modulate the HPA axis (Hill and Tasker, 2012). Glucocorticoid application to PVN slices results in a rapid suppression of glutamate-mediated excitatory synaptic currents in CRH neurons whereas this effect is completely abolished with co-application of a CB_1R antagonist (Di et al., 2003; Malcher-Lopes et al., 2006; Wamsteeker et al., 2010b). Furthermore, glucocorticoids increase AEA and 2-AG content in PVN slices *in vitro* (Malcher-Lopes et al., 2006) as well as in whole tissue punches of the hypothalamus *in vivo* (Hill et al., 2010a). Moreover, 30 min exposure to acute restraint stress increases hypothalamic 2-AG content (Evanson et al., 2010). Lastly, genetic deletion of CB_1Rs result in elevated ACTH and CORT responses to acute stress, indicating that

the loss of these receptors diminishes negative feedback, thus yielding an increase in HPA axis activity in response to acute stress exposure (Aguado et al., 2005; Barna et al., 2004; Haller et al., 2002; Hill et al., 2011b).

Beyond the PVN, previous work from our laboratory has demonstrated a clear role for eCB signaling in the PFC in the regulation of glucocorticoid-mediated feedback inhibition of HPA axis activity. Acute stress exposure elicits a glucocorticoid-dependent delayed increase in PFC 2-AG content through genomic actions as the increase in 2-AG was blocked by administration of a GR antagonist (RU-486; Hill et al., 2011b). Furthermore, local administration of a CB_1R antagonist in the PFC results in a protracted CORT response, although the peak magnitude of the response was not affected (Hill et al., 2011b). PFC CB_1Rs were found situated on GABAergic terminals that impinged on layer V pyramidal neurons and PFC slices exposed to CORT exhibited a reduction in GABA release, which was blocked with CB_1R antagonist treatment (Hill et al., 2011b). Collectively, these results suggest that glucocorticoids increase PFC 2-AG content via genomic mechanisms, which reduces inhibitory GABA tone allowing increased PFC outputs to contribute to termination of the stress response and negative feedback (Hill et al., 2011b).

There are also multiple lines of evidence indicating that amygdalar eCB signaling (particularly in the BLA) is also key in the regulation of basal and stress-induced HPA axis activity, functioning as a "gatekeeper" over the HPA axis (Hill and Tasker, 2012). Acute stress consistently decreases AEA content of the BLA by a rapid increase in FAAH activity (Hill et al., 2009; Patel et al., 2005b). Furthermore, local infusion of a FAAH inhibitor specifically into the BLA attenuates, while infusion of a CB_1R agonist suppresses stress-induced activation of the HPA axis (Hill et al., 2009). Collectively, the emerging picture indicates that BLA AEA tone gates excitatory glutamatergic inputs to principal neurons and when stress exposure disrupts this

tone, this increases principal neuron activity, activation of the HPA axis and subsequent release of glucocorticoid hormones into circulation (Hill and Tasker, 2012).

Amygdalar eCB signaling has also been shown to be a critical regulator of HPA axis stress habituation (Hill et al., 2010b). Repeated exposure to 9 consecutive days of 30 min sessions of restraint stress produces reductions in corticolimbic AEA, contributing to a basal hypersecretion of CORT (Hill et al., 2010b) whereas a region-specific elevation of 2-AG in the amygdala induces habituation of the HPA axis (i.e., reduced circulating CORT levels; Hill et al., 2010b; Patel et al., 2009; Patel et al., 2005b). These findings suggest that repeated exposure to the same stressor results in an enhanced capacity to elevate amygdalar 2-AG content which acts to dampen HPA axis activity (Hill et al., 2010b). Given the previously discussed evidence indicating that the BLA gates HPA axis activity by regulating excitatory inputs to principal neurons in this structure, it is likely that repeated stress exposure induced transient increases of 2-AG content to dampen these excitatory inputs, thus reducing amygdalar principal neuron activity and resulting in HPA axis habituation (Hill et al., 2010b). This theory is corroborated by a study demonstrating that CORT inhibits glutamatergic inputs to the BLA via an eCB-dependent mechanism, but only in rodents with a previous history of stress exposure (Karst et al., 2010). Collectively, the body of research discussed in this section indicates that adult corticolimbic eCB signaling exerts tight regulation over HPA activity and negative feedback through temporal-, region- and eCB ligand-dependent mechanisms.

1.8 Early Life Stress and Cannabinoid Treatment Engage the Endocannabinoid System to Modulate HPA Functioning

The SHRP coincides with relatively low CB_1R density in limbic and midbrain structures (de Fonseca et al., 1993), increasing AEA concentrations that peak at PND 1, and relatively low yet constant 2-AG levels in the whole brain (Berrendero et al., 1999). Due to a lack of empirical

data, the exact mechanisms of eCB regulation of HPA axis responsivity in this SHRP are not clearly defined. However, several studies discussed below (see Table 1.2) have observed that stress-induced increases in the eCB system mediate the effects of early life stress (~PND 10), which then affects future stress-induced HPA axis functioning and relevant neural and behavioural processes.

Table 1.2. Effects of stress and/or cannabinoid treatment on behavioural, neural and HPA axis function during early development of the rat

Age of testing	Species/ Sex	Cannabinoid Treatment	Stressor	Behavioural Effects	Neural and HPA Axis Function Effects	Reference
PND 10	Sprague-Dawley (♂)	Phospolipid-derived arachidnoic acids in maternal diet; AM-251 on PND 10	3 min ether stress on PND 10	—	High-fat n-3 ↓2-AG in hippocampus; High-fat diet ↑ basal CORT; diet-specific insensitivity to ether stress; AM-251 ↑ HPA responsivity	(D'asti et al., 2010)
PND 12; 35; 80	Wister (♂)	THC; gestational day 15 – PND 9; maternal 2.5-5 mg/kg	—	5 mg/kg dose ↑ultrasonic vocalizations; ↓adolescent play and social behaviour; ↑ anxiety in adulthood	—	(Trezza et al., 2008)
PND 13	Albino Wister (♀,♂)	AA-5-HT or OMDM-2; PND 7-12; 5mg/kg	MDEP; 24 hr on PND 9	—	MDEP ↑2-AG, astrocytes in hippocampus; CB treatment reversed effects; MDEP ↑ CORT in ♀,♂; CB treatment reversed effects	(Llorente et al., 2008)
PND 13	Albino Wister (♀,♂)	—	MDEP; 24 hr on PND 9	—	MDEP ↑ MAGL and DAGLα in hippocampus; ♀,♂	(Suárez et al., 2010)
PND 13	Albino Wister (♀,♂)	—	MDEP; 24 hr on PND 9	—	MDEP ↑CB_1 and CB_2 receptor density in hippocampus; ♀,♂	(Suárez et al., 2009)
PND 21-30	Sprague Dawley (♂)	—	1 or 3-10 consecutive days 30 min restraint	—	↓ DSI and CB1R density in parvocellular neurons of PVN	(Wamsteeker et al., 2010b)

(Table 1.2 continued)

Age of testing	Species/ Sex	Cannabinoid Treatment	Stressor	Behavioural Effects	Neural and HPA Axis Function Effects	Reference
PND 30-34	Albino Wister (♀,♂)	WIN 55,212-2; PND 10; 0.1 mg/kg	MDEP; 24 hr on PND 9	CB treatment ↓ locomotion, ↑ anxiety in ♂; MDEP reversed these effects. MDEP and CB treatment ↑ depression-like behaviour in ♀,♂	—	(Llorente et al., 2007)
"Adult" Age not provided	Wister (♀,♂)	CP 55,940; PND 28-42; 0.4 mg/kg	MDEP; 24 hr on PND 9	CB treatment ↓ anxiety in ♂; impaired Prepulse inhibition in ♀; no effect on depression-like behaviour	CORT levels were not affected by MDEP or CB treatment	(Llorente-Berzal et al., 2011)
> PND 70	Wister (♀,♂)	THC; gestational day 5 to PND 24; maternal exposure; oral 5mg/kg	—	↑ exploration in ♀,♂	↓medial basal hypothalamic CRH and CORT in ♂, but ↑ in ♀	(Rubio et al., 1995)
> PND 70	Wister (♂)	HU-210; maternal exposure; 1, 5, or 25 µg/kg	—	—	Dose dependent ↑ basal CORT; high dose ↓HPA responsivity to acute HU-210 (20 µg/kg) challenge; low dose ↑ HPA responsivity to acute HU-210 challenge	(del Arco et al., 2000)
> PND 75	Albino Wister (♀,♂)	CP 55,940; PND 35-45; 0.4 mg/kg	—	↓ anxiety in ♀,♂;	—	(Biscaia et al., 2003)

CB – Cannabinoid; MDEP – Maternal deprivation; PND – Post-natal day

One 24 hr session of maternal deprivation (PND 9) can induce several changes in the hippocampus (Llorente et al., 2008): increased MAGL and increased DAGL–α immunoreactivity, but no change in DAGL-β (DAGL–α and -β are enzymes involved in 2-AG synthesis; Suárez et al., 2010) in male and female rats. Maternal deprivation also increases hippocampal astrocyte number and 2-AG content in males but not females (Llorente et al., 2008) by PND 13. Another study using the same stress paradigm revealed reduced hippocampal CB_1R and increased CB_2 receptor immunoreactivity in both male and female neonates (Suárez et al., 2009). Furthermore, sub-chronic administration of a FAAH inhibitor, AA-5-HT, or eCB reuptake inhibitor, OMDM-2, between PND 7 - 12 reversed maternal separation stress-induced increases in CORT levels, hippocampal 2-AG levels and astrocyte counts in the male and female rats (Llorente et al., 2008). Taken together, an episode of maternal separation stress on PND 9 induces intermediate-term upregulation of HPA axis activity, with corresponding changes in hippocampal eCB signaling, namely a decrease in CB_1R density coupled to an increase in 2-AG content. The combination of reduced CB_1R immunoreactivity in a structure central to regulation of HPA functioning and elevated CORT levels, is suggestive of early glucocorticoid negative regulation on CB_1R signaling.

These early life findings are in agreement with previous work demonstrating direct eCB regulation by glucocorticoids in adult rats. Chronic CORT administration reduced hippocampal CB_1R density, without affecting AEA and 2-AG content (Hill et al., 2008c), while the same treatment did not affect CB_1R density but elevated 2-AG in the amygdala (Hill et al., 2005a). As there is evidence for direct glucocorticoid regulation of CB_1R expression as early as 1-2 weeks of life (Suárez et al., 2009), this may reveal a critical, yet somewhat fragile glucocorticoid-regulated mechanism guiding normative development, particularly in light of findings that while circulating basal free cortisol levels are low during early life, a significant proportion exists bound to cortisol binding globulin (i.e. CBG; Stahl et al., 1979). Thus, if an infant is subjected

to excessive stress, even small circulating glucocorticoid elevations may cause significant downregulation of CB_1R density and thus alter eCB signaling. This glucocorticoid-mediated alteration in eCB signaling, could then modify subsequent neurodevelopmental trajectories, HPA axis functionality, and conceivably lead to added vulnerability to stress-related disease.

Longer term effects of maternal separation stress in adolescence demonstrate a depression- and anxiety-like behavioural phenotype in male and female rats (~ PND 40; Marco et al., 2009a). However, maternal deprivation seems to exert protection against long-term anxiogenic effects of an acute dose of cannabinoid agonist, WIN 55,212-2 (PND 10) in males but not females. The neonatal drug treatment reduced adolescent stress-coping behaviour in both adult male and female rats, with no influence of maternal deprivation (Llorente et al., 2007). In comparison, adolescent treatment (PND 35-37) with the cannabinoid agonist, WIN 55, 212-2, in male and female rats failed to reverse the increase in depression-like behaviour in the forced swim test that was induced by one 24 hr maternal deprivation session (PND 12; Macri and Laviola, 2004). Lastly, relatively modest effects were reported in a "two-hit" model of susceptibility to neuropsychiatric disease: chronic adolescent CP-55,940 treatment (PND 28-42) increased ACTH and CORT levels in adult males regardless of maternal stress condition, while females were unaffected (Llorente-Berzal et al., 2011). Taken together, these results lend support to the idea that neonatal and adolescent eCB system development, which are both periods of enhanced vulnerability, are also distinct and likely possess differential sensitivities to cannabinoid manipulation and stress.

Another model of perinatal cannabinoid exposure via placental transmission in gestation and maternal milk in early life (Hutchings et al., 1989) also indicates that these offspring are at risk for abnormal eCB system development, thus affecting HPA axis functioning and stress-related behaviours. Perinatal THC exposure increases ultrasonic vocalizations following removal from the nest, reduces social interactions and play, and increases anxiety-like behaviour in the

elevated plus maze with no impairment in general locomotor activity (Trezza and Vanderschuren, 2008). Preclinical examination of the long lasting consequences of perinatal cannabinoid exposure also suggests alterations to normal maturation of other neurotransmitter systems. For example, μ-opioid receptor density and sensitivity to the reinforcing effects of opioids are altered, thus amplifying the potential for maladaptive development and contributing to a greater likelihood of developing psychiatric disorders in adulthood (Campolongo et al., 2011; Vela et al., 1998). It is hypothesized that perinatal cannabinoid treatment initiates a short term surge in CORT levels which sets in motion long term HPA axis hypoactivity in compensation (Campolongo et al., 2011). This theory is partially supported by findings that adult males exposed to perinatal THC treatment exhibit reductions in medial basal hypothalamic CRH and CORT levels while adult females exhibit increases in these markers (Rubio et al., 1995). This theory of HPA axis compensation is further supported by data showing that male rats exposed to high perinatal doses of the highly potent CB_1R agonist, HU-210, exhibit elevated adult basal CORT levels, but reduced responsivity to a subsequent HU-210 injection challenge in adulthood and males exposed to a low perinatal dose exhibited smaller elevations in basal CORT levels with an elevated response to acute HU-210 challenge (del Arco et al., 2000).

Whereas concerns over perinatal exposure to cannabinoids are relevant to a relatively small subset of mothers, perinatal eCB system dysregulation by quality and quantity of fat in maternal diet is relevant to all. eCBs are products of phospholipid-derived arachidonic acids, and availability of these molecules is reliant on maternal diet. An elegant set of studies (D'asti et al., 2010) demonstrated that mothers fed a high fat diet, regardless of quality, yielded offspring with elevated basal CORT levels. Basal AEA and 2-AG content were also altered in a region-specific manner in the hypothalamus and hippocampus, two neural structures critical to regulating the HPA axis stress response. Furthermore, offspring of mothers fed a high fat diet rich in fish oils exhibited resistance to the effects of CB_1R antagonism while this pretreatment

increased stress-induced ACTH secretion in control pups. These studies provide evidence that the neonatal eCB system is mature enough that it is capable of being recruited in the negative regulation of stress-induced HPA axis stress responsivity and further, that this developing system is vulnerable to dysregulation by maternal diet.

1.9 HPA Axis Function in Adolescence: A Role for the Endocannabinoid System?

To our knowledge, mechanisms subserving interactions between adolescent development and stress-induced corticolimbic and HPA axis responsivity in relation to the eCB system remain to be determined. In research with humans, mostly descriptive rather than experimental evidence has been employed to learn about eCB system regulation of HPA axis functioning and development. From what is known, adolescent male cannabis users exhibit region-dependent alterations in thickness of the PFC relative to healthy controls (Lopez-Larson et al., 2011). Moreover, cannabis use can often be used as a stress coping strategy and chronic use may potentiate dependence or its abuse, as well as alter the impact of stressful events in the developing adolescent (Hyman and Sinha, 2009), potentially leading to dysregulation of HPA axis functioning, and ultimately leaving an individual at greater risk in developing other stress related disorders.

The rodent literature (see Table 2), while it has fewer gaps than the human literature, nonetheless raises many questions about potential mechanisms underlying age-dependent effects of HPA axis stress responsivity. In an electrophysiological study demonstrating age-dependent recruitment of the eCB system in stress-induced HPA axis activity, prepubertal male rats (PND 21-30) revealed a transient loss of depolarization-induced suppression of inhibition (DSI), in parvocellular neuroendocrine cells of the PVN immediately following 5 consecutive days of 30 min restraint stress (Wamsteeker et al., 2010a; Wamsteeker et al., 2010b). These effects were only observed in the prepubertal, but not adult rats exposed to repeated restraint (Wamsteeker et

al., 2010a). The loss in eCB signaling (as revealed by reduced DSI) was found to be a result of stress-induced CORT activating intracellular GR receptors to cause a downregulation of presynaptic CB_1R activity (Wamsteeker et al., 2010b). Thus, this *in vitro* study provides early evidence of divergent age-dependent downregulation of CB_1Rs in parvocellular neuroendocrince cells of the hypothalamus as a result of repeated stress exposure. Moreover, the fact that repeated restraint fails to produce the neuroendocrine habituation response in adolescent rats (Romeo, 2010a) coupled with downregulation of hypothalamic PVN CB_1Rs, suggests that CB_1R signaling also plays a role in launching this adaptive response and indicates this mechanism is not fully functional in adolescent HPA axis stress responsivity.

In another study, adolescent (PND 35-45) male and female rats were chronically administered the synthetic THC analogue, CP 55,940. CORT levels and behavioural assays of emotionality and anxiety were assessed in adulthood. Chronic adolescent CP 55,940 treatment reduced emotionality and anxiety-like behaviour in the hole board, open field tests and elevated plus maze, but had no long term effect on CORT levels (Biscaia et al., 2003). Overall, females exhibited higher basal levels of CORT relative to males (Biscaia et al., 2003), consistent with other literature (McCormick et al., 2010; Romeo, 2010a). Thus, results of this experiment suggest that chronic adolescent cannabinoid exposure has long-term anxiolytic effects, but no lasting consequences on HPA axis function. This is surprising in light of other evidence to be described below.

1.10 Long-term Consequences of Adolescent Endocannabinoid System Dysregulation on the Developing Brain and Behaviour

Despite several investigations of the short and long term impact of adolescent chronic cannabinoid exposure on the adult brain and behaviour, very few have directly assessed HPA axis stress responsivity. However, previous work from our laboratory has demonstrated that

adolescent treatment (PNDs 35-47) with escalating doses of the CB_1R agonist, HU-210, reduces adult hippocampal neurogenesis and increases adult HPA axis stress responsivity to acute restraint stress in male but not female rats (Lee et al., 2014b). Beyond that, the reported long term neural and behavioural changes produced by adolescent cannabinoid treatment are consistent with the effects of stress exposure, making it conceivable that a paradigm aimed at modeling adolescent cannabis use can induce dysregulation of HPA axis functioning. In the studies described below, results from employing such a paradigm suggest that escalating doses of THC exposure in adolescent rats (PND 35-45) induce several long-term sex-dependent neural and behavioural consequences associated with stress-induced HPA axis dyregulation. On a behavioural level, this drug regimen reduces stress coping behaviour in the forced swim test in female rats, impairs spatial working memory in males, and leads to anhedonia in both males and females as measured by the sucrose preference test (Rubino et al., 2008b). Neural consequences include differential reductions in amygdalar and hippocampal CB_1R expression and $[^{35}S]GTP\gamma S$ binding in males and females (Rubino et al., 2008b), decreases in markers of neuroplasticity such as synaptophysin and PSD 95 in the PFC of females (Rubino et al., 2009a), dendritic atrophy and reduced number of spines in hippocampal neurons in males (Rubino et al., 2009b), and reductions in cell proliferation in the dentate gyrus of the hippocampus in females (Realini et al., 2011). Research from the same laboratory also demonstrated that chronic adolescent administration of the FAAH inhibitor, URB-597, decreased hippocampal CB_1R expression in male rats (Marco et al., 2009b) and reversed the THC-induced reduction in stress-coping and increase in anxiety behaviours in females (Realini et al., 2011). In contrast, adolescent administration of CP 55,940 (PND 28-43) resulted in increased CB_1R activity in the PFC of adult male but not female rats (Mateos et al., 2011).

Recent work has demonstrated that the detrimental impact of adolescent THC treatment is due, at least in part, to disruption of normative adolescent eCB signaling that regulates PFC

maturation (Rubino et al., 2014). Furthermore, multiple studies by the Parolaro laboratory (Rubino et al., 2014; Rubino et al., 2009a; Rubino et al., 2009b; Rubino et al., 2008b; Zamberletti et al., 2014) have demonstrated that chronic pharmacological enhancement of adolescent eCB signaling results in long term modulation of corticolimbic CB_1R density and binding as well as other long term neural and behavioural changes similar to those produced by chronic stress (via glucocorticoid hypersecretion; e.g. McEwen, 2005) and prolonged glucocorticoid exposure (Hill et al., 2008c). If the theory of Campolongo and colleagues (2011) that long term compensatory alterations of HPA axis activity to perinatal cannabinoid exposure also hold true for adolescent cannabinoid exposure, we postulate that the increasingly high doses would correspondingly provoke high glucocorticoid levels in the short term, possibly leading to permanent hypoactivity or dysregulation of HPA axis activity and reduced responsivity to cannabinoid-induced HPA axis challenge. It is also reasonable to postulate that the downregulation of hippocampal CB_1R density and binding represent a long-term compensatory mechanism of the eCB system in response to high and prolonged exposure to THC. However, these hypotheses remain largely unexplored.

Other studies employing cannabinoid agonists during adolescence support the conclusion that the relative expression of emotional and anxiety-like behaviour is sex-dependent (O'Shea et al., 2004; Romero et al., 2002; Rubino and Parolaro, 2008; Schneider, 2008; Schneider et al., 2005; Schneider and Koch, 2003). These reports also provide evidence that adolescence is a period of particular susceptibility to the effects of chronic cannabinoid exposure as adult rats with the same treatment did not exhibit these behavioural phenotypes. However, there are some contrasting reports that chronic adolescent exposure to CP 55, 940 either decreased or had no effect on anxiety-like behaviour as measured in the open field test, elevated plus maze (Biscaia et al., 2003) and social interaction test (O'Shea et al., 2006). As others have suggested, this is at

least partly due to differences in the type of cannabinoid agonist used, drug doses and administration schedule (Rubino and Parolaro, 2008; Schneider, 2008).

1.11 Summary and Conclusions

Review of the evidence supports the idea that early life and adolescence are periods of heightened susceptibility to disturbances such as stress as well as cannabis use and abuse; yet at the same time, they are very distinct and separate developmental windows. This is likely due, at least in part, to age-dependent differences in corticolimbic eCB signaling as well as HPA axis development and functionality. Adolescent neurodevelopment consists of maturational processes that are both time-dependent and region-specific. This provides the adolescent brain with remarkable plasticity yet may also confer vulnerability to developing mental illness as a result of biological, environmental, and genetic disruptions that exacerbate transient imbalances occurring between these region- and time-dependent maturational processes (Lee et al., 2014a).

Given the eCB system's ability to exert regulation over HPA axis activity and neurodevelopment, it is reasonable to propose that this system plays an important role in adolescent vulnerability to mental illness. In this chapter, evidence has been presented that demonstrates eCB regulation on neurodevelopment, particularly with respect to corticolimbic circuitry, as well as how this system governs HPA axis stress responsivity. Moreover, evidence has been presented indicating that early life and adolescent stress exposure can alter eCB signaling both in the immediate and long-term and that developmental cannabinoid exposure generally results in behavioural and neural consequences similar to that of stress exposure. However, there is a significant gap in the adolescent literature investigating the role of normative eCB signaling in the corticolimbic circuit and regulation of stress responsivity in the immature HPA axis. Uncovering the relationship between eCB mediation of HPA functioning and

development are of special importance given growing health concerns of vulnerability to cannabis use in adolescents.

1.12 Objectives and Hypotheses

The current collective body of work attempts to address some of these gaps in knowledge with the overarching hypothesis that normative adolescent corticolimbic eCB signaling is an important mediator of age-dependent differences in HPA activity and the development of normative adult HPA axis stress responsivity and emotional behaviour. Furthermore, the specific objectives of the proposed research are as follows:

1. **The primary objective of Chapter 2 is to determine normative corticolimbic eCB system activity and content across the adolescent period in the male rat.** Given evidence suggesting that AEA represents a tonic signal and 2-AG signifies a phasic signal in the regulation of adult HPA axis activity, we hypothesize that adolescent corticolimbic AEA content would maintain relatively stable levels whereas 2-AG content would be more likely to fluctuate and possibly increase into adulthood.
2. **The primary objective of Chapter 3 is to determine how adult and adolescent corticolimbic eCB signaling, HPA activity, and pubertal maturation differs with acute and repeated restraint exposure in male rats**. Our hypothesis is that any age-dependent differences in HPA activity will correspond with differences in eCB signaling. In particular, previous research indicates that amygdalar 2-AG mobilization is essential for the expression of HPA axis habituation in adult male rats. Given that prepubertal rats do not exhibit this habituation response to repeated restraint stress, it is predicted that the adolescent male rats in the current study will not exhibit elevated amygdalar 2-AG levels with repeated restraint exposure. **The secondary objective of this chapter is to determine the short- and long-term**

effects of adolescent stress experience on CB_1R expression in male rats. We hypothesize that adolescent stress experience will yield greater short- and long-term effects on corticolimbic CB_1R expression. **A third objective of this chapter is to determine whether dorsal and ventral hippocampal GR and MR protein levels are also modulated by age and stress condition (i.e., acute versus repeated restraint).** We hypothesize that MR and GR protein levels will be modulated by age and stress condition in the ventral hippocampus, but not the dorsal hippocampus.

3. **The primary objective of Chapter 4 is to investigate the role of sustained adolescent CB_1R blockade on the subsequent development of adult male HPA axis stress responsivity and emotional behaviour.** Based on evidence suggesting that pharmacological and genetic deletion of CB_1Rs produces neural and behavioural effects indicative of chronic stress exposure, we hypothesize that adolescent CB_1R antagonism will yield detrimental consequences to adult corticolimbic eCB signaling, emotional behaviour and HPA axis stress reactivity.

2. Temporal Changes in *N*-acylethanolamine Content and Metabolism Throughout the Adolescent Period

2.1 Introduction

N-Acylethanolamines (NAE) are a class of fatty acid-derived signalling lipids that are generated by the transfer of a sn-1 fatty acid from a donor phospholipid to phosphatidylethanolamine (PE) to form *N*-acyl PE (NAPE) by a currently undefined calcium-activated *N*-acyltransferase (Ahn et al., 2008; Ueda et al., 2010). NAPE is then converted to the active NAE through several potential mechanisms, including NAPE-specific phospholipase D (NAPE-PLD), α/β-hydrolase-4 or phospholipase C/tyrosine phosphatase pathways (Ahn et al., 2008; Ueda et al., 2010). With respect to the central nervous system, however, it is unknown which of these mechanisms (or an alternative) is the primary pathway mediating NAE biosynthesis. In fact, the possibility exists that different NAE molecules may utilize distinct biosynthetic pathways (Ueda et al., 2010). While there are many species of NAEs, the most studied of the NAE molecules is the endocannabinoid (eCB), *N*-arachidonylethanolamine (also known as anandamide; AEA), which is an endogenous partial agonist of the cannabinoid type 1 receptor (CB_1R;(De Petrocellis and Di Marzo, 2009). AEA also possesses non-CB_1R signaling actions to L-type Ca^{2+} channels, transient changes in intracellular Ca^{2+}, and disruption to gap function. Lastly, the most well studied of AEA non-eCB actions is the activation of vanilloid receptors (see review, Howlett and Mukhopadhyay, 2000), which has been reported to enhance fear and anxiety-related responses (Moreira et al., 2012). In addition to AEA, two other prominent NAEs are oleoylethanolamine (OEA) and palmitoylethanolamine (PEA). In contrast to AEA, OEA and PEA do not act as agonists at the cannabinoid receptors (Hansen, 2010) and it appears that their synthesis is significantly driven by NAPE-PLD (Leung et al., 2006). OEA and PEA have been found to activate peroxisome proliferator-activated receptor-α (PPARα; Fu et al., 2003; Lo Verme et al., 2005) and OEA is also an agonist at the orphan G-protein coupled

receptor 119 (GPR119; Overton et al., 2006). Notwithstanding differences in their biosynthetic pathways and functional targets, all three molecules are hydrolyzed by fatty acid amide hydrolase (FAAH), resulting in the formation of ethanolamine and the parent fatty acid (i.e., AEA is metabolized into ethanolamine and arachidonic acid; Ueda et al., 2010).

A second, more ubiquitous endogenous ligand of the eCB system is known as 2-arachidonoylglycerol (2-AG), which acts as a full agonist at the CB_1R (Ueda et al., 2011). There are two proposed pathways of 2-AG synthesis; however, the hydrolysis of inositol phospholipids containing arachidonic acid at the sn-2 position by phospholipase C into diacylglycerol, which is then further hydrolyzed to 2-AG by diacylglycerol lipase-α (DAGLα) is likely the most important (Hillard, 2000; Sugiura et al., 2002; Ueda et al., 2011). The pathways leading to breakdown of 2-AG are less clear, with at least 8 participating enzymes, although 2-AG hydrolysis by monoacylglycerol lipase (MAGL) to arachidonic acid and glycerol is considered to be the major pathway of 2-AG breakdown (Ueda et al., 2011).

While the determination of the functional targets of NAE and 2-AG signaling are progressing, relatively little is known about the ontogeny of this system. With respect to synthesis, it has been demonstrated that the enzymatic activity of the N-acyl transferase responsible for NAPE synthesis dramatically declines from birth to the onset of adolescence and also remains low in adulthood (Moesgaard et al., 2000). Similarly, FAAH mRNA expression and activity are found to be very high at birth and decline into adulthood (Thomas et al., 1997). These data suggest that the capacity for both synthesis and metabolism of NAEs is high in early life, but declines with age. NAPE-PLD and FAAH expression were found to steadily increase from early life to adulthood in the human dorsolateral PFC (Long et al., 2012b). Interestingly, DAGLα mRNA expression was observed to peak from school age to young adulthood and MAGL expression declines from infancy to adulthood in the dorsolateral PFC (Long et al., 2012a). Whether NAE and 2-AG synthesis, contents or hydrolysis change during the rodent

adolescent period remains unknown. This question may be of particular relevance for the regulation of eCB signaling, as CB_1R activation is known to be important for many neurodevelopmental processes (Harkany et al., 2008), some of which continue in adolescence (e.g. Gogtay et al., 2004; Gogtay et al., 2006). Furthermore, profound changes in behavioural repertoire and physiological status occur during the adolescent period; it is during this stage that mammals achieve sexual maturation, leave their primary caregivers and establish a sense of independence (McCormick et al., 2010; Pattwell et al., 2011; Romeo, 2010a; Spear, 2000). This process of development involves a fine balance of several behavioural processes, including stress perception, reward sensitivity and higher order cognition, all of which are processes that are influenced by NAE signaling, particularly AEA (Hill and Tasker, 2012; Solinas et al., 2008; Zanettini et al., 2011).

The aim of the current experiment was to characterize developmental patterns of 2-AG, AEA, PEA and OEA, and their metabolism by FAAH, in forebrain structures primarily involved in emotion, cognition and decision-making, structures that are also known to undergo functional and architectural changes during the adolescent period. To achieve this, we measured both 2-AG, NAE content and FAAH activity at time points representing pre-adolescence (post natal day [PND] 25), early to mid-adolescence (PND 35), late adolescence (PND 45) and adulthood (PND 70). Our data indicate time-specific changes in NAE content and metabolism throughout these forebrain structures, the functional significance of which remains to be determined.

2.2 Materials & Methods

2.2.1 Subjects

Male Sprague Dawley rats (Charles River, QC, Canada) were received at the University of British Columbia on post-natal (PND) 21. Rats were pair housed by age in clear polyurethane cages (48 x 27 x 20 cm) filled with cedar bedding and paper towels for enrichment, and

maintained on a 12h/12h light/dark cycle (lights on at 9 am). Food (Purina rat chow) and water were provided *ad libitum.* All protocols were carried out in accordance with the Canadian Council for Animal Care guidelines and were approved by the Animal Care Committee at the University of British Columbia. Four ages were compared: PND 25 (pre- adolescence; n=8), PND 35 (early/mid-adolescence; n=8), PND 45 (late adolescence; n=8), and PND 70 (adulthood, n=8).

2.2.2 Endocannabinoid extraction and analysis

All animals were sacrificed by rapid decapitation during the first third of the light cycle and brain tissue was collected for eCB content analysis. PFC, hippocampus, amygdala, and hypothalamus were dissected within 5 min, as previously described (Hill et al., 2010a), frozen on dry ice, and stored at -80° C until analysis.

Brain regions underwent a lipid extraction process as previously described (Patel et al., 2003). The tissue samples were weighed then placed in borosilicate glass culture tubes containing 2 mL of acetonitrile with 84 pmol of [$^2H_8$] anandamide and 5 nmol of [$^2H_8$] 2-AG. These samples were homogenized with a glass rod and sonicated for 30 min, at which point they were incubated overnight at -20°C to precipitate proteins. The samples were centrifuged at 1500 g to remove particulates. Supernatants were removed to a new glass culture tube and evaporated to dryness under N_2 gas, then re-suspended in 300 µL of methanol to recapture any lipids adhering to the tube and re-dried again under N_2 gas. The final lipid extracts were suspended in 20 µL of methanol and stored at -80°C until analysis. 2-AG and NAE contents (AEA, PEA and OEA) within lipid extracts were determined using isotope-dilution, liquid chromatography-mass spectrometry as described earlier (Patel et al., 2005a).

2.2.3 FAAH activity assay

Frozen brain tissue samples (i.e. amygdala, hippocampus, hypothalamus, PFC) were homogenized in 10 volumes of TME buffer (50 mM Tris HCl, pH = 7.4; 1 mM EDTA, and 3 mM $MgCl_2$). Homogenates were then centrifuged at 18, 000 x g for 20 min and the resulting crude membrane fraction-containing pellet was re-suspended in 10 volumes of TME buffer. Protein concentrations were determined using the Bradford method (Bio-Rad, Hercules, CA, USA).

FAAH activity was measured by conversion of AEA labelled with [^{3}H] in the ethanolamine portion of the molecule ([3H]AEA; Omeir et al., 1995) to [^{3}H] ethanolamine preparations as reported earlier (Hillard et al., 1995). Membranes were incubated in a final volume of 0.5 mL TME buffer (50 mM Tris-HCl, 3.0 mM $MgCl_2$, and 1.0 mM EDTA, pH 7.4) that contained 1.0 mg/ml fatty acid-free bovine serum albumin and 0.2 nM [^{3}H]AEA. Isotherms were constructed using eight concentrations of AEA at concentrations between 10 nM and 10 mM. Incubation was carried out at 37 °C and the enzymatic reaction was stopped by the addition of 2 ml of chloroform/methanol (1:2). After remaining at room temperature for 30 min with frequent mixing, 0.67 ml of chloroform and 0.6 ml of water were added and aqueous and organic phases were separated by centrifugation at 1000 rpm for 10 min. The amount of [^{3}H] in 1 ml each of the aqueous and organic phases was determined by liquid scintillation counting and conversion of [^{3}H]AEA to [^{3}H]ethanolamine was calculated. The K_m and V_{max} values for this conversion were determined by fitting the data to a single site Michaelis-Menten equation using Prism.

2.2.4 Statistics

2-AG, AEA, OEA, and PEA contents and FAAH activities were analyzed using one-way analysis of variance (ANOVA), as a function of age for each brain region. Bonferroni post-hoc

tests were used to determine whether there were any significant differences between age groups in each brain structure.

2.3 Results

One way ANOVA revealed significant effects of age on AEA content in the amygdala ($F_{(3, 15)} = 15.54$, $p=0.0003$; Figure 2.1), hypothalamus ($F_{(3,15)} = 10.01$, $p=0.001$; Figure 2.1), PFC ($F_{(3,15)} = 11.43$, $p=0.0008$; Figure 2.1) and hippocampus ($F_{(3,15)} = 5.63$, $p=0.01$; Figure 2.1). In the amygdala, post-hoc analyses revealed that AEA content was significantly lower on PND 25 relative to PND 35 ($p < 0.05$) and 70 ($p < 0.005$). PND 70 AEA content was also significantly higher than on PND 45 ($p < 0.005$). Post-hoc comparisons of AEA content in the hypothalamus also demonstrated significantly higher AEA content on PND 70 relative to PND 25 ($p < 0.05$) and 45 ($p < 0.005$), whereas AEA content on PND 35 was significantly higher than on PND 45 ($p < 0.05$). Examination of AEA content in the PFC also revealed that on PND 70, content was significantly higher than on PND 25 ($p < 0.005$) and 45 ($p < 0.005$). Furthermore, AEA content on PND 35 was significantly higher than on PND 25 ($p <0.005$) and PND 45 ($p < 0.05$). Post-hoc analyses revealed that AEA content in the hippocampus was significantly greater on PND 70 than on PND 25 ($p < 0.05$) and 45 ($p < 0.05$).

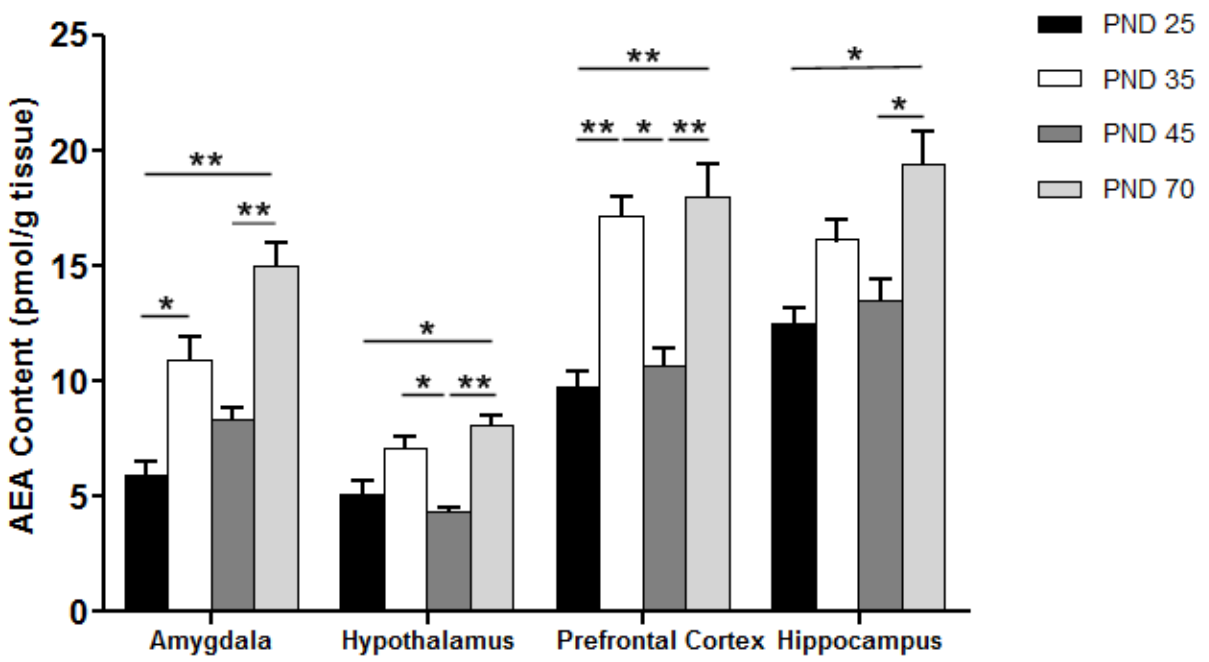

Figure 2.1. Mean (+ SEM) anandamide (AEA) concentrations throughout structures of the corticolimbic circuit in adolescent and adult male rats. AEA generally increased from PND 25 to 35, then decreased by PND 45. By adulthood, AEA concentration was significantly higher than PNDs 25 and 45 in the amygdala, hypothalamus, prefrontal cortex and hippocampus. * denotes $p < 0.05$; ** denotes $p < 0.005$; *** denotes $p < 0.0005$.

One way ANOVA revealed an age effect on OEA content in the amygdala ($F_{(3,15)}$ = 20.67, p=0.0001; Figure 2.2), hypothalamus ($F_{(3,15)}$ = 5.08, p=0.02; Figure 2.2), PFC ($F_{(3,15)}$ = 13.41, p=0.0004; Figure 2.2) and hippocampus ($F_{(3,15)}$ = 12.13, p=0.0006; Figure 2.2). Adult (i.e., PND 70) OEA content in the amygdala was significantly higher relative to content on PND 25 ($p < 0.0005$), 35 ($p < 0.05$) and 45 ($p < 0.0005$). Furthermore, PND 35 OEA content was significantly higher than on PND 25 ($p < 0.05$). In the hypothalamus, OEA content at PND 35 was significantly greater than on PND 25 ($p<0.05$). Post-hoc analyses of OEA content in the PFC revealed that relative to animals on PND 70, animals that were 25 and 45 days old had significantly lower OEA content ($p < 0.005$ for both comparisons). OEA content on PND 35 was also significantly greater than on PND 25 ($p < 0.005$) and 45 ($p < 0.005$) in the PFC. OEA content in the hippocampus on PND 70 was significantly higher than on PNDs 25 ($p < 0.005$) and 45 ($p < 0.005$). OEA content within the hippocampus on PND 25 was significantly lower relative to content on PND 35 ($p < 0.05$).

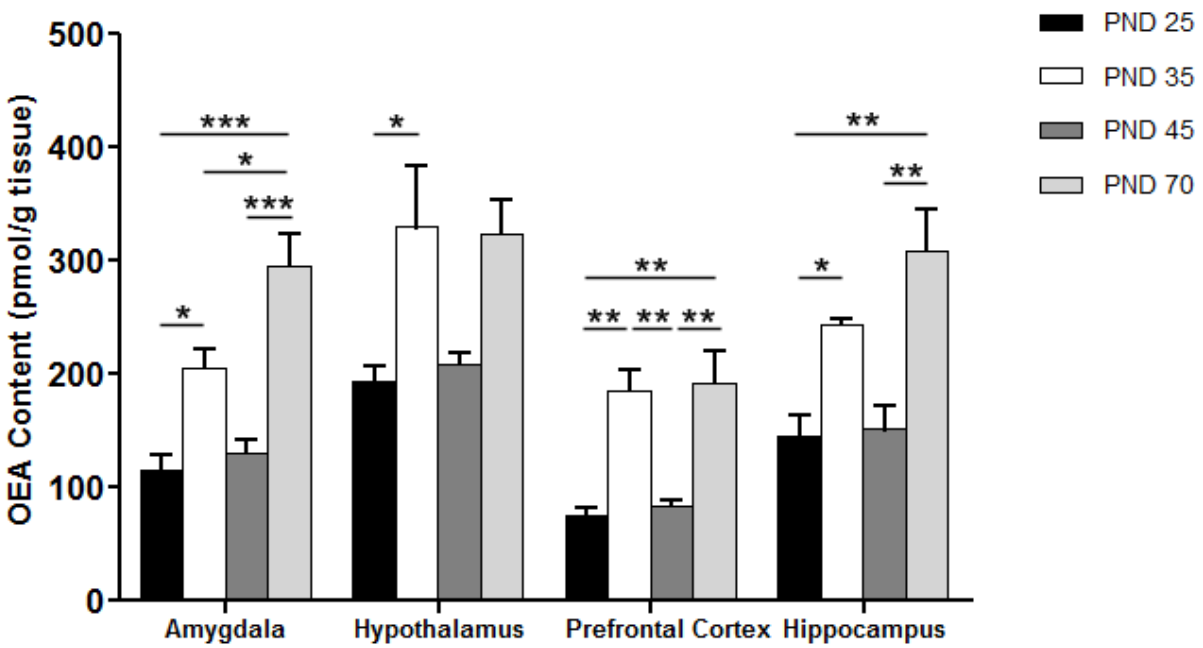

Figure 2.2. Mean (+ SEM) oleoylethanolamine (OEA) concentrations throughout structures of the corticolimbic circuit in adolescent and adult male rats. OEA generally increased from PND 25 to 35, then decreased by PND 45. By adulthood, OEA concentration was significantly higher than PNDs 25 and 45 in the amygdala, prefrontal cortex and hippocampus. * denotes $p < 0.05$; ** denotes $p < 0.005$; *** denotes $p < 0.0005$.

One way ANOVA revealed a significant effect of age on PEA content in the amygdala ($F_{(3,15)} = 6.26$, $p=0.009$; Figure 2.3), PFC ($F_{(3,15)} = 6.92$, $p=0.006$; Figure 2.3) and hippocampus ($F_{(3,15)} = 9.26$, $p=0.002$; Figure 2.3); however, no significant age dependent effects were observed in PEA content of the hypothalamus ($F_{(3,15)} = 1.852$, $p=0.19$; Figure 2.3). Amygdalar PEA content at PND 70 was significantly higher than on PND 25 ($p < 0.05$) and 45 ($p < 0.05$). Furthermore, PEA content in the PFC was significantly greater on PND 70 relative to PND 25 ($p < 0.05$) and 45 ($p < 0.05$), and PEA content on PND 35 was higher relative to PND 25 ($p < 0.05$). In the hippocampus, PEA content was higher on PND 70, relative to content on PND 25 ($p < 0.005$) and PND 45 ($p < 0.005$).

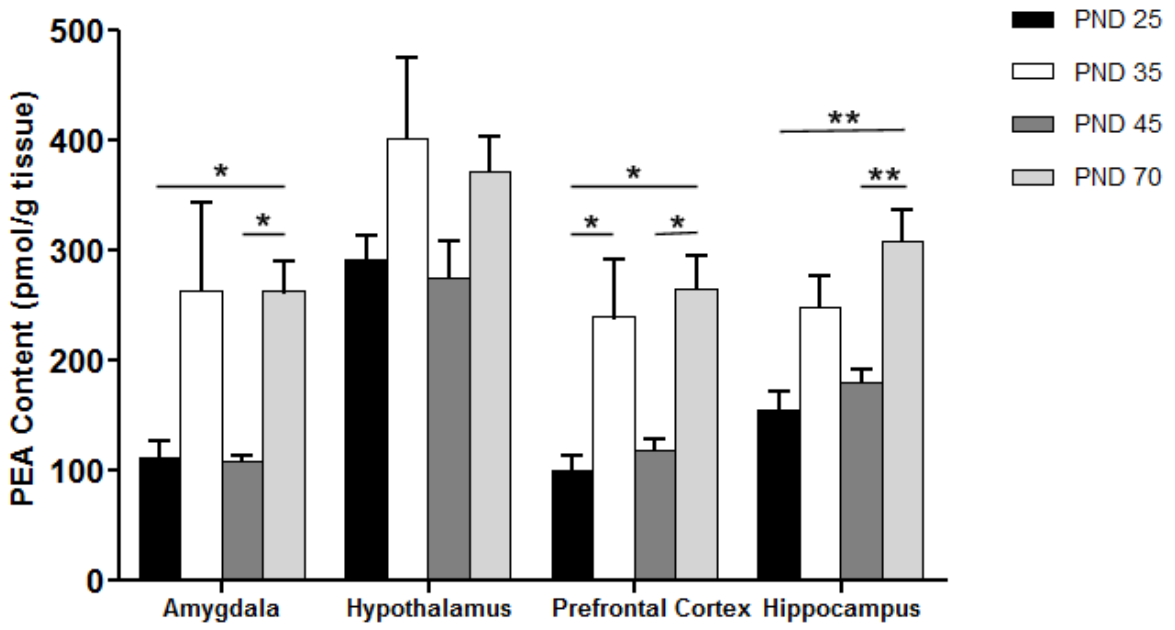

Figure 2.3. Mean (+ SEM) palmitoylethanolamide (PEA) concentrations throughout structures of the corticolimbic circuit in adolescent and adult male rats. PEA generally increased from PND 25 to 35, then decreased by PND 45. By adulthood, PEA concentration was significantly higher than PNDs 25 and 45 in the amygdala, prefrontal cortex and hippocampus. * denotes $p < 0.05$; ** denotes $p < 0.005$; *** denotes $p < 0.0005$.

One way ANOVAs of maximal hydrolytic activity (Vmax) of FAAH revealed a significant age-dependent effect that was the inverse of that seen in NAE content in the amygdala ($F_{(3,15)} = 6.50$, $p=0.007$; Figure 2.4), hypothalamus ($F_{(3,15)} = 4.48$, $p=0.03$; Figure 2.4), PFC ($F_{(3,14)} = 5.00$, $p=0.02$; Figure 2.4) and hippocampus ($F_{(3,14)} = 4.26$, $p=0.03$; Figure 2.4). Post-hoc comparisons of FAAH activity in the amygdala revealed that FAAH activity on PND 25 was significantly higher than on PND 35 ($p < 0.05$) and 70 ($p < 0.05$). In the hypothalamus, FAAH activity was greater on PND 45 than on PND 70 ($p < 0.05$). Post-hoc analyses of FAAH activity in the PFC revealed significantly higher levels of FAAH activity on PND 45 relative to activity on PND 70 ($p < 0.05$). In the hippocampus, FAAH activity was significantly greater on PND 25 than on PND 70 ($p < 0.05$).

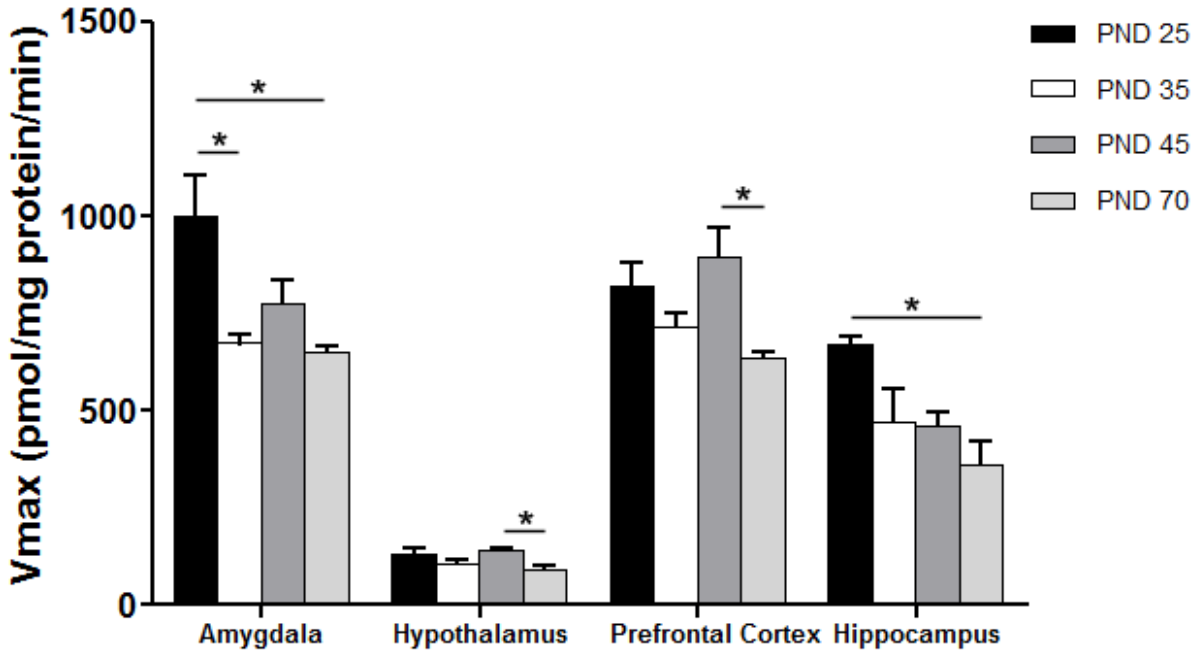

Figure 2.4. Mean (± SEM) fatty acid amide hydrolase (FAAH) activity (Vmax values) throughout structures of the corticolimbic circuit in adolescent and adult male rats. FAAH activity generally decreased from PND 25 to 35, then increased by PND 45. By adulthood, FAAH activity was generally reduced relative to activity during adolescence. * denotes $p < 0.05$.

A one-way ANOVA indicated that there were no age effects on the Km values of FAAH activity (see Table 2.I) in the amygdala ($F_{(3,15)} = 0.72$, $p = 0.56$), hypothalamus ($F_{(3,15)} = 0.36$, $p = 0.79$) and hippocampus ($F_{(3,14)} = 0.41$, $p = 0.75$). However, there was a significant effect of age on Km values in the PFC ($F_{(3,14)} = 4.25$, $p = 0.03$) such that Km values were significantly lower on PND 35 relative to PND 25 ($p < 0.05$; see Table 2.I).

Table 2.1. Binding affinity (Km) of anandamide for fatty acid amide hydrolase (FAAH) throughout the corticolimbic stress circuit during adolescence.

	PND 25	PND 35	PND 45	PND 70
Amygdala	0.54	0.45	0.46	0.45
Hypothalamus	0.41	0.48	0.54	0.39
Prefrontal Cortex	0.47	0.29*	0.44	0.40
Hippocampus	670.20	470.25	463.03	362.60

All values represented are in nM and * indicate significantly different from PND 25, $p < 0.05$.

Lastly, one way ANOVA revealed no age effects on 2-AG content in the amygdala ($F_{(3,11)} = 1.87$, $p=0.19$; Figure 2.5), hypothalamus ($F_{(3,12)} = 0.37$, $p=0.77$; Figure 2.5), PFC ($F_{(3,12)} = 0.48$, $p=0.70$; Figure 2.5) and hippocampus ($F_{(3,12)} = 2.71$, $p=0.10$); Figure 2.5).

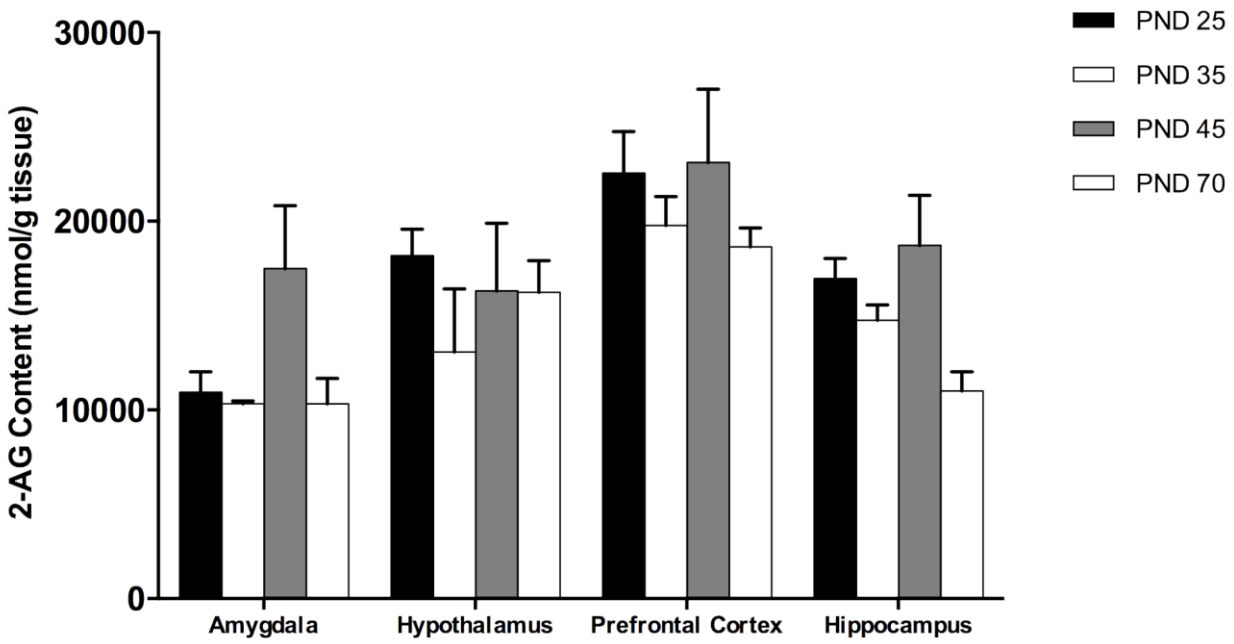

Figure 2.5. Mean (± SEM) 2-arachidonoylglycerol (2-AG) content throughout structures of the corticolimbic circuit in adolescent and adult male rats. There were no significant effects of age on 2-AG content in all four corticolimbic structures.

2.4 Discussion

The results of the current study reveal significant age-dependent changes in NAE content (i.e., AEA, OEA and PEA) and FAAH activity, but there were no significant differences in 2-AG throughout the adolescent period into adulthood. Generally, the contents of all of the NAEs increased from PND 25 to 35, and then decreased between PND 35 and 45. By PND 70, these ligands had increased to adult concentrations. These temporal changes in tissue NAE content were accompanied by opposite changes in FAAH activity. FAAH was generally high on PND 25, declined by PND 35, and then increased again by PND 45. By PND 70, FAAH activity was significantly reduced relative to the earlier time points, especially relative to PNDs 25 and 45. Collectively, these data would indicate that there is a peak in NAE content in the brain during the early phases of adolescence, which is likely mediated by a transient reduction in FAAH-mediated metabolism. Furthermore, considering the similarity amongst the NAE content throughout adolescence, it seems likely that these age-dependent changes in NAEs are influenced by changes in FAAH activity. However, we have not explored age-related changes in rates of NAE biosynthesis.

While there is much debate over a specific age range for the adolescent period, both in the human and animal model literature, it is agreed that pubertal onset and development occur during this phase (Morishita et al., 2005; Spear, 2000). This raises the possibility that the observed changes in AEA, PEA and OEA contents may also be related to shifts in gonadal hormone concentrations that facilitate pubertal maturation. In support of this, Wenger and colleagues (2002) reported peak AEA contents immediately prior to pubertal onset (PND 30-31) in the hypothalamus of female rats which declined with vaginal opening. Furthermore, prepubertal administration of an exogenous CB_1R agonist was shown to delay pubertal onset relative to that of vehicle-injected female rats (Wenger et al., 1988). Evidence that estradiol

reduces the expression of FAAH and elevates AEA content in the brain and periphery (De Petrocellis and Di Marzo, 2009; Hill et al., 2007; Maccarrone et al., 2000; Scorticati et al., 2003), suggests the possibility that an increase in estradiol during the onset of puberty can modulate FAAH activity and AEA content in females. Our current understanding of male pubertal onset and testosterone in relation to eCBs and related NAEs is far less developed. A frequently accepted estimate of the adolescent period in males is PND 28-55, with pubertal onset at about PND 42 (Spear, 2000). We observed peak content of AEA throughout the corticolimbic circuit at PND 35. Since measurements of AEA, PEA and OEA were not performed between PND 35 and 45, the timing of the changes in NAEs in relationship to the onset of puberty is not known. Similarly, the relationship of these changes to circulating levels of testosterone are unknown; although previous studies have shown that male rodents have little to no testosterone on PND 25 but exhibit circulating testosterone on PND 35 (Romeo et al., 2006; Romeo et al., 2004a). Future studies should include concise temporal measures during this period of pubertal onset to determine whether changes in FAAH activity or AEA, PEA and OEA content correlate with the emergence of circulating gonadal androgens and maturation of the testes.

The pattern of FAAH activity observed throughout adolescent development is in agreement with that previously reported in rat whole brain samples where FAAH activity increased from birth to PND 21, decreased moderately by PND 42 then increased again by PND 56 (Morishita et al., 2005). In that same study, NAPE-PLD exhibited a sharp increase in specific activity from birth to PND 21 and a slower increase to PND 56, which was also consistent with NAPE-PLD mRNA expression in the rat brain during development (Morishita et al., 2005). The modest change in NAPE-PLD activity and expression during adolescence suggests AEA biosynthesis is not completely linked, or only provides a partial contribution to the clear pattern of age-dependent NAE changes reported here. It should be noted that there are at least 3 other AEA biosynthetic pathways (Di Marzo, 2011) that do not include NAPE-PLD which may

exhibit other age-dependent changes that contribute to the observed peak NAE levels at PND 35; however, methods of assessing these mechanisms do not yet exist. Furthermore, Thomas and colleagues (1997) reported a progressive increase in FAAH mRNA contents between embryonic day 14 to PND 10, which remained relatively high until PND 30 and subsequently decreased by adulthood (although no measures were taken between PND 30 and 60). This study also demonstrated that the enzymatic activity of FAAH was strongly correlated with the time-course of mRNA in the same regions (Thomas et al., 1997). Taken together with the current data, there are age-dependent changes in FAAH activity, which are likely mediated by transcriptional regulation of FAAH during development.

In this study, OEA and PEA increased from PND 25, peaking at PND 35 and then decreased to adult levels (PND 70). The temporal changes in OEA and PEA during adolescence parallel previous reports of age-dependent effects on AEA content throughout the different structures of the brain (e.g. Berrendero et al., 1999; Ellgren et al., 2008; Wenger et al., 2002). In particular, a spike in AEA content has been observed during early adolescence within both the hypothalamus of females (first day of proestrus [PND 30-31]; Wenger et al., 2002) and the nucleus accumbens of males (PND 38; Ellgren et al., 2008). Temporal changes in CB_1R density are also seen during early development and into adolescence (see Lee and Gorzalka, 2012). However, while AEA content exhibits a general trend of increasing from early life into adulthood, CB_1R density declines between adolescence and adulthood (see Lee and Gorzalka, 2012). As such, early adolescence provides a unique temporal window in which, despite opposite trajectories, there is a combined peak of AEA/CB_1R activity.

Given that AEA/CB_1R signalling in corticolimbic circuits is known to regulate reward sensitivity (Mahler et al., 2007) and stress responsivity (Hill and Tasker, 2012), these changes may be key to some of the neurobehavioural changes which occur during the adolescent period. For example, adolescence is a period of sensation seeking, impulsivity and reduced contextual

fear (Casey et al., 2011; Pattwell et al., 2011; Spear and Brake, 1983), in which greater exploration and risk taking occurs, presumably in an effort to establish independence (Spear, 2000). Inhibition of eCB signalling has been reported to improve inhibitory control and reduce impulsivity (Pattij et al., 2007; Wiskerke et al., 2011), suggesting that tonic eCB signalling may facilitate impulsive behaviours. Similarly, inhibition of FAAH has been shown to reduce anxiety in an array of animal models (Hill et al., 2013; Kathuria et al., 2003; Kinsey et al., 2011; Rossi et al., 2010), although one caveat of these findings is that the anxiolytic effects of FAAH inhibition are dependent on factors such as dose, route of administration, timing of exposure and context (see review Zanettini et al., 2011). Of interest, human carriers of the C385A polymorphism of FAAH (which compromises cellular FAAH expression and increases circulating AEA levels; Chiang et al., 2004; Sipe et al., 2010) exhibit blunted activation of the amygdala in response to threat and an increase in ventral striatal activation in response to reward (Hariri et al., 2009). Collectively, these data suggest that lower FAAH activity and increased AEA/CB_1R signaling subserve a shift in corticolimbic circuits promoting a state of low anxiety, high responsivity to rewards and reduced inhibitory control. Given that the emergence to adolescence represents a period when this behavioural profile is maximal, which is coincident with a peak in AEA/CB_1R activity and reduced FAAH activity, it is tempting to speculate that these temporal changes in FAAH activity and AEA content represent a neural substrate of the adolescent phenotype. Further research is required to delineate the contributions these changes in FAAH activity and AEA content play with respect to this phenotype.

In contrast, 2-AG content did not exhibit any temporal-specific patterns across all four corticolimbic structures. While it is not clear why there are two endogenous ligands of the eCB system, the available literature suggests that AEA contributes to a “tonic-like” mechanism whereas 2-AG promotes a phasic mechanism in CB_1R activation (Ahn et al., 2008; Gorzalka et al., 2008; Hill et al., 2010b). This is particularly relevant to eCB regulation of adult HPA axis

stress responsivity, with tonic AEA levels constraining HPA axis activity and 2-AG mobilization necessary for termination of the stress response (Hill and Tasker, 2012). Given these functional differences, it is possible that although adolescent corticolimbic AEA content fluctuates, maintaining the same temporal-specific pattern throughout these structures is somehow maturationally significant to the adolescent phenotype and/or establishing a stable adult HPA axis tone, whereas maintaining a tone for 2-AG may be less relevant. These divergent findings for NAEs and 2-AG across the adolescent period certainly deserve further research delineating the functional relevance of these differences.

It is only relatively recently that researchers have demonstrated adolescence as a distinct maturational period. The fact that the functionality of corticolimbic circuits during early adolescence is unique compared to that during younger or older age intervals further highlights the idea that this maturational stage has its own unique demands including behavioural and neural adaptations to facilitate establishing independence. The data presented herein, demonstrate that the hydrolytic activity of FAAH and the tissue content of its NAE substrates: AEA, PEA and OEA, exhibit discrete, temporal-specific changes throughout the adolescent period while corticolimbic 2-AG content does not. Future research will endeavour to determine the functional role of these changes in the onset of puberty, development of the adolescent phenotype and maturation into adulthood.

3. Age-dependent Profiles of Corticolimbic Endocannabinoid Signaling and HPA Axis Stress Responsivity and the Long-term Effects of Adolescent Repeated Stress Exposure

3.1 Introduction

Stress is considered a state of strain elicited by a real or perceived threat to homeostatic functioning (McEwen, 2007). Exposure to stress results in a cascade of physiological changes, including secretion of adrenal hormones, changes in immune function and alterations in motivation and emotion, which act to facilitate survival of an organism. Acutely, these responses are time limited and an array of negative feedback processes act to terminate these responses once the stressful stimulus has been removed (Pecoraro et al., 2006). Sustained exposure to stress, however, is known to exert adverse effects on neural structure and function, in part, through excessive secretion of glucocorticoid hormones (McEwen, 2008). Specifically, preclinical studies have demonstrated that in adult rodents, exposure to chronic stress can increase behavioural indices of anxiety, cause neuronal remodeling in limbic circuits (decreased dendritic branching in the hippocampus and prefrontal cortex (PFC) and increased dendritic branching in the amygdala) and up- or downregulate several classes of neurochemical receptor systems (Hill et al., 2012; McEwen et al., 2012; McLaughlin et al., 2009). Interestingly, if adult animals are given a recovery period following the cessation of stress, most, if not all, of these changes return to basal levels (Bloss et al., 2011; Hoffman et al., 2011,; Lin et al., 2009 but see, Vyas et al., 2004). Consistent with this, human studies have found that chronic stress can modulate corticolimbic structure and function (Ansell et al., 2012; Gianaros et al., 2007; Liston et al., 2009; Soares et al., 2012), and similar to the rodent studies, most of these effects are found to normalize following a recovery period (Liston et al., 2009; Soares et al., 2012). These data indicate that the adult brain exhibits a high degree of plasticity in response to the effects of chronic stress.

Converging animal and human studies have reliably demonstrated that unlike the transient effects of stress in adulthood, stress exposure during discrete developmental windows can have very profound and sustained effects on corticolimbic circuits (Koenig et al., 2011). For instance, early life stress experienced in the neonatal and juvenile phase of life can result in a reprogramming of stress systems in the body, resulting in glucocorticoid resistance, heightened inflammation and increased susceptibility to anxiety and depression in adulthood (Danese et al., 2008; Ladd et al., 2000; Miller et al., 2009). Given that adolescence is a period of dramatic neuronal reorganization (characterized by synaptic pruning, dendritic remodeling and the formation of neural circuits subserving mood and reward (Brenhouse and Andersen, 2011; Spear, 2000), there is increasing evidence that stress during this period can have sustained effects on the adult brain (Andersen and Teicher, 2008). Specifically, it has been found that exposure to various forms of stress during the adolescent period can result in a suppression of hippocampal neurogenesis in female, but not in male rats (Barha et al., 2011), a reduction in synaptic density within the PFC of male rats (Leussis et al., 2008) and reduced dopaminergic function in the PFC of male rats in adulthood (Watt et al., 2009; Wright et al., 2008). Concomitant with these sustained effects on corticolimbic function, stress during the adolescent period has also been shown to cause long-lasting changes in emotional behaviour, with increased levels of anxiety and depression reported in adulthood in both preclinical and clinical studies (Andersen and Teicher, 2008; McCormick et al., 2008; Wright et al., 2008). As such, the plasticity that the adult brain exhibits in response to chronic stress exposure appears to be more established, while chronic stress exposure during various developmental periods can leave a residual biological effect on neural systems subserving mood and anxiety.

An important and adaptive form of stress responsivity occurs in situations of repeated exposure to a homotypic (same) stressor, which involves a gradual dampening of hypothalamic-pituitary-adrenal (HPA) axis output, presumably in an effort to limit subsequent adrenocortical

secretion (Calogero et al., 1991). Many real-life stressors are recurrent or persistent in nature, making this form of HPA axis plasticity particularly relevant to avoiding excessive glucocorticoid exposure. While the exact mechanisms subserving this habituation response have yet to be determined, previous work has demonstrated it is achieved by a systems-level adaptation in which there is a decrease in neuronal activation in sensory-limbic structures and glucocorticoid synthesis and release (Girotti et al., 2006; Jaferi and Bhatnagar, 2006). In this way, the HPA axis avoids needless secretion of glucocorticoids, yet maintains the ability to mount a neuroendocrine response when necessary. Otherwise, excessive and prolonged secretion of glucocorticoids in the system can lead to harmful psychological, neural, cardiovascular, metabolic and immune consequences (Chrousos, 2009).

Despite comparable basal stress hormone levels between pubertal and adult humans, rats, mice and hamsters, a divergence in stress hormone levels occurs in response to acute and repeated homotypic stress exposure (Romeo, 2010b). Preclinical studies report that following acute restraint exposure, prepubertal male and female rodents exhibit a similar peak in HPA axis activity as adults; however, a protracted adrenocorticotropic hormone (ACTH) and corticosterone (CORT) stress response occurs in prepubertal animals relative to adults (Romeo et al., 2004a; Romeo et al., 2004b). With repeated restraint exposure, prepubertal male rats fail to exhibit blunted CORT and ACTH levels characteristic of adult HPA axis habituation but recovery to basal levels of stress hormone is accelerated (Romeo et al., 2006; Romeo et al., 2004a; Romeo et al., 2004b). Subsequent studies have demonstrated that it is not until the late adolescent period (post-natal day (PND) 40 and 50 in the male rodent) that the HPA axis begins to approximate the adult stress response to acute restraint exposure (Foilb et al., 2011); however it is currently unknown which specific maturational processes occur to ensure HPA axis habituation. Taken together, prepubertal animals appear to have greater stress-induced

glucocorticoid secretion than adults, which leads to the possibility that repeated exposure to the same stressor has greater detrimental long-term impacts on adolescents than adults.

One potential candidate underlying age-dependent differences in HPA axis stress responsivity and the long term impact of stress exposure is the endocannabinoid (eCB) system which buffers the effects of stress exposure by constraining activation of the HPA axis (see review, Hill and Patel, 2013; Hill et al., 2010c). The eCB system's two major endogenous ligands, N-arachidonoylethanolamine (anandamide; AEA) and 2-arachidonylglycerol (2-AG), signal via activation of the cannabinoid receptor type 1 (CB_1R) to inhibit neurotransmitter release. Fatty acid amide hydrolase (FAAH) primarily breaks down AEA while monoacylglyercol lipase (MAGL) primarily degrades 2-AG. Work from our laboratory and others has revealed divergent actions of the two eCB ligands, particularly with respect to amygdalar eCB regulation of the HPA axis; AEA behaves by "tonic-like" means to constrain HPA axis activity and anxiety in non-stressful environments, whereas 2-AG functions as more of a "phasic" mechanism on CB_1R activation to restrict the magnitude of the HPA axis stress response and aid in its recovery to basal levels (Gorzalka et al., 2008; Hill and Patel, 2013). Following exposure to chronic stress, however, CB_1R expression exhibits widespread down-regulation and desensitization throughout limbic structures such as the hippocampus (Hill et al., 2005b; Hu et al., 2011; Reich et al., 2009), hypothalamus (Hill et al., 2008b; Wamsteeker et al., 2010b), amygdala (Patel et al., 2009) and nucleus accumbens (Wang et al., 2010) in male rodents. This loss of CB_1R function is thought to contribute to many of the adverse effects of chronic stress, such as sensitization of the HPA axis, alterations in cognitive-emotional function and changes in dendritic morphology (Gorzalka et al., 2008).

Other evidence suggesting that the eCB system may underlie age-dependent differences in HPA axis stress responsivity and the long term effects of repeated stress exposure derives from findings that eCBs regulate neural development (see review, Maccarrone et al., 2014) as

well as mediates interactions between HPA axis stress responsivity and maturational stage (see review, Lee and Gorzalka, 2012). During the adolescent period, the male and female eCB system undergoes temporal-specific dynamic changes; CB_1R expression peaks with the onset of the adolescent period then decreases to adult levels (de Fonseca et al., 1993; Heng et al., 2011) while adolescent corticolimbic AEA and FAAH levels fluctuate before maintaining adult levels (Ellgren et al., 2008; Lee et al., 2013; Rubino et al., 2014; Wenger et al., 2002). The available evidence suggests that disruption to normative adolescent eCB signaling may provoke alterations to normative neurodevelopmental processes which alter the developmental trajectory of the adult brain (Rubino et al., 2014) and modulate adult stress-coping and anxiety behaviour, cognition, and HPA axis stress responsivity (Lee et al., 2014b; Lee et al., under review; Rubino et al., 2008b). Taking into account evidence of eCB signaling involvement in development and the neuroendocrine regulation of the adult stress response, it is reasonable to suggest that differences in eCB signaling may also account for the divergent adolescent and adult HPA axis response to repeated restraint stress.

Furthermore, glucocorticoids, such as CORT and cortisol, exert genomic and non-genomic feedback regulation on the stress response. This feedback is mediated by glucorticoid receptors (GR) and mineralocorticoid receptors (MR) expressed in limbic forebrain regions (Ahima and Harlan, 1990; Ahima et al., 1991; Ulrich-Lai and Herman, 2009). In particular, MR and GR are densely expressed within the hippocampus and are likely co-localized in some neurons (Ulrich-Lai and Herman, 2009). MR is known to have a high binding affinity for corticosteroids and is bound even during the circadian nadir of CORT secretion. In contrast, GR has a lower binding affinity for glucocorticoids and therefore, only binds abundantly under conditions of relatively high corticosteroid circulation, such as during stress exposure (Reul and de Kloet, 1985; Ulrich-Lai and Herman, 2009). MR and GR are ligand-gated transcription factors that modulate gene transcription and are believed to have complementary functions, with

MR contributing to setting a threshold for HPA axis activation (genomic) as well as contributing to cognitive aspects in the acute stress response such as appraisal of stressful stimuli (non-genomic; Joëls et al., 2008; ter Heegde et al., in press). Moreover, pharmacological studies demonstrate that MR participates in regulating the HPA axis habituation response to repeated homotypic stressors (Cole et al., 2000). Activation of GRs by CORT contributes to regulation of basal HPA axis tone as well as termination of the stress response (Ulrich-Lai and Herman, 2009).

Furthermore, because of these differences in binding affinity, the hippocampus is responsive to both basal and stress-induced elevations in CORT (de Kloet et al., 1998). Interestingly, there is strong genetic, connectivity and behavioural evidence supporting a functional separation of the dorsal and ventral hippocampus (see review, Fanselow and Dong, 2010). These data indicate that the dorsal hippocampus primarily contributes to cognitive processes such as memory function. In contrast, the ventral hippocampus supports processes related to emotion and HPA axis stress responsivity. Hippocampal lesions impair control of the HPA axis stress response (Dedovic et al., 2009). Similarly, elevations in CORT levels result in hippocampal dysfunction such as reduced dendritic morphology (McEwen et al., 1997) and is associated with affective disorders such as depression and post-traumatic stress disorder in humans (Fanselow and Dong, 2010). Furthermore, prepubertal exposure to chronic variable stress has been reported to alter adult hippocampal volume, hippocampal-dependent learning, and decrease GR mRNA in the dentate gyrus and CA1 (Isgor et al., 2004). One theory suggests that age-dependent differences in HPA axis stress responsivity are a result of immaturity of glucocorticoid-dependent negative feedback processes. It is possible that changes in stress-induced eCB system activity may also mediate differences in hippocampal GR and/or MR expression and thus, modulate HPA axis stress responsivity.

In Chapter 2, we showed that basal corticolimbic AEA content and FAAH activity age-dependently fluctuated across the adolescent period, while 2-AG did not change appreciably. Following up on this in Chapter 3, we now assess stress-induced changes in corticolimbic eCB content and activity as a function of age in Experiment 1. GR and MR protein levels are also assessed in the dorsal and ventral hippocampus to determine whether age dependent differences in HPA axis stress responsivity are associated with immature negative feedback processes involving these receptors. Furthermore, since there is little known about the ability of the eCB system to recover following exposure to repeated stress, Experiment 2 sought to determine the immediate and sustained effects of repeated restraint stress during early to mid-adolescence (the period in which corticolimbic AEA content was shown to decline in Chapter 2) on corticolimbic CB_1R binding as a function of age at stress exposure.

3.2 Materials & Methods

3.2.1 Experiment 1

3.2.1.1 Subjects

Juvenile male Sprague Dawley rats (Charles River, Canada, QC) were received on post-natal (PND) 21. Animals were pair housed in standard size clear polyurethane cages (48 x 27 x 20 cm) filled with cedar bedding and paper towels for enrichment. A 12h/12h light/dark cycle (lights on at 9 am) was maintained and access to food (Purina rat chow) and water was provided *ad libitum.* All protocols in Experiment 1 were carried out in accordance with the Canadian Council for Animal Care guidelines and were approved by the Animal Care Committee at the University of British Columbia.

3.2.1.2 Stress paradigm, pubertal maturation, blood and tissue collection

Adolescent and adult rats were randomly assigned to one of three conditions such that a total of 6 experimental groups were used in this study: 1.) Adolescent/Naive (no stress), 2.) Adolescent/Acute stress (30 min restraint stress session on PND 45, 3.) Adolescent/Repeated stress (daily 30 min restraint stress from PND 35-45), 4.) Adult/Naive (no stress), 5.) Adult/Acute stress (30 min restraint stress session on PND 85), and 6.) Adult/Repeated stress (daily 30 min restraint stress from PND 75-85). Following stress sessions, all prepubertal adolescent rats were inspected daily for the prepuce to completely retract from the head of the penis (balano-preputial separation; Lewis et al., 2002) as an external sign of change in peri-pubertal androgen secretion and index of male pubertal development (Korenbrot et al., 1977). All of the adolescent rats were prepubertal prior to restraint stress exposure and all had reached balano-preputial separation by PND 43, which is consistent with previous reports (Korenbrot et al., 1977; Lewis et al., 2002).

All restraint stress sessions were conducted in a separate testing room, and occurred in the first third of the light cycle, during the daily nadir of HPA axis activity. During these 30 min sessions, rats were put into a polystyrene tube (diameter 6 cm, length 20 cm) with breathing holes for up to 10 consecutive days. Animals were sacrificed by rapid decapitation during the first third of the light cycle to allow for collection of trunk blood for plasma ACTH, CORT and testosterone level measurements and brain tissue for eCB content and activity analysis. PFC, hippocampus, amygdala, and hypothalamus were dissected within 5 min, as previously described (Hill et al., 2010a; Lee et al., 2013), frozen on dry ice, and stored at -80° C until analysis.

3.2.1.3 Radioimmunoassay of ACTH, CORT and testosterone

Total corticosterone (CORT; 5 uL), ACTH (50 uL) and testosterone (50 uL) concentrations were measured using commercial ImmuChem RIA kits (MP Biomedicals

Canada), using [^{125}I] as a tracer. For CORT, plasma samples were diluted 1:100 and 1:200 for basal and stress conditions, respectively, to render hormone detection within the linear part of the standard curve (3.125-1000 ng/mL). Plasma ACTH levels were determined according to manufacturer instructions, from a standard curve derived from 8 standards ranging from 0 – 1000 pg/mL. Testosterone concentrations were also determined according to manufacturer instructions, from a standard curve derived from 8 standards ranging from 0-100 pg/mL. The intra- and interassay coefficient of variations were under 12% for all assays.

3.2.1.4 Endocannabinoid extraction and analysis

Brain regions underwent a lipid extraction process as previously described (Patel et al., 2003). Tissue samples were weighed and placed in borosilicate glass culture tubes containing 2 mL of acetonitrile with 5 pmol of [$^{2}H_{8}$] AEA and 5 nmol of [$^{2}H_{8}$] 2-AG for extraction. These samples were homogenized with a glass rod, sonicated for 30 min, incubated overnight at -20°C to precipitate proteins, then centrifuged at 1500 g for 5 min to remove particulates. Supernatants were removed to a new glass culture tube and evaporated to dryness under N_2 gas, re-suspended in 300 µL of methanol to recapture any lipids adhering to the tube and re-dried again under N_2 gas. The final lipid extracts were suspended in 20 µL of methanol and stored at -80°C until analysis. AEA and 2-AG contents within lipid extracts were determined using isotope-dilution, liquid chromatography-mass spectrometry.

Liquid chromatography mass spectrometry (LC-MS/MS) analyses were carried out on an Eksigent ekspert™ micro LC 200 coupled with an AB Sciex Qtrap™ 5500 mass spectrometer, installed with a Turbo V™ Spray ion source (AB Sciex, Ontario, Canada). The LC was equipped with a temperature-controlled CTC autosampler. An Eksigent HALO C18 HPLC column (1 x 50 mm, 2.7 µm particle diameter, 90Å pore size) was used. Samples were analyzed isocratically, at a flow rate of 30 µL/minute and a solvent composition of 15% mobile phase A (10 mM

ammonium acetate in water), and 85% mobile phase B (acetonitrile). After 3.25 minutes, the column was regenerated with 100% B. Before each injection, the column was re-equilibrated at the initial mobile phase condition for 2 minutes. Following each LC-MS/MS run, a blank was run. The sample injection loop (5 µL loop size) was rinsed with 40 µL of methanol, and the column purged for 5 min with 100% B for 4.5 min and followed by 85% B for 0.5min. This was intended to mitigate cross contaminations due to carryover from preceding sample injections. The LC column was maintained at 25°C, and the samples at 10°C. The mass spectrometer was operated in positive ion mode, with the ion-spray voltage set at 5500V, curtain gas at 20 (arbitrary units), source gas 1 and gas 2 both at 40, and source temperature at 300°C. Protonated molecular ions of AEA (m/z 348) and AEA-d8 (m/z 356), and ammonium adduct ions of 2-AG (m/z 396) and 2-AG-d8, (m/z 404) were selected as the respective precursor ions for CID. MRM scan modes were used with Q1 and Q3 both at unit resolution. Optimized collision energies for the transitions were as follows: AEA (348 to 62) CE 22V, AEA-d8 (356 to 62) CE 22V, 2-AG (396 to 287) CE 15V; and 2-AG-d8 (404 to 294) CE 17V.

Each brain tissue extract sample was further diluted in ACN to yield a 100-fold diluted sample, for the LC-MS/MS quantification of 2-AG, while the undiluted extract sample was analyzed directly for quantification of AEA. It has been reported that 2-AG undergoes spontaneous isomerization converting to 1-AG by acyl group migration during tissue extraction and reconstitution procedures. We also observed that 1-AG was present in authentic standard solutions of 2-AG, as well as brain tissue extracts. Peak areas of 1-AG and 2-AG were combined to establish a standard calibration curve for 2-AG. The data processing was accomplished using Analyst® 1.5.2 software (AB Sciex). Linear regressions of relative peak areas (analyte to IS ratios) were performed over analyte concentrations from 0.00025 to 0.25 pmol/µL (AEA), and 0.0025 to 2.5 pmol/µL (2-AG). Analyte levels were normalized to tissue weight.

3.2.1.5 Membrane preparation

Membrane preparation was conducted as previously described (Lee and Hill, 2013). Briefly, membranes were collected from isolated brain regions by homogenization of frozen tissue in 20 volumes of TME buffer (50 mM Tris HCl, pH 7.4; 1 mM EDTA and 3 mM $MgCl_2$) and centrifuged at 18,000 *x g* for 20 min. The resulting pellet was re-suspended in 20 volumes of TME buffer. Protein concentrations were determined using a commercially available BCA kit (Pierce Biotechnology, Rockville, IL). Hydrolysis rates of AEA (Omeir et al., 1995) and 2-oleoylglycerol (2-OG; Dinh et al., 2002) labelled with [^{3}H] were determined for FAAH as previously reported (Hillard et al., 1995) and MAGL using a slightly modified procedure previously described (Nithipatikom et al., 2004), respectively.

3.2.1.6 FAAH and MAGL assays

Membranes were incubated in a final volume of 0.5 mL TME buffer (50 mM Tris-HCl, 3.0 mM $MgCl_2$, and 1.0 mM EDTA, pH 7.4) that contained 1.0 mg/ml fatty acid-free bovine serum albumin and 0.2 nM [^{3}H]AEA or 0.2 nM [^{3}H]2-OG. For FAAH activity, isotherms were constructed using eight concentrations of AEA at concentrations between 10 - 10 mM. For MAGL activity, isotherms were constructed using six 2-OG concentrations between 10-500 uM. Incubation was carried out at 37 °C (FAAH) or 30 °C (MAGL) and the enzymatic reaction was stopped by the addition of chloroform/methanol (1:2). After remaining at room temperature with frequent mixing, chloroform and water were added. Aqueous and organic phases were separated by centrifugation at 1000 rpm for 10 min. The amount of [^{3}H] in 1 ml of the aqueous and organic phases was determined by liquid scintillation counting and conversion of [^{3}H]AEA to [^{3}H]ethanolamine by FAAH or conversion of [^{3}H]2-OG to [^{3}H]glycerol by MAGL were calculated. The K_m and V_{max} values for these conversions were determined by fitting the data to a single site Michaelis-Menten equation using Prism.

3.2.1.7 MR and GR protein levels

Animals were exposed to stress and brain tissue harvested as described in section 3.2.2.1. Dorsal and ventral hippocampus were dissected and stored at -80° C until analysis. Whole cell proteins were extracted by dounce homogenization using a RIPA based lysis buffer with proteinase inhibitor (Roche). Homogenized samples were then spun for 30 min at 16,000 g at 4° C and the supernatant was immediately collected. Protein concentrations were determined using the BCA protein assay kit and adjusted to ~1-2 ug/ul. Samples were denatured with Laemmli sample buffer (Biorad) with **β**-mercaptoethanol and frozen at -20 °C.

20 µg of protein was loaded into TGX 4-15% gradient gels (Biorad), then wet transferred and blocked with Odyssey blocking buffer. Membranes were then probed for GR (Santa Cruz GR-H300, 1:000, Santa Cruz MCR, 1:1000, and LI-COR anti-rabbit IRDye secondary, 1:10,000) and loading controls (Sigma **β**-actin, 1:50,000, and LI-COR anti-mouse IRDye secondary, 1:10,000). Blots were then scanned and analyzed (by optical density) using an Odyssey V3.0.

3.2.1.8 Statistics

Separate 2-way ANOVAs were used to analyze AEA content and 2-AG content, FAAH and MAGL activity in the amygdala as well as serum CORT, ACTH and testosterone levels, with age and stress condition as the between-subjects factors. A one-way ANOVA was used to determine whether stress condition significantly altered age of pubertal onset. Bonferroni corrections were used for all post-hoc comparisons. All statistical procedures were set at $\alpha = 0.05$. All data presented are listed as mean values $\pm$ standard error.

3.2.2 Experiment 2

3.2.2.1 Subjects

Male Sprague-Dawley rats (Charles River, QC, Canada) were used in this study. For the adolescent cohort, rats were at post-natal day (PND) 35 at the onset of the study, and for the

adult cohort, rats were at PND 75 at the onset of the study. All rats were pair housed with age-matched cage mates 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 1000 hr. All rats were given ad libitum access to Purina Rat Chow and tap water. All protocols in Experiment 2 were approved by the Canadian Council for Animal Care and the Animal Care Committee of the University of Calgary. All manipulations occurred during the first third of the light cycle, during the daily nadir of HPA axis activity.

3.2.2.2 Stress paradigm

Animals were randomly assigned to unstressed conditions or exposed to a 30 min episode of restraint, in which rats were put into a transparent acrylic Broome rodent restrainer (178 mm long, 51 mm high; Harvard Apparatus, St. Laurent QC Canada) each day, for 10 consecutive days. Unstressed animals were handled three times a week during cage changing, but left undisturbed for the duration of the experiment. For studies examining the immediate effects of repeated stress on CB_1R binding, animals were euthanized via rapid decapitation 24 hr following the final stressor. For studies examining recovery from stress, animals were left undisturbed for a period of 40 days following the final stressor and then euthanized via rapid decapitation. Thus, for the effects of immediate stress, the adolescent animals were at PND 45 when they were euthanized, while the adults were at PND 85. For the recovery portion of the study, the animals that had been stressed in adolescence were at PND 85 when they were euthanized, while the subjects that had been stressed as adults were at PND 125 when they were euthanized. Two separate cohorts of animals were used for the immediate and recovery arms of the study to account for differences in age and exposure to colony environment.

3.2.2.3 Blood collection and CORT analysis

To assess the immediate effects of repeated stress on circulating levels of CORT, blood samples were obtained via tail nick at the termination of 30 min restraint on the first and last days of restraint stress. To assess if the exposure to repeated restraint had sustained effects on basal CORT following a 40-day recovery period, blood samples were obtained via collection of trunk blood following decapitation 40 days following the cessation of stress. All samples were centrifuged at 3000 x *g* for 15 min, after which plasma was removed and stored at -80 ºC. CORT levels in plasma were measured in duplicate using commercial ELISA kits (Cayman Chemical, Ann Arbor, MI USA). Samples were diluted 1:1000 for stress conditions and 1:100 for basal conditions to render hormone detection in the linear part of the standard curve.

3.2.2.4 Tissue extraction and membrane preparation

Following rapid decapitation, the PFC, amygdala and hippocampus were dissected as previously described and flash frozen on dry ice (Hill et al., 2010a). Membranes were collected from isolated brain regions by homogenization of frozen tissue in 20 volumes of TME buffer (50 mM Tris HCl, pH 7.4; 1 mM EDTA and 3 mM $MgCl_2$). Homogenates were centrifuged at 18,000 *x g* for 20 min and the resulting pellet, which contains a crude membrane fraction, was resuspended in 20 volumes of TME buffer. Protein concentrations were determined by the BCA method using a commercially available kit (Pierce Biotechnology. Rockville IL, USA).

3.2.2.5 CB_1 receptor radioligand binding assay

CB_1R agonist binding parameters were determined using radioligand binding using a Multiscreen Filtration System with Durapore 1.2-µM filters in 96 well filter plates (Millipore, Bedford, MA). 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 in triplicate to wells containing 0.1, 0.25, 0.5, 1.0, 1.5 or 2.5 nM [^{3}H]CP

55,940 (American Radiochemicals, St. Louis, MO USA), a cannabinoid CB_1R agonist, and incubated for 1 hr at room temperature on an orbital shaker. Ten µM AM251 (Tocris Biosciences, Minneapolis MN, USA) was used to determine non-specific binding. B_{max} (maximal binding site density) and K_D (binding affinity) values were determined by nonlinear curve fitting of specific binding data to the single site binding equation using GraphPad Prism (San Diego, CA, USA).

3.2.2.6 Statistics

CORT data comparing the effects of the first and last days of restraint were analyzed using a between and within subjects ANOVA with day of stress as the within factor and age as the between factor. Basal CORT and CB_1R binding data were analyzed using a two way ANOVA with age and stress exposure as fixed factors. Post hoc analysis was performed using a corrected Bonferroni's test to examine differences between experimental conditions. Significance for all tests was established at $p < 0.05$. All data presented are listed as mean values $\pm$ standard error of the mean.

3.3 Results

3.3.1 Experiment 1

3.3.1.1 Corticolimbic 2-AG content increases with repeated restraint exposure in adult, but not adolescent rats

There was a significant interaction effect on 2-AG content in the amygdala ($F(2,40) = 3.73$, $p = 0.03$; Figure 3.1A). Post-hoc analyses revealed that adolescent rats exposed to repeated restraint had significantly lower 2-AG levels in the amygdala than their adult counterparts ($p < 0.05$). Furthermore, adults exposed to repeated restraint had significantly higher amygdalar 2-AG

content than naïve adults ($p < 0.05$). There were no significant main effects of age ($F(1,40) = 0.91$, $p = 0.34$) or stress condition ($F(2,40)=0.43$, $p=0.65$).

In the hippocampus, there were trends for an interaction between age and stress condition ($F(2,37)=2.78$, $p=0.07$; Figure 3.1B) as well as a main effect of stress condition ($F(2,37)=2.69$, $p=0.08$), but no significant main effect of age ($F(2,37)=1.62$, $p=0.21$). With the trending interaction, adults exposed to repeated restraint stress tended to have higher hippocampal 2-AG levels than naïve adults and adolescents exposed to repeated restraint. Furthermore, with the trending main effect, stress exposure increased hippocampal 2-AG content, regardless of age.

There was a significant interaction ($F(2,39)=3.50$, $p=0.04$; Figure 3.1C) on hypothalamic 2-AG content such that adults exposed to repeated restraint had higher hypothalamic 2-AG content than naïve adults ($p<0.01$) whereas stress exposure did not significantly affect adolescent hypothalamic 2-AG content. Additionally, naïve adults and adolescents did not significantly differ in hypothalamic 2-AG content ($p<0.05$); however, adults in the acute and repeated stress conditions had higher hypothalamic 2-AG content than their adolescent counterparts. There was also a significant main effect of age ($F(1,39)=41.36$, $p<0.0001$) with adults exhibiting generally higher hypothalamic 2-AG content than adolescents. Furthermore, a trend emerged for a main effect of stress condition ($F(2,39)=2.87$, $p=0.07$) in which repeated restraint stress exposure tended to increase hypothalamic 2-AG levels relative to naïve animals.

Lastly, two-way ANOVA of PFC 2-AG content revealed that there was no significant interaction ($F(2,39)=0.60$, $p=0.55$; Figure 3.1D) or main effect of age ($F(1,39)=1.00$, $p=0.32$). However, a main effect of stress condition emerged such that stress exposure generally increased 2-AG content in the PFC ($F(2,39)=5.16$, $p=0.01$).

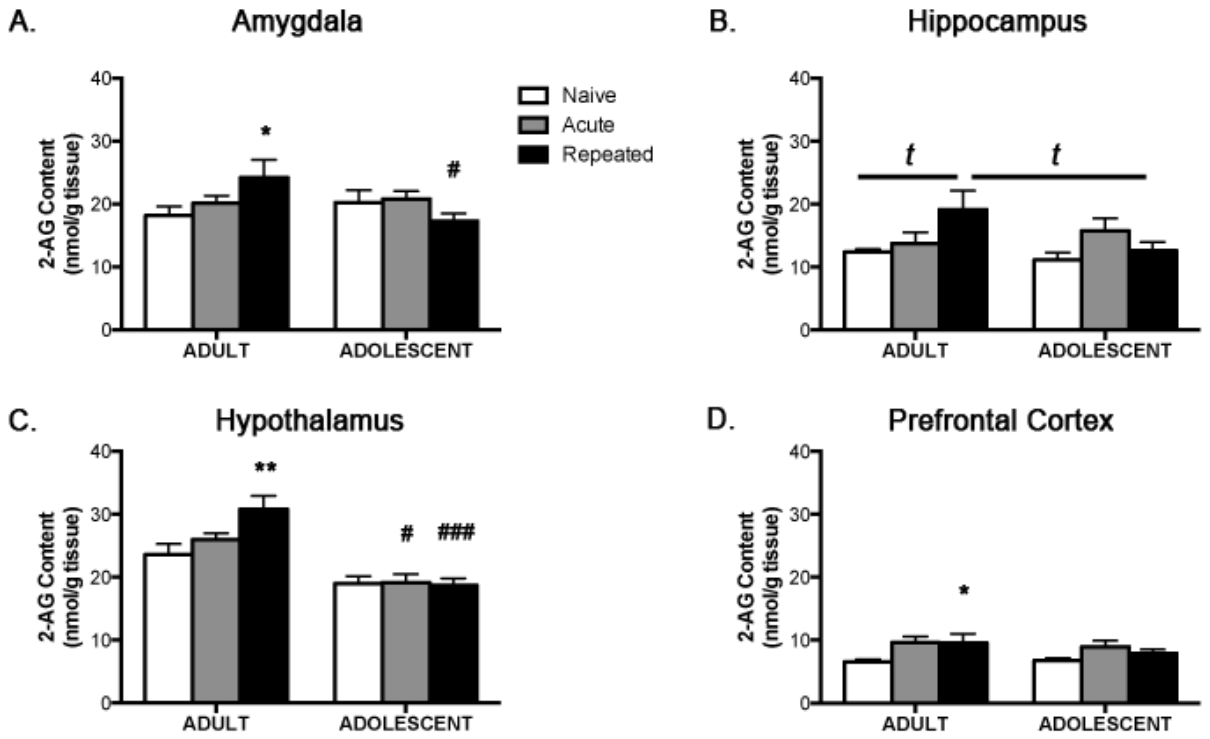

Figure 3.1. Corticolimbic 2-AG content increases with repeated restraint exposure in adult, but not adolescent rats. (A) Adolescent male rats exposed to repeated restraint fail to exhibit an increase in mean (± SEM) amygdalar 2-AG levels compared to adult male rats exposed to repeated restraint. Instead, amygdalar 2-AG levels were elevated in adolescent male rats in response to repeated restraint stress. (B) Mean hippocampal 2-AG content tended to increase in adult but not adolescent rats. (C) Repeated restraint increased 2-AG content in the hypothalamus of adult, but not adolescent rats. (D) Mean prefrontal cortical 2-AG content increased with repeated restraint exposure in adults, but not adolescents. *, ** denotes significantly different comparison ($p<0.05$ and $p<0.01$, respectively) to age-matched naïve group; #, ### denotes significantly different from adults in the same stress condition ($p<0.05$ and $p<0.001$, respectively); ***t*** indicates a trend, $p=0.07$.

3.3.1.2 Repeated restraint exposure reduces corticolimbic monoacylglycerol lipase activity in adult, but not adolescent rats

Given that statistical analyses revealed significant differences in corticolimbic 2-AG content between naïve animals and those exposed to repeated restraint, possible differences in adult and adolescent corticolimbic MAGL activity were examined between these stress conditions.

A significant interaction effect ($F(1,11)=19.37$, $p=0.001$; Figure 3.2A) emerged such that naïve adult rats had significantly higher amygdalar MAGL V_{max} values than naïve adolescents ($p<0.05$). Furthermore, adults exposed to repeated restraint had significantly lower amygdalar MAGL activity than naïve adults ($p<0.01$). There were no significant main effects of age ($F(1,11)=0.70$, $p=0.42$) or stress condition ($F(1,11)=1.09$, $p=0.32$). Analyses of amygdalar K_m values yielded no significant interactions ($F(1,11)=3.32$, $p=0.10$; Table 3.1) or main effects of age ($F(1,11)=1.78$, $p=0.21$) and stress condition ($F(1,11)=0.55$, $p=0.47$).

There was a significant interaction of age and stress condition on hippocampal MAGL V_{max} values ($F(1,11)=15.29$, $p=0.002$; Figure 3.2B) such that adults exposed to repeated restraint had significantly lower hippocampal MAGL activity than naïve adults ($p<0.01$) whereas stress condition did not affect adolescent MAGL activity ($p>0.05$). There were no significant main effects of stress condition ($F(1,12)=2.85$, $p=0.12$) and age ($F(1,12)=0.34$, $p=0.57$) on hippocampal V_{max} values. Finally, there was no significant interaction effects ($F(1,10)=1.55$, $p=0.24$; Table 3.1) or main effects of age ($F(1,10)=0.60$, $p=0.46$) or stress condition ($F(1,10)=3.00$, $p=0.11$) on hippocampal K_m values.

There were no significant interactions $F(1,11)=0.39$, $p=0.55$; Figure 3.2C), nor main effects of age ($F(1,11)=0.59$, $p=0.46$) or stress condition ($F(1,11)=0.46$, $p=0.51$) on hypothalamic MAGL V_{max} values. There were also no significant interactions ($F(1,11)=0.06$, $p=0.82$; Table

3.1), nor main effects of age ($F(1,11)=0.71$, $p=0.42$) or stress condition ($F(1,11)=3.37$, $p=0.12$) on hypothalamic MAGL K_m values.

In the PFC, there was a significant interaction of age and stress condition on MAGL V_{max} values ($F(1,10)=8.52$, $p=0.02$; Figure 3.2D) such that naïve adult rats had significantly higher V_{max} values than naïve adolescents ($p<0.05$). In contrast, repeated restraint failed to decrease MAGL activity in adolescent rats ($p>0.05$). There was no significant main effect of age ($F(1,10)=2.47$, $p=0.15$) but there was a trend for a main effect of stress condition ($F(1,10)=3.85$, $p=0.08$) such that repeated restraint exposure reduced MAGL activity. There was no significant interaction on K_m values in the PFC ($F(1,10)=0.27$, $p=0.62$; Table 3.1) nor were there significant main effects of age ($F(1,10)=1.58$, $p=0.24$) or stress condition ($F(1,10)=3.73$, $p=0.09$).

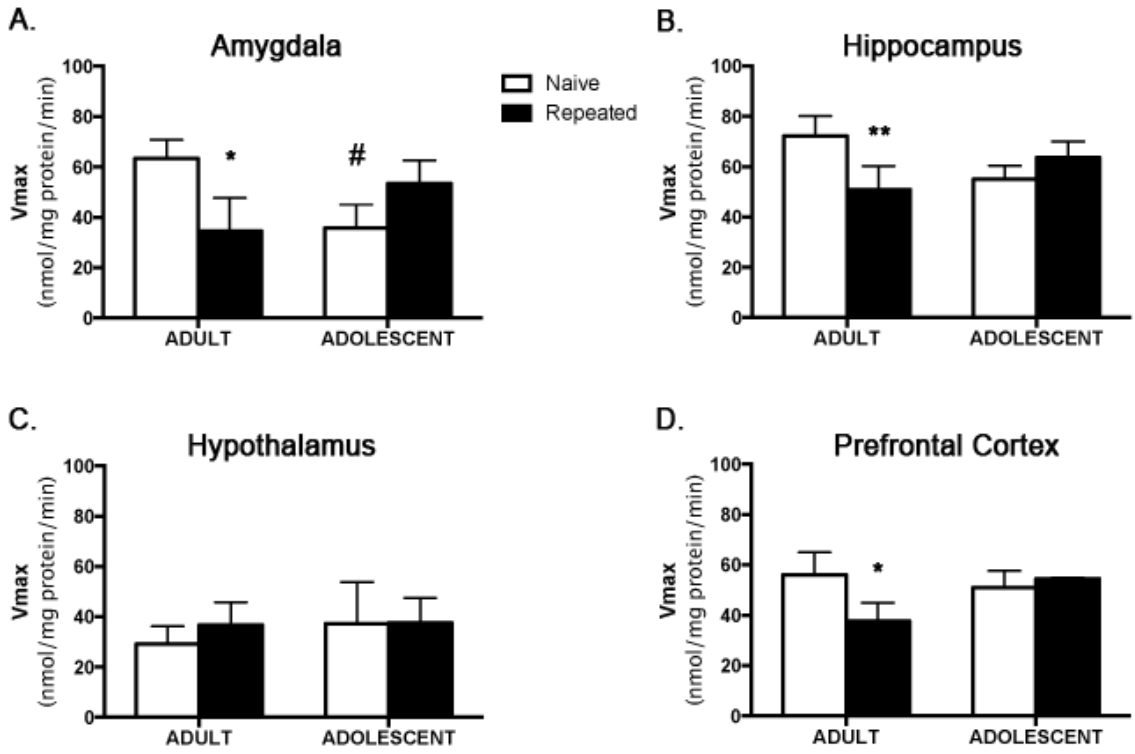

Figure 3.2. Repeated restraint exposure reduces corticolimbic monoacylglycerol lipase (MAGL) activity in adult, but not adolescent rats in the (A) amygdala, (B) hippocampus and (D) prefrontal cortex, but not the hypothalamus (C). *, ** denotes statistically significant differences ($p<0.05$ and $p<0.01$, respectively) to age-matched naïve group; # denotes significantly different from adults in the same stress condition ($p<0.05$).

Table 3.1. Age of preputial separation, monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH) binding affinity (K_m) values in the amygdala (AMY), hippocampus (HIPPO), hypothalamus (HYPO) and prefrontal cortex (PFC) of adult and adolescent rats exposed to restraint stress

		MAG Lipase Activity K_m Values (nM)				FAAH Activity K_m Values (nM)			
Treatment Group	Age of Preputial Separation (days old)	AMY	HIPPO	HYPO	PFC	AMY	HIPPO	HYPO	PFC
Naïve Adult	-	88 ± 11	81 ± 30	114 ± 29	65 ± 8	0.75 ± 0.1	0.73 ± 0.07	0.51 ± 0.07	0.80 ± 0.07
Acute Adult	-	-	-	-	-	0.79 ± 0.03	0.91 ± 0.03*	0.74 ± 0.07	0.94 ± 0.06
Adult Repeated	-	48 ± 21	38 ± 3	166 ± 52	36 ± 5	0.95 ± 0.03	1.13 ± 0.13*	0.79 ± 0.12	0.94 ± 0.08
Naïve Adolescent	41.9 ± 0.3	38 ± 16	52 ± 14	102 ± 9	73 ± 14	0.83 ± 0.08	0.87 ± 0.01	0.75 ± 0.04	0.84 ± 0.05
Acute Adolescent	42.1 ± 0.3	-	-	-	-	0.85 ± 0.07	0.83 ± 0.1*	0.79 ± 0.06	0.75 ± 0.11
Repeated Adolescent	42.3 ± 0.3	55 ± 11	45 ± 14	140 ± 11	57 ± 17	0.87 ± 0.05	0.95 ± 0.05*	0.83 ± 0.21	0.97 ± 0.07

* indicates significantly different from Naïve Adult group, $p \leq 0.05$.

3.3.1.3 Amygdalar AEA content is reduced with stress exposure in adult but not adolescent rats

Analyses of anandamide content in the amygdala revealed a significant interaction effect between age and stress condition ($F(2,38) = 4.68$, $\underline{p} = 0.01$; Figure 3.3A). Bonferroni post-hoc analyses revealed that adults exposed to repeated restraint had significantly lower amygdalar AEA content than naïve adult and adolescent rats ($p<0.005$) and there was a trend for the same effect when comparisons were made between acutely stressed adults and naive adults ($p=0.08$). However, amygdalar AEA levels were not significantly different in adolescent rats, regardless of stress condition ($p>0.05$). There was also a main effect of age ($F(2,38)=5.66$, $p=0.02$) in which adults had higher overall amygdalar AEA levels than adolescents.

In the hippocampus, there was no significant interaction between age and stress condition on AEA content in the hippocampus ($F(2,38)=0.02$, $p=0.98$; Figure 3.3B). However, there was a main effect of age ($F(1,38)=21.16$, $p<0.0001$) such that overall hippocampal AEA content was lower in adolescents relative to adults. There was also a main effect of stress condition such that AEA content was reduced with acute and repeated restraint exposure ($F(2,38)=3.27$, $p=0.04$).

There was no significant interaction between stress condition and age on hypothalamic AEA content ($F(2,39)=0.20$, $p =0.82$; Figure 3.3C). However, there were significant main effects of age ($F(1,39)=101.5$, $p<0.0001$) such that, regardless of stress condition, adolescent rats had significantly lower hypothalamic AEA content than adults ($p<0.0001$). Furthermore, there was a main effect of stress condition ($F(2,39)=5.52$, $p=0.008$) in which animals exposed to acute and repeated restraint stress had reduced hypothalamic AEA content relative to naïve animals, irrespective of age.

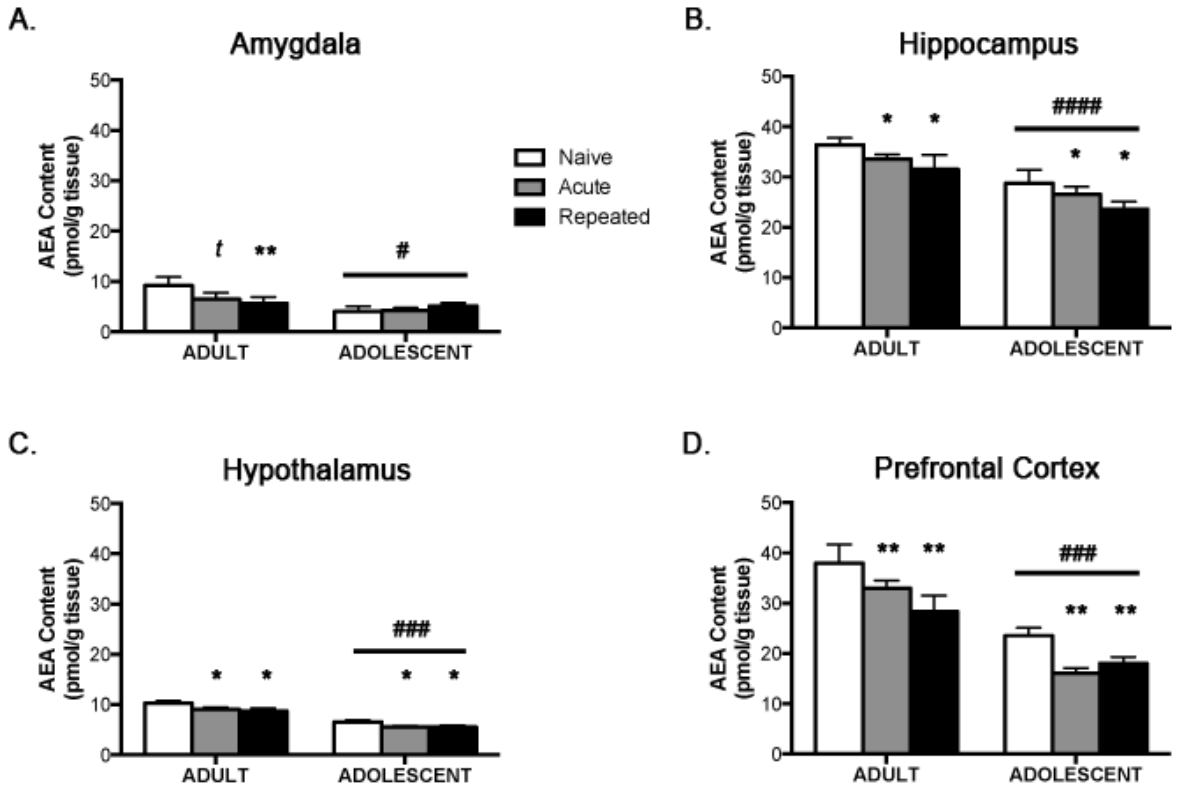

Figure 3.3. Adult male rats have higher mean (± SEM) corticolimbic anandamide content than adolescent male rats. Restraint stress exposure decreases corticolimbic anandamide (AEA) content in adult, but not adolescent rats in the (A) amygdala. Restraint exposure reduced AEA content in the (B) hippocampus, (C) hypothalamus, and (D) prefrontal cortex regardless of age. *, ** denotes significantly different comparison ($p<0.05$ and $p<0.01$, respectively) to age-matched naïve group; #, ###, #### denotes significantly different from adults in the same stress condition ($p<0.05$, $p<0.001$, $p<0.0001$, respectively)); ***t*** indicates a trend, $p=0.08$.

Finally, there was no significant interaction between age and stress condition on PFC AEA content ($F(2,39)=1.14$, $p=0.33$; Figure 3.3D).However there was a main effect of age ($F(1,39)=60.07$, $p<0.0001$) and stress condition ($F(2,39)=6.83$, $p<0.003$). Again, adult rats had higher PFC AEA content than adolescent rats. Furthermore, stress exposure generally reduced AEA levels in the PFC.

3.3.1.4 Corticolimbic fatty acid amide hydrolase (FAAH) activity increases with stress exposure in adult, but not adolescent rats

In the amygdala, there was a significant interaction between age and stress condition ($F(2,27)=5.33$, $p=0.01$; Figure 3.4A) such that relative to the naïve animals, repeated restraint exposure increased FAAH V_{max} values in adult rats ($p<0.01$), but not adolescent rats ($p>0.05$). Furthermore, post-hoc analyses revealed that FAAH activity was significantly higher in naïve adolescents than naïve adult rats ($p<0.05$). There was a main effect of stress condition ($F(2,27)=5.87$, $p=008$) in which FAAH V_{max} values generally increased with stress exposure; however, there was no main effect of age ($F(1,27)=2.54$, $p=0.12$). There were no significant interactions ($F(2,27)=0.84$, $p=0.44$; Table 3.1), nor main effects of stress condition ($F(2,27)=1.90$, $p=0.17$) and age ($F(1,27)=0.20$, $p=0.66$) on amygdalar K_m values.

In the hippocampus, a significant interaction ($F(2,28)=7.48$, $p=0.002$; Figure 3.4B) emerged such that repeated restraint exposure increased FAAH V_{max} values relative to naïve adult rats ($p<0.01$), but not adolescents ($p>0.05$). There was also a main effect of stress condition, in which FAAH V_{max} values generally increased with stress exposure ($F(2,28)=3.41$, $p=0.05$), but no main effect of age ($F(1,28)=0.02$, $p=0.90$). There were no significant interaction effects ($F(2,28)=2.10$, $p=0.14$; Table 3.1) nor main effect of age ($F(1,28)=0.34$, $p=0.57$) on hippocampal K_m values. However, there was a main effect of stress condition ($F(2,28)=4.93$, $p=0.01$) in which K_m values were increased with stress exposure.

There was a significant interaction between stress condition and age ($F(2,26)=14.20$, $p<0.0001$; Figure 3.4C) in the hypothalamus such that repeated restraint exposure increased FAAH activity relative to naïve animals in the adult ($p<0.05$), but not adolescent rats ($p>0.05$). Furthermore, there was a main effect of age in which naïve adolescent rats had significantly higher hypothalamic FAAH V_{max} values than naïve adult rats ($F(1,26)=22.66$, $p<0.0001$). There was no main effect of stress condition ($F(2,26)=0.34$, $p=0.72$). There were no significant interaction effects ($F(2,26)=0.56$, $p=0.57$; Table 3.1), nor main effects of age ($F(1,26)=1.65$, $p=0.21$) and stress condition ($F(2,26)=1.47$, $p=0.25$) on hypothalamic K_m values.

Lastly, there was a significant interaction effect on PFC FAAH activity ($F(2,28)=6.32$, $p=0.005$; Figure 3.4D) in which acute and repeated restraint stress exposure increased V_{max} values in adult rats (both $p<0.05$), but not adolescent rats (both $p>0.05$). No main effects of age ($F(1,28)=1.04$, $p=0.32$) nor stress condition ($F(2,28)=1.74$, $p=0.19$) on PFC FAAH activity were detected. There were no significant interaction effects ($F(2.28)=1.52$, $p=0.24$; Table 3.1), nor main effects of age ($F(1,28)=0.39$, $p=0.54$) and stress condition ($F(2,28)=2.03$, $p=0.15$) on PFC K_m values.

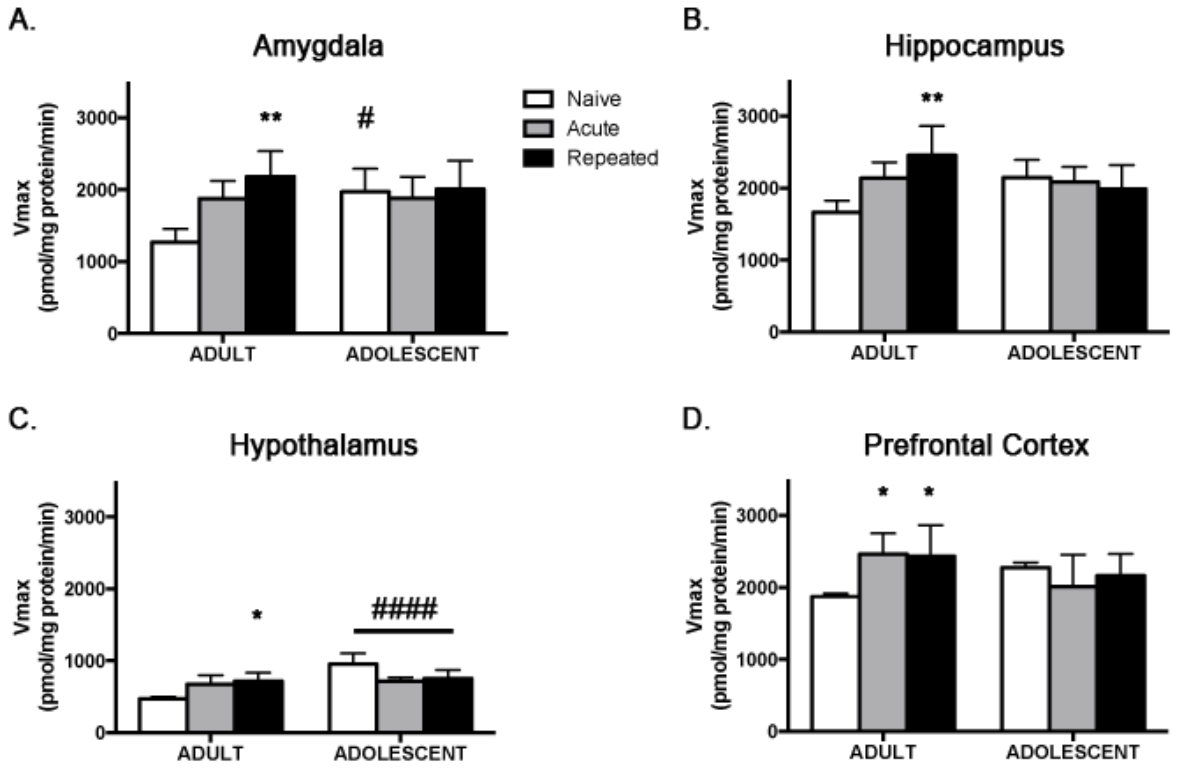

Figure 3.4. Repeated restraint exposure increases mean (± SEM) corticolimbic fatty acid amide hydrolase (FAAH) activity in adult, but not adolescent rats in the (A) amygdala, (B) hippocampus, (C) hypothalamus, and (D) prefrontal cortex. Corticolimbic FAAH activity is also elevated in adolescent male rats relative to adult male rats. *, ** denotes significantly different ($p<0.05$ and $p<0.01$, respectively) from age-matched naïve group; #, #### denotes significantly different from adults in the same stress condition ($p<0.05$ and $p<0.0001$, respectively).

3.3.1.5 Adolescent male rats do not exhibit HPA axis habituation and have lower basal testosterone levels than adult male rats

A significant interaction effect ($F(2,28)=3.58$, $p=0.04$; Figure 3.5A) emerged such that acute ($p<0.01$) and repeated restraint ($p<0.05$) exposure in adolescents increased plasma ACTH levels relative to naïve rats, whereas only acute stress increased adult ACTH levels ($p<0.001$). Acute restraint-induced increases in adult ACTH levels were also significantly higher than those induced in adults by repeated restraint ($p<0.01$), indicating that the repeated restraint group had achieved stress-induced ACTH habituation. There was also a significant main effect of stress condition ($F(2,28)=19.64$, $p<0.0001$) such that stress exposure generally increased ACTH levels regardless of age. However, there was no significant main effect of age ($F(1,28)=0.0005$, $p=0.98$).

Analyses of variance revealed a significant interaction effect of age and stress condition on plasma CORT levels ($F(2,27) = 25.66$, $p <0.0001$; Figure 3.5B). Post-hoc analyses revealed that adolescent acute ($p<0.0001$) and repeated restraint ($p<0.0001$) exposure similarly increased plasma CORT levels relative to naïve rats. There was no significant difference between acute and repeated restraint-induced increases in CORT levels in adolescents ($p>0.05$). Acute ($p<0.0001$) and repeated restraint ($p<0.01$) exposure in adults also increased plasma CORT levels relative to naïve adult rats; however, acute restraint elevated CORT levels more than repeated restraint in adults ($p<0.0001$), indicating stress-induced CORT habituation had occurred. Lastly, despite no significant effect of age on elevated peak CORT levels (30 min following restraint onset) induced by acute restraint ($p>0.05$), adults had significantly lower CORT levels than adolescents exposed to repeated restraint ($p<0.0001$), indicating that adults exhibited HPA axis habituation whereas adolescents did not. A significant main effect of age ($F(1,27)=5.37$, $p=0.03$) emerged in which adolescent rats had higher overall CORT levels than

adults. There was also a significant main effect of stress condition ($F(2,27)=152.3$, $p<0.0001$) such that stress exposure generally increased CORT levels regardless of age.

Finally, there was no significant interaction effect between age and stress condition on plasma testosterone levels ($F(2,30)=0.67$, $p=0.52$; Figure 3.5C) and no main effect of stress condition ($F(2,30)=1.12$, $p=0.34$). There was a significant main effect of age ($F(1,30)=35.99$, $p<0.0001$) in which adult male rats had higher testosterone levels than the adolescent rats. A one-way ANOVA performed on age of preputial separation in the adolescent rats revealed no significant effect of stress condition ($F(2,33)=0.42$, $p=0.66$; Table 3.1).

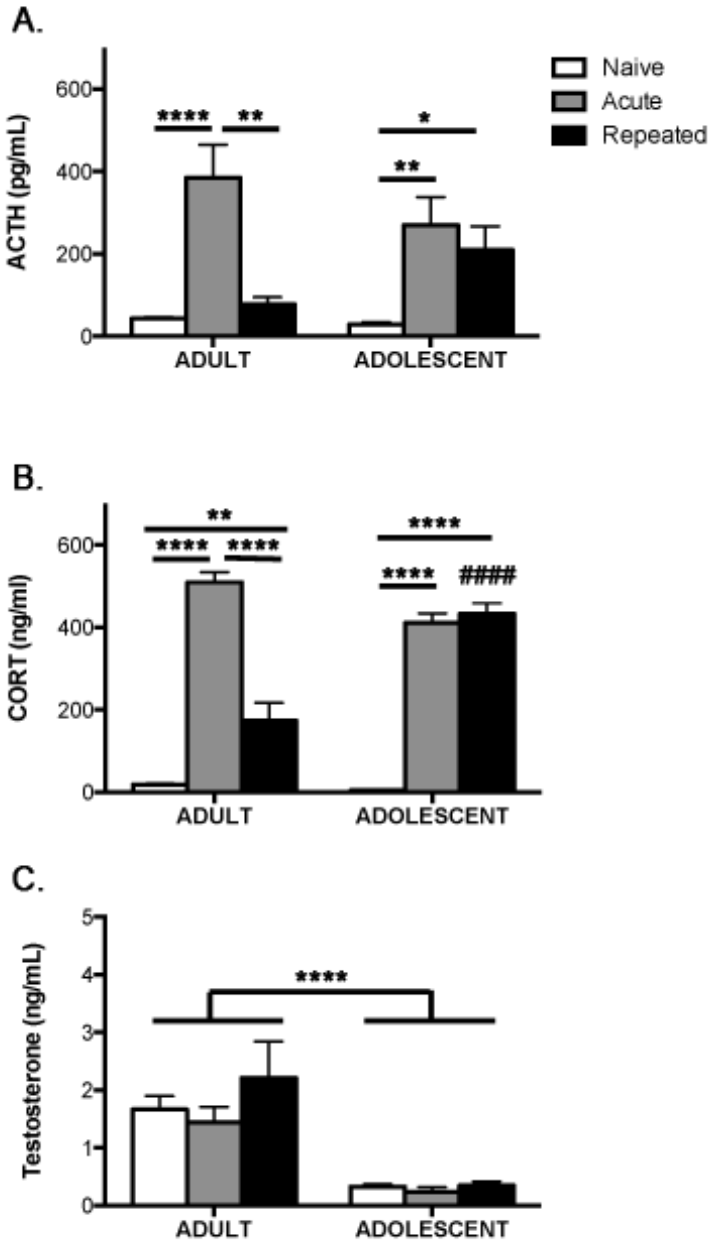

Figure 3.5. Adolescent male rats exposed to repeated restraint fail to exhibit HPA axis stress habituation relative to adult male rats, as measured by (A) ACTH (mean ± SEM) and (B) CORT (mean ± SEM) levels. (C) Adult male rats have higher overall testosterone levels (mean ± SEM) than adolescents. . *, **, **** denotes significantly different ($p<0.05$, $p<0.01$, $p<0.0001$, respectively) from age-matched naïve group; #### denotes significantly different from adults in the same stress condition ($p<0.0001$).

3.3.1.6 Stress exposure and age do not affect whole cell mineralocorticoid (MR) and glucocorticoid (GR) protein Levels

Two-way ANOVA revealed no significant interaction ($F(2,30)=0.72$, $p = 0.49$; Table 3.2), nor main effects of age ($F(1,30)=0.22$, $p=0.64$) and stress ($F(2,30)=0.06$, $p=0.94$) on GR/Actin ratio in the ventral hippocampus. There was also no significant interaction ($F(2,30)=0.06$, $p = 0.94$; Table 3.2), or main effects of age ($F(1,30)=0.37$, $p=0.55$) and stress ($F(2,30)=1.27$, $p=0.30$) on MR/Actin ratio within the ventral hippocampus. No significant interaction ($F(2,29)=0.24$, $p=0.79$; Table 3.2), nor main effects of age ($F(1,29)=1.66$, $p=0.21$) and stress ($F(2,29)=0.40$, $p=0.68$) were detected on the ratio of GR/MR in the ventral hippocampus.

Two-way ANOVA revealed no significant interaction ($F(2,28)=0.13$, $p = 0.88$; Table 3.2), nor main effects of age ($F(1,28)=0.48$, $p=0.50$) and stress ($F(2,28)=0.54$, $p=0.59$) on GR/Actin ratio in the dorsal hippocampus. There was also no significant interaction ($F(2,28)=0.29$, $p = 0.75$; Table 2), nor main effects of age ($F(1,28)=1.06$, $p=0.31$) and stress ($F(2, 28)=0.01$, $p=0.99$) on MR/Actin ratio within the dorsal hippocampus. No significant interaction ($F(2,27)=1.04$, $p=0.37$; Table 3.2), nor main effects of age ($F(1,27)=0.27$, $p=0.60$) and stress ($F(2,27)=0.24$, $p=0.79$) were detected on the ratio of GR/MR in the dorsal hippocampus.

Table 3.2. Mean (± SEM) relative optical density of glucocorticoid and mineralocorticoid receptor expression of adult and adolescent rats exposed to acute and repeated restraint stress

	Ventral Hippocampus			Dorsal Hippocampus		
Treatment Group	GR/Actin	MR/Actin	GR/MR	GR/Actin	MR/Actin	GR/MR
Adult-Naïve	0.033 ± 0.006	0.17 ± 0.01	0.22 ± 0.02	0.078 ± 0.01	0.18 ± 0.03	0.47 ± 0.03
Adult-Acute Stress	0.031 ± 0.002	0.17 ± 0.02	0.22 ± 0.02	0.091 ± 0.014	0.16 ± 0.02	0.49 ± 0.06
Adult-Repeated Stress	0.038 ± 0.006	0.15 ± 0.01	0.21 ± 0.02	0.078 ± 0.007	0.18 ± 0.02	0.45 ± 0.02
Adolescent-Naïve	0.037 ± 0.006	0.17 ± 0.01	0.18 ± 0.02	0.085 ± 0.012	0.19 ± 0.03	0.46 ± 0.07
Adolescent-Acute Stress	0.037 ± 0.003	0.18 ± 0.01	0.21 ± 0.02	0.091 ± 0.005	0.20 ± 0.03	0.46 ± 0.05
Adolescent-Repeated Stress	0.033 ± 0.003	0.16 ± 0.02	0.19 ± 0.02	0.087 ± 0.009	0.18 ± 0.03	0.54 ± 0.03

3.3.2 Experiment 2

3.3.2.1 Immediate effects of repeated stress on corticosterone and CB_1 receptor binding

Stress-induced increases in CORT levels were measured on the first and last day of restraint stress exposure to determine the magnitude of HPA axis habituation to the repeated stressor. Analysis demonstrated a significant interaction between age and stress [$F(1, 12) = 5.09$, $p < 0.05$; Table 3.3], such that adult rats exposed to repeated restraint stress exhibited a significant reduction in stress-induced increases in CORT levels on day 10 relative to day 1 ($p < 0.05$). Adolescent rats, however, did not exhibit any significant differences in CORT secretion between the first and last day of restraint ($p > 0.05$), indicating that they did not exhibit habituation to the stressor, a phenomenon consistent with previous reports (Romeo, 2010b).

Table 3.3. Adolescent rodents do not exhibit habituation of corticosterone secretion (ng/mL) to repeated restraint stress

	Day 1	Day 10
Adolescent	240.7 ± 12.6	233.8 ± 29.0
Adult	253.9 ± 32.1	142.2 ± 11.4*

Ten days of repeated restraint stress (30 min/day) resulted in a significant reduction in corticosterone secretion from day 1 to day 10 in adult, but not adolescent, rats. Data are presented as mean values ± SEM (n = 4 / condition). * denotes significant differences between corticosterone levels on day 1 and day 10.

With respect to CB_1R binding, within the PFC, there was no significant interaction between age and stress on the B_{max} of the CB_1R [$F(1, 12) = 1.19$, $p > 0.05$; Figure 3.6], nor was there a main effect of age [$F(1, 12) = 0.01$, $p > 0.05$]; there was, however, a very significant main effect of stress [$F(1, 12) = 14.67$, $p < 0.005$] such that stress increased CB_1R binding site densities in the PFC regardless of age. There was no significant interaction between age and stress [$F(1, 12) = 1.46$, $p > 0.05$; Table 3.4], nor a main effect of age [$F(1, 12) = 1.44$, $p > 0.05$] or stress [$F(1, 12) = 2.61$, $p > 0.05$] on the K_d of the CB_1R.

Within the hippocampus, there was a significant interaction between age and stress on the B_{max} of the CB_1R [$F(1, 12) = 6.26$, $p < 0.03$; Figure 3.6], such that adult rats exposed to repeated restraint stress exhibited a significant reduction in CB_1R binding site densities relative to age matched controls ($p < 0.05$), while there was no effect of stress on CB_1R binding densities in the hippocampus of adolescent rats ($p > 0.05$). There was also a significant interaction between age and stress exposure on the K_d of the CB_1R in the hippocampus [$F(1,12) = 5.98$, $p < 0.05$; Table 3.4]; however, post-hoc analysis demonstrated that there were no significant differences between any of the experimental conditions.

There was a significant interaction between age and stress on the B_{max} of the CB_1R in the amygdala [$F(1,12) = 5.85$, $p < 0.04$; Figure 3.6]. Post hoc analysis revealed that in non-stressed animals, adults exhibited higher levels of CB_1R binding in the amygdala relative to adolescents ($p < 0.05$). Interestingly, stress exposure in adult animals had no effect on CB_1R binding ($p > 0.05$), while in adolescent rats, stress exposure produced an increase in CB_1R binding relative to their age-matched controls ($p < 0.01$). With respect to K_d of the CB_1R, there was no interaction between age and stress [$F(1, 12) = 0.01$, $p > 0.05$; Table 3.4], nor significant main effects of stress [$F(1, 12) = 0.29$, $p > 0.05$] or age [$F(1, 12) = 2.64$, $p > 0.05$].

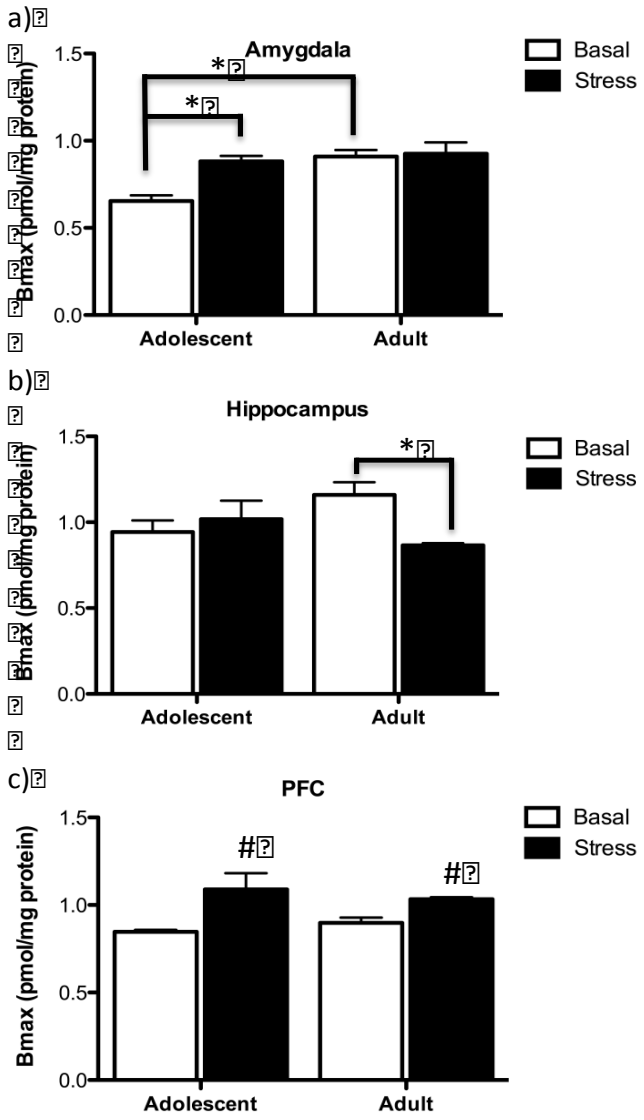

Figure 3.6. Repeated stress produces immediate region specific effects on CB_1 receptor binding in an age dependent manner. The effects of repeated restraint stress (30 min/day for 10 days) on the maximal binding site density (B_{max}) of the CB_1 receptor in the a) amygdala, b) hippocampus and c) prefrontal cortex (PFC), 24 hr after the final stressor. Stress exposure in adolescent rats spanned from post-natal day (PND) 35-44 and tissue was collected on PND 45. Stress exposure in adult rats spanned from PND 75-84 and tissue was collected on PND 85. Data are presented as mean $\pm$ SEM; n=4 / treatment condition. * denotes significant differences ($p < 0.05$) between identified groups; # denotes significant main effect ($p < 0.05$) of stress relative to non-stressed animals.

Table 3.4. The effects of repeated restraint stress on the binding affinity of the cannabinoid CB_1 receptor.

		Immediate (24 hours)		Recovery (40 days)	
		Stress Naive	Stress	Stress Naive	Stress
Amygdala	Adolescent	1.19 ± 0.12	1.29 ± 0.19	0.97 ± 0.11	0.79 ± 0.09
	Adult	1.47 ± 0.14	1.55 ± 0.20	1.01 ± 0.08	1.14 ± 0.13
Hippocampus	Adolescent	0.75 ± 0.13	1.03 ± 0.9	0.75 ± 0.07	0.87 ± 0.11
	Adult	1.00 ± 0.10	0.71 ± 0.14	0.71 ± 0.15	0.99 ± 0.11
Prefrontal cortex	Adolescent	1.13 ± 0.06	1.13 ± 0.03	0.92 ± 0.09	0.59 ± 0.10*
	Adult	1.16 ± 0.07	1.31 ± 0.09	0.88 ± 0.06	1.01 ± 0.12

Rats were exposed to repeated restraint stress (30 min/day for 10 days), either as adolescents (stress from post-natal day [PND] 35-44) or adults (stress from PND 75-84), and then examined immediately after (24 hr following final stressor) or following a recovery period (40 days following final stressor). There were minimal effects of age or stress exposure on the binding affinity (K_d) of ^{3}H-CP55,940 for the CB_1 receptor. Data are presented as mean values ± SEM (n = 4 / condition). * denotes significant differences ($p < 0.05$) between stressed adolescents and stressed adult rats.

3.3.2.2 Sustained effects of stress on cannabinoid CB_1 receptor binding

There was neither a significant interaction [$F(1, 12) = 0.15$, $p > 0.05$; Table 3.5], nor a main effect of age [$F(1, 12) = 2.08$, $p > 0.05$] or stress [$F(1,12) = 0.07$, $p > 0.05$] on basal CORT levels, indicating that stress exposure in adolescence and adulthood does not exert sustained effects on basal HPA axis function.

Within the PFC, there was a significant interaction between stress and age on the B_{max} of the CB_1R [$F(1, 12) = 5.11$, $p < 0.05$; Figure 3.7], with post-hoc analysis demonstrating that animals which had been exposed to repeated stress in adolescence exhibited a significant reduction in CB_1R binding site densities in the PFC relative to all other treatment conditions ($p < 0.01$). Animals that had been exposed to stress in adulthood had no significant difference in their B_{max} values of the CB_1R in the PFC relative to their non-stressed controls ($p > 0.05$). With respect to the K_d of the CB_1R, there was a significant interaction between stress and age [$F(1, 12) = 5.81$, $p < 0.05$; Table 3.4]. However, post-hoc analysis revealed that this effect was due to a significant difference in K_d between animals that had been exposed to stress as adolescents and animals which had been exposed to stress as adults ($p < 0.05$). Lastly, there were no significant differences in K_d between stressed animals and their age-matched counterparts.

Table 3.5. Prior history of stress does not alter basal corticosterone levels (ng/mL) after a recovery period of forty days.

	Stress Naive	Stress
Adolescent	16.5 ± 8.9	15.0 ± 4.8
Adult	27.5 ± 10.2	34.3 ± 15.3

Forty days following the conclusion of repeated restraint stress (30 min/day for 10 days), there was no difference in basal corticosterone levels relative to unstressed control rats, regardless of whether this stress exposure occurred in adolescence or adulthood. Data are presented as mean values ± SEM (n = 4 / condition).

There was no significant interaction between age and stress [$F(1, 12) = 0.01$, $p > 0.05$; Figure 3.7], nor a main effect of age [$F(1, 12) = 0.25$, $p > 0.05$} on the B_{max} of the CB_1R in the hippocampus. There was, however, a significant main effect of stress [$F(1, 12) = 6.02$, $p < 0.04$] on the B_{max} of the CB_1R in the hippocampus, such that, regardless of age of exposure, animals which had been exposed to repeated stress exhibited a significant increase in CB_1R binding site densities in the hippocampus when examined after a 40 day recovery period. There was no significant interaction between age and stress [$F(1, 12) = 0.55$, $p > 0.05$; Table 3.4], nor a main effect of age [$F(1, 12) = 0.12$, $p > 0.05$] and stress [$F(1, 12) = 3.08$, $p > 0.05$] on the K_d of the CB_1R in the hippocampus.

Finally, within the amygdala there was no significant interaction between stress and age on the binding site density of the CB_1R [$F(1, 12) = 1.80$, $p > 0.05$; Figure 3.7], or main effect of stress [$F(1, 12) = 2.08$, $p > 0.05$]. There was, however, a main effect of age [$F(1, 12) = 5.36$, $p < 0.04$] such that PND125 animals had higher levels of CB_1R binding sites than animals at PND 85, regardless of previous stress exposure. There was no interaction [$F(1, 12) = 2.20$, $p > 0.05$; Table 3.4], nor main effects of age [$F(1, 12) = 3.68$, $p > 0.05$] and stress [$F(1, 12) = 0.05$, $p > 0.05$] on the K_d of the CB_1R in the amygdala.

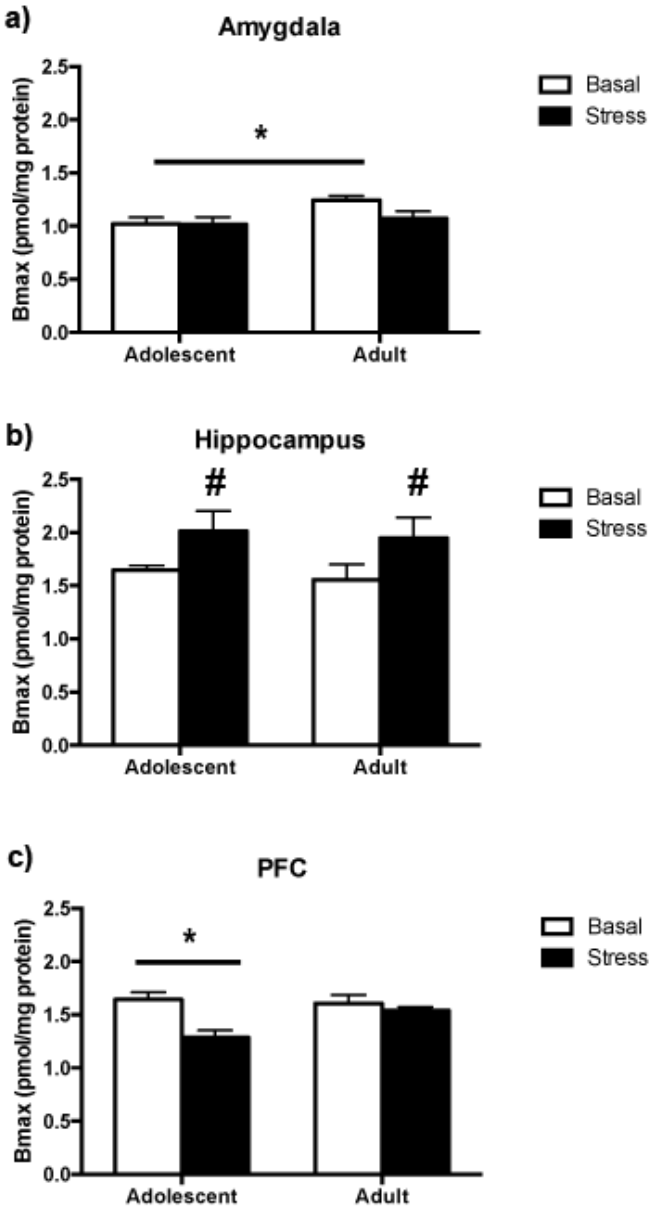

Figure 3.7. Sustained effect of repeated stress on CB_1 receptor binding. The effects of repeated restraint stress for 10 days (30 min/day) on the maximal binding site density (B_{max}) of the CB_1 receptor in the a) amygdala, b) hippocampus and c) prefrontal cortex (PFC), when examined 40 days after the final stressor. Stress exposure for adolescent rats spanned from post-natal day (PND) 35-44 and tissue was collected on PND 85. Stress exposure for adult rats spanned from PND 75-84 and tissue was collected on PND 125. Data are presented as mean $\pm$ SEM; n = 4 / treatment condition. * denotes significant differences ($p < 0.05$) between identified groups; # denotes significant main effect ($p < 0.05$) of stress relative to non-stressed animals.

3.5 Discussion

3.5.1 Experiment 1

Results indicate that corticolimbic eCB signaling is not mature during adolescence and supports the notion that the eCB system is an important mediator of interactions between HPA axis stress responsivity and maturational stage. While adult rats exhibit a characteristic habituation of stress-induced CORT and ACTH secretion following repeated restraint, adolescent rats do not exhibit such habituation. The current study extends these findings by demonstrating that in addition to a lack of neuroendocrine habituation to stress exposure, adolescent rats do not exhibit an increase in amygdalar 2-AG signaling, a biochemical signature we have previously demonstrated is critical for manifesting stress habituation (Hill et al., 2010b). Furthermore, adolescent rats also failed to exhibit a reduction in amygdalar AEA content following either acute or repeated stress.

2-AG content in the remaining adult corticolimbic structures (hippocampus, hypothalamus, PFC) of adult rats was also increased with repeated restraint exposure, an effect that was also absent in adolescent rats. In contrast, adolescent and adult rats exhibited a similar general reduction in AEA content within the hippocampus, hypothalamus and PFC. The stress-induced alterations in corticolimbic 2-AG and AEA content were observed to be at least partially due to corresponding changes in MAGL and FAAH enzyme activity. Moreover, it is worth noting that regardless of stress condition, adult rats generally exhibited greater AEA content and less FAAH activity across all four corticolimbic structures than 45-day old adolescent rats, confirming our findings from Chapter 2. Collectively, these findings suggest that differential age-dependent crosstalk occurs between the stress axis and the eCB system. Somewhat surprisingly, age and stress conditions were not found to significantly alter MR and GR protein

levels. Furthermore, stress exposure in the adolescent rats did not affect pubertal onset and as expected, adult male rats exhibited higher testosterone levels than adolescent male rats.

These data lend further support to previous work demonstrating that 2-AG elevation in the basolateral amygdala (BLA) following repeated stress induces habituation of HPA axis activation (Hill et al., 2010b). This recruitment of amygdalar 2-AG is believed to be a neural locus contributing to stress adaptation in the form of the habituation response by dampening activation of the BLA to affect HPA function (Hill et al., 2010b). The BLA possesses indirect efferent projections (via the medial amygdala and bed nucleus of the stria terminalis) to the paraventricular nucleus (PVN) of the hypothalamus as well as connections facilitating communication with other corticolimbic structures such as the prefrontal cortex and hippocampus (Lopez et al., 1999). Thus, reduced firing in the BLA results in a more general dampening of activity in these neural structures, to culminate in a lack of HPA response to the previously stress-inducing stimulus. CB_1R activation also appears integral to the expression of habituation, as when the CB_1R antagonist, AM-251, was locally infused into the BLA, the habituation response was blocked (Hill et al., 2010b) whereas CB_1R activation in the BLA appears to reduce firing of efferents to impinge on HPA function (Hill et al., 2009).

We observed an absence of amygdalar 2-AG mobilization and no reduction in CORT and ACTH secretion in adolescent male rats exposed to repeated restraint stress, suggesting a mechanism related to impaired stress habituation in adolescents. The habituation response is thought, at least in part, to be mediated by glucocorticoid feedback (Jaferi and Bhatnagar, 2006). While the mechanism driving the increase in amygdalar 2-AG is unknown, sustained elevations in glucocorticoids appear to be sufficient to recruit this process (Hill et al., 2005a). As such, 2-AG in the amygdala may be recruited by sustained glucocorticoid receptor activation, in a manner that has not reached full maturity in adolescence, ultimately affecting the dampening of the neural stress axis and the manifestation of habituation. One proposed mechanism underlying

the adolescent's inability to launch a habituation response posits that this is due to region specific GR expression and/or developmental stage-dependent expression of steroid receptor co-activators which modulate glucocorticoid receptor gene transcription (Romeo et al., 2008). Thus, immature glucocorticoid-mediated feedback in adolescence could subsequently impair the recruitment of amygdalar 2-AG signaling and result in a lack of stress habituation. Moreover, shifts in adrenal sensitivity to ACTH may also contribute to age-dependent differences in HPA axis stress responsivity (Romeo et al., 2014). Indeed, we found that repeated restraint stress-induced 2-AG content elevations in the hippocampus, hypothalamus and PFC (in addition to the amygdala) were absent in adolescent rats. Corticolimbic 2-AG elevations are reported to contribute to the recovery of stress-induced HPA axis levels to basal levels (Hill and Patel, 2013; Roberts et al., 2014). It seems likely that the general lack of 2-AG mobilization in the adolescent hippocampus, hypothalamus and PFC reflect immature regulation of stress-induced HPA axis recovery to basal ACTH and CORT levels from repeated stress exposure. However, this possibility requires further investigation.

Converging lines of evidence indicate that adult AEA-CB_1R signaling within the BLA of the amygdala function as a gatekeeper of HPA axis activity. Acute stress exposure produces a reduction of AEA content in the BLA as a result of an increase in FAAH activity (Hill et al., 2009; Patel et al., 2005b). Furthermore, local infusion of a FAAH inhibitor or a CB_1R agonist specifically into the BLA similarly suppress stress-induced activation of the HPA axis (Hill et al., 2009). Thus, AEA-CB_1R tone in the BLA appears to gate excitatory glutamatergic inputs to principal neurons and when stress exposure disrupts this tone (by reducing AEA or CB_1R blockade), this increases principal neuron activity, activation of the HPA axis and subsequent release of glucocorticoid hormones into the circulation (Hill and Tasker, 2012). In the current study, corticolimbic AEA content was generally reduced by acute and repeated restraint exposure, regardless of age, suggesting that in some respects, adolescent eCB - HPA axis

interactions function similarly to those of adults in that activation of the HPA axis requires reductions in corticolimbic AEA content.

However, adolescent amygdalar AEA content failed to exhibit a reduction with acute and repeated restraint stress exposure. Previous reports indicate that stress-induced decreases in CB_1R activation by AEA contribute to the stimulation of the HPA axis stress response (Hill et al., 2009). Moreover, stress exposure consistently results in a reduction of AEA content in multiple corticolimbic structures, but is most robustly detected in the amygdala (Dubreucq et al., 2012; Hill et al., 2008a; Hill et al., 2013; Hill et al., 2010b; Hill et al., 2009; Patel et al., 2005b), with magnitude of the amygdalar AEA reduction negatively correlated with circulating CORT levels (Hill et al., 2009). Adolescent amygdalar AEA was not reduced with stress exposure, indicating some immaturity in eCB regulation of the HPA axis stress response. It is possible that this observation is related to the protracted nature of the adolescent acute stress response or accelerated recovery to non-stress levels from repeated restraint exposure (Romeo et al., 2006); however, these possibilities seem unlikely given that the adult literature implicates a role for 2-AG, rather than AEA, in the termination of the neuroendocrine stress response.

In conjunction with the lack of AEA response to stress exposure, adolescent rats also exhibited generally lower corticolimbic AEA content and elevated FAAH enzyme activity across the amygdala, hippocampus, hypothalamus and PFC, relative to adult rats. This supports previous findings from Chapter 2 that basal corticolimbic AEA content at 45 days of age is significantly lower than at 70 days of age (Lee et al., 2013). It is conceivable that lower basal AEA levels (and elevated FAAH activity) in adolescent rats, particularly in the amygdala, functionally translate to lower AEA tone in adolescent rats and thus, a reduced ability to gate HPA axis activity. However, age-dependent differences in corticolimbic AEA content did not translate to elevations in basal or stress-induced increases in ACTH or CORT levels that would be expected from differences in ability to constrain HPA axis activity. Therefore, it seems more

likely that the normative fluctuations in adolescent corticolimbic AEA content and FAAH activity and the lack of stress-induced reduction in amygdalar AEA reflect some degree of instability or immaturity of eCB regulation over the HPA axis in light of relatively stable corticolimbic eCB signaling and HPA axis stress responsivity profiles associated with adulthood.

The available literature indicates AEA constrains HPA axis activity primarily via the CB_1R (Hill and Patel, 2013). Developmentally, CB_1R expression increases from early life (e.g., PND 10 and 25), plateauing just before pubertal onset (PND 30-35), then declines to adult levels (PND 70) in limbic structures (e.g. hippocampus, amygdala and hypothalamus; de Fonseca et al., 1993) and the PFC (Heng et al., 2011). In Experiment 1, adolescent rats were 45 days old on the final day of repeated restraint, leading to the possibility that normative declines in CB_1R expression could mediate age-dependent responses to repeated restraint stress. To further support this idea, electrophysiological study of prepubertal rats (PND 21-30) subjected to 5 consecutive days of restraint stress revealed a transient loss of depolarization-induced suppression of inhibition (DSI), a relatively consistent real time assay of activity dependent eCB production (Kreitzer and Regehr, 2001), in parvocellular neuroendocrine cells of the PVN (Wamsteeker et al., 2010a; Wamsteeker et al., 2010b). These effects were only observed in the prepubertal, but not adult rats exposed to repeated restraint (Wamsteeker et al., 2010a). The loss in eCB signaling (i.e., reduced DSI) was found to be a result of a CORT-mediated downregulation in presynaptic CB_1Rs (Wamsteeker et al., 2010b). Taken together with previous studies, age-dependent differences in AEA-CB_1R signaling, particularly in the amygdala, appear at least partially responsible for the observed age-dependent HPA axis stress responsivity profiles.

However, we found that adolescent and adult dorsal and ventral hippocampal GR and MR protein levels were remarkably similar between ages and neither area responded to stress exposure, despite robust age-related differences in eCB signaling and the hormonal HPA axis stress response. These findings are generally consistent with previous work demonstrating no

age-related differences in hippocampal GR and MR expression (Dziedzic et al., 2014; Romeo et al., 2008; Vazquez, 1998). However, the current findings failed to detect a stress-induced decline in GR expression, which has been previously reported in the rat (Isgor et al., 2004; Liberzon et al., 1999; Nishimura et al., 2004; Paskitti et al., 2000; Romeo et al., 2008; Tritos et al., 1999). Human and non-human primate studies have reported no differences in GR mRNA between adolescence and adulthood, but slight decreases in MR mRNA have been observed (Perlman et al., 2007; Pryce, 2008).

Romeo and colleagues (2008) have reported a significant decline in GR mRNA in the dorsal hippocampus and dentate gyrus of the ventral hippocampus 45 min following an acute 30 min restraint stress session. Given that in the current study, tissue was collected immediately following the onset of the acute or repeated restraint stress session, it is possible that this time point was too early to be able to detect any stress-induced changes in GR and/or MR protein levels. Similarly, there was no effect of repeated restraint stress exposure on hippocampal GR protein levels in adult and adolescent rats, which is consistent with previous findings (Romeo et al., 2008). This may be due to the fact that 30 min restraint is a relatively mild stressor (Romeo et al., 2008). Moreover, repeated restraint stress-induced alterations in MR and GR protein levels are known to be region-dependent and vary as a function of the repeated restraint paradigm employed (Gądek-Michalska et al., 2013). Lastly, although potential differences in functionality of these receptors remain to be determined, the accumulating evidence suggests that any pubertal-related change in negative feedback of the HPA axis is not dependent on genomic changes in GR and/or MR expression (Eiland and Romeo, 2013).

Lastly, our neuroendocrine findings are in line with the work of several others, with adolescent rats demonstrating divergent HPA axis stress responsivity from adult rats (e.g. CORT and ACTH; Doremus-Fitzwater et al., 2009; Romeo et al., 2006; Romeo et al., 2004a; Romeo et al., 2004b; Vázquez and Akil, 1993) independent of differences in circulating testosterone levels

(Romeo et al., 2006; Romeo et al., 2004a). Furthermore, it is well documented that stress exposure can modulate hypothalamic-pituitary-gonadal axis activity to alter age of pubertal onset (Grachev et al., 2013) by altering circulating gonadal hormone levels and ultimately modifying the developmental trajectory of the adolescent brain. However, 10 consecutive days of 30 min restraint exposure did not affect male pubertal onset in the current study, indicating that the observed age-dependent neuroendocrine and eCB signaling profiles are probably autonomous from age of pubertal onset and its related rise in testosterone levels.

3.5.2 Experiment 2

The results of Experiment 1 replicate and extend previous studies regarding the regulation of cannabinoid CB_1Rs by stress in the adult brain. Consistent with previous reports (Hill et al., 2008b; Hill et al., 2005b; Reich et al., 2009) exposure to repeated stress in adult rats resulted in an immediate decrease in CB_1R binding in the hippocampus, increase in CB_1R binding in the PFC and no effect in the amygdala. Interestingly, we found that adolescent rats exposed to repeated stress exhibited a similar immediate increase in CB_1R binding in the PFC, increase in CB_1R binding in the amygdala but not the hippocampus. These data demonstrate that some of the effects of repeated stress on CB_1R densities are age-specific. This finding is not without precedent, though. For example, it has previously been shown that repeated stress can desensitize CB_1Rs on GABAergic and glutamatergic terminals in the PVN of adolescent (Wamsteeker et al., 2010b), but not adult rats (Wamsteeker et al., 2010a). Further, repeated stress exposure does not alter CB_1R signaling on GABAergic terminals in the hippocampus of adolescents (Wamsteeker et al., 2010b) but is desensitizing in adults (Hu et al., 2011), which is consistent with the current findings. Outside of stress-induced regulation of CB_1Rs, there have been a wide array of differences between adolescent and adult animals in the manner in which they respond to repeated stress, such as habituation of the HPA axis (Barha et al., 2011;

Doremus-Fitzwater et al., 2009; current data; Romeo et al., 2006). As such, these data reinforce the concept that adolescents and adults exhibit differential adaptive responses to chronic stress.

Interestingly, regardless of the age of stress exposure (and in conjunction with observations that adolescent rats did not exhibit a downregulation of hippocampal CB_1Rs when examined immediately following repeated stress exposure), all animals with a history of repeated stress exposure were found to have increased hippocampal CB_1R densities following a 40-day recovery period. One interpretation of this receptor upregulation is that it is a compensatory response to the stress-induced downregulation (observed in the immediate repeated stress adult rats). However, this cannot account for why the adolescent repeated stress animals, which did not exhibit immediate downregulation, similarly showed an upregulation of CB_1R density following a 40-day recovery period. Counter-regulatory responses to chronic stress, sometimes even in the absence of an initial change, following a delayed recovery are quite common. For example, in the PFC, an increase in neuropeptide Y levels was found following a delayed recovery, but not immediately after chronic stress (McGuire et al., 2011). Additionally, within the PFC it has been found that chronic stress produces dendritic retraction in layer V neurons; however, during a recovery phase, there is a compensatory growth of dendritic arbors and spines in proximal regions of the neuron that do not exhibit retraction (Goldwater et al., 2009). Thus, the emergence of delayed changes from chronic stress is not unprecedented; however, their role has yet to be established as adaptive or maladaptive to the recovery response. Hippocampal CB_1Rs are known to promote neuroplastic phenomena, such as long-term potentiation (Carlson et al., 2002), growth factor secretion (Khaspekov et al., 2004) and neurogenesis (Aguado et al., 2005; Jin et al., 2004), all of which are compromised following chronic stress (McEwen, 2008). Taken together, the upregulation of CB_1Rs in the hippocampus may serve as a mechanism to facilitate the reinstatement of neuroplasticity and aid in the recovery from stress.

The ability of repeated stress exposure to immediately increase CB_1R density in the PFC, regardless of age, is consistent with several recent reports (Hill et al., 2008b; Zoppi et al., 2011). This response is believed to represent an adaptive response that helps to constrain the effects of stress on excitotoxicity, dendritic retraction and inflammation within the PFC (Hill et al., 2011a; Zoppi et al., 2011) as well as maintain active coping responses to stress (McLaughlin et al., 2013). This hypothesis is consistent with data indicating that activation of CB_1Rs in the PFC produces anxiolytic and antidepressant-like effects and promotes recovery from stress (Bambico et al., 2007; Hill et al., 2011b; Lafourcade et al., 2011; McLaughlin et al., 2012; Rubino et al., 2008a; Rubino et al., 2008b). Thus, this presumably adaptive response to chronic stress appears to be present regardless of age of exposure.

Repeated stress during adolescence produced a sustained downregulation of CB_1Rs in the PFC in adulthood. This response is not seen in adult rodents that were exposed to repeated restraint stress and given a recovery period, indicating that this residual effect is specific to stress exposure during adolescence. There is substantial evidence that the PFC undergoes dramatic neuronal reorganization and maturation during adolescence, rendering it especially vulnerable to long-term neural changes (Crews et al., 2007; Spear, 2000). For example, rodents exposed to repeated stress during adolescence have been shown to exhibit reduced synaptic densities in the PFC in adulthood (Leussis et al., 2008) and humans exposed to abusive stress during adolescence have been found to exhibit a significant decrease in cortical gray matter volume in adulthood (Andersen et al., 2008). Concurrent with this, there are many reports of enduring effects of stress during adolescence on emotional behaviour and impaired recovery from stress (Andersen and Teicher, 2008; Isgor et al., 2004; McCormick et al., 2008; Wright et al., 2008), both of which are associated with integrity of the PFC (Etkin et al., 2011; Radley, 2012). We have recently demonstrated that impairments in adult CB_1R signaling can reduce dendritic arbors within the PFC (Hill et al., 2011a), suggesting that the enduring effects of adolescent stress on

prefrontal cortical CB_1R density could contribute to sustained effects on neuronal architecture. Furthermore, as described above, adult CB_1R signaling in the PFC can regulate mood and anxiety (Bambico et al., 2007; Hill et al., 2011b; Lafourcade et al., 2011; McLaughlin et al., 2012; Rubino et al., 2008a; Rubino et al., 2008b). Following this idea, compromised CB_1R signaling in the PFC due to adolescent stress exposure may represent a substrate underlying the association of stress during this developmental window and the emergence of neurobehavioural changes in adulthood.

Outside of the amygdala, where CB_1R density was found to increase with advancing age, we did not detect any significant age related differences. This finding is in contrast to other reports of ontogenetic changes in CB_1R levels throughout corticolimbic structures (see Table 1.1; Lee and Gorzalka, 2012). Within the PFC (Heng et al., 2011), however, the change in CB_1R levels between immature and mature animals appears to be more pronounced at younger ages (PND 25 and below) than with animals at the age investigated in this study (PND 45). Further, CB_1R densities within the PFC have been shown to fluctuate throughout stages of adolescence (Ellgren et al., 2008; Heng et al., 2011). Taken together, it is possible that by only investigating CB_1R densities at a single time point during adolescence, developmental differences in receptor levels throughout corticolimbic structures were missed.

The findings of Experiments 1 and 2 indicate that stress exposure elicits age-dependent eCB and HPA axis responses and these differences at least partially contribute to the basis by which stress exposure exerts differential age-dependent immediate and long-term effects on the brain. In Experiment 1, corticolimbic eCB system maturation, particularly in the amygdala, appears key to achieving adult regulation of the HPA axis given that: 1.) there was a general lack of amygdalar 2-AG elevation in adolescents exposed to repeated restraint which also corresponded with an inability to exhibit HPA axis habituation and 2.) significantly lower corticolimbic AEA content and higher FAAH activity as well as a lack of stress-induced AEA

reduction in the amygdala suggest an immature eCB regulatory tone on the HPA axis in adolescent compared to adult rats. The results of Experiment 2 demonstrate that the age of stress exposure can dramatically alter both the immediate and enduring effects of repeated stress on corticolimbic CB_1R binding site densities. In particular, the ability of stress during adolescence to produce a residual and sustained downregulation of PFC CB_1Rs reinforces the hypothesis that the PFC is particularly vulnerable to stress during adolescence, due to its ongoing development and maturation. Furthermore, this persistent downregulation also provides a putative substrate linking adolescent stress to long-lasting increases in vulnerability to an array of psychiatric disorders, such as mood and anxiety disorders. Future research will continue to examine the full ontogenetic development of the corticolimbic eCB system, how this trajectory can be influenced by stress exposure at various developmental windows and mechanisms of age-dependent differences in the HPA axis to better understand the role of the eCB system as a mediator between development and stress responsivity.

4. Disruption of Adolescent Endocannabinoid Signaling Modulates Neuroendocrine and Behavioural Responses to Stress in Adulthood

4.1 Introduction

Accumulating evidence suggests that the adolescent brain undergoes region-dependent maturation in key higher-order processing structures, such as the prefrontal cortex (PFC; e.g. Casey and Jones, 2010; Gogtay et al., 2004). However, one consequence of the highly plastic nature of these maturational neural processes is that developing neural circuits are vulnerable to perturbation, such as stress and activation of the hypothalamic-pituitary-adrenal (HPA) axis. Preclinical studies suggest that adolescent stress exposure elicits detrimental long-term behavioural and neural alterations, depending on stressor type, duration and age of exposure. Indeed, adolescent stress exposure is known to alter HPA axis stress responsivity (Ver Hoeve et al., 2013), decrease social interaction (McCormick et al., 2015), increase anxiety and depressive-like behaviour (McCormick et al., 2013), facilitate amphetamine- and ethanol-stimulated locomotion, preference and self-administration (Burke and Miczek, 2014), alter reproductive behaviour (McCormick et al., 2013) and impair working memory (Novick et al., 2013) during adulthood. Similarly, adolescent stress exposure leads to long-term neural changes including modifications to adult hippocampal electrophysiological and morphological profiles (Buwalda et al., 2005), reduced hippocampal neurogenesis in female, but not in male rats (Barha et al., 2011), and reduced prefrontal cortical synaptic density in male rats (Leussis et al., 2008).

The endocannabinoid (eCB) system, which interacts with the psychoactive constituents of cannabis, regulates a variety of processes in adulthood, including HPA axis stress responsivity (Hill and Tasker, 2012), emotional behaviour (Kathuria et al., 2003; McLaughlin and Gobbi, 2011), reproductive behaviour (Gorzalka et al., 2010) and drug dependence (Parolaro et al., 2007). The eCB system consists of two G-protein coupled receptors, CB_1 and CB_2. The CB_1 receptor (CB_1R) is widely expressed in the brain on neuron terminals (Herkenham et al., 1991),

where it plays a regulatory role in synaptic function and plasticity (Castillo et al., 2012). CB_2 receptors are predominantly found in peripheral tissues, although recent reports have documented CB_2 receptors in the central nervous system (Van Sickle et al., 2005). The eCB system also possesses two major endogenous ligands, *N*-arachidonylethanolamine (anandamide; AEA) and 2-arachidonoylglycerol (2-AG), which are synthesized "on demand" and act as retrograde messengers to regulate the release of other neurotransmitters (Ahn et al., 2008). These actions contribute to both short and long term forms of synaptic plasticity (Mackie, 2006). Lastly, eCB signaling is highly regulated by metabolic enzymes such as fatty acid amide hydrolase (FAAH) and monoacylglyceride lipase which hydrolyze AEA and 2-AG, respectively.

Moderate to high densities of CB_1Rs are found in adult male rodent corticolimbic structures regulating stress responsivity and emotional behaviour (Herkenham et al., 1991) and evidence suggests that pharmacological antagonism and genetic deletion of CB_1Rs emulate a phenotype similar to that of chronic stress exposure, including heightened emotionality (Haller et al., 2002), hypothalamic-pituitary-adrenal (HPA) axis dysregulation (Barna et al., 2004; Hill et al., 2011a) and shorter, less complex pyramidal neurons in the medial PFC (Hill et al., 2011a). Accumulating evidence also indicates that CB_1R activation plays a regulatory role in neural development (Díaz-Alonso et al., 2012). Previous work on embryonic neuronal development and maturation has revealed that the CB_1R, AEA and 2-AG jointly regulate neural progenitor proliferation, guiding axonal growth, and differentiation (Díaz-Alonso et al., 2012).

Moreover, CB_1R expression peaks in male and female rodents across several corticolimbic brain regions with the onset of adolescence (PND 30) and then gradually decreases to adult levels by PND 70 (de Fonseca et al., 1993; Heng et al., 2011). Simultaneously, AEA content and FAAH activity dynamically fluctuate throughout the adolescent period to adulthood (Ellgren et al., 2008; Lee et al., 2013; Rubino et al., 2014; Wenger et al., 2002). Thus, perturbations to these normative fluctuations in eCB signaling may compromise

neurodevelopmental processes resulting in sustained changes in the adult brain (Rubino et al., 2014). The results of several studies indicate that adolescent cannabinoid administration alters the developmental trajectory of corticolimbic structures, provoking profound, permanent, and often deleterious effects on cognition, emotionality and stress responsivity (Lee and Gorzalka, 2012; Rubino and Parolaro, 2008). Recently, these effects have been attributed to the possibility that excess exposure to cannabinoids during adolescence impairs ongoing eCB signaling through a desensitization of the system, and it is this disruption that mediates the adverse effects of excess exposure to cannabinoids during adolescence (Rubino et al., 2014).

However, the long-term consequences of adolescent CB_1R antagonism in the development of adult HPA axis stress responsivity and emotional behaviour remain unclear. To this end, we sought to unmask the contribution of normative adolescent CB_1R signaling in emotional behaviour and HPA axis stress responsivity via pharmacological disruption of adolescent eCB signaling. In Chapters 2 and 3, we established differential patterns of: 1.) basal corticolimbic AEA and 2-AG content during the adolescent period (Chapter 2), and 2.) stress-induced changes in corticolimbic AEA and 2-AG content which also varied as a function of age (Chapter 3, Experiment 1). Furthermore, in Experiment 2 of Chapter 3, repeated restraint stress exposure was found to cause age- and region-specific alterations in CB_1R binding. Following up on these findings, in Chapter 4, we sought to determine the long-term consequences of pharmacological blockade of CB_1Rs (which is known to produce similar behavioural and physiological effects as stress exposure) during adolescence on adult eCB signaling, HPA axis stress responsivity and emotional behaviour.

4.2 Materials & Methods

4.2.1 Subjects

Male Sprague-Dawley rats (Charles River, QC, Canada) were received on post-natal day (PND) 21. Animals were pair housed in clear polyurethane cages (48 x 27 x 20 cm) filled with cedar bedding and paper towels for enrichment. A 12h/12h light/dark cycle (lights on at 9 am) was maintained and access to food (Purina at Chow) and water was provided *ad libitum.* All protocols were carried out in accordance with the Canadian Council for Animal Care guidelines and were approved by the Animal Care Committee at the University of British Columbia.

4.2.2 Drug administration

The CB_1R inverse agonist/antagonist, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (Cayman Chemicals, Pittsburgh, PA; AM-251), was dissolved in a vehicle of 10% DMSO, 10% Tween-80 and 80% 0.9% saline. The rats were randomly assigned to AM-251 and vehicle (VEH) groups, in which they received daily intraperitoneal (IP) injections of AM-251 (5 mg/kg) or an equivalent volume of vehicle from PND 35-45. Animals were left undisturbed until PND 75, at which time they were randomly assigned to three different cohorts for analyses of: 1) emotional behaviour, 2) HPA axis stress reactivity and 3) eCB content and expression throughout corticolimbic structures (all n = 10; see Figure 4.1).

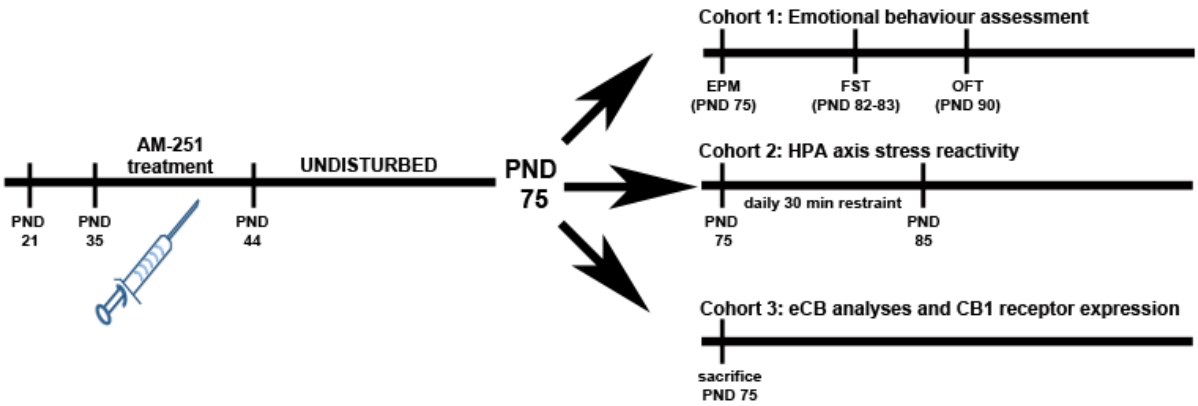

Figure 4.1. Schematic diagram of the adolescent treatment period and subsequent biochemical, behavioural and neuroendocrine measures.

4.2.3 Emotional behaviour assessment

Adult male rats were exposed to three behavioural tests (same sequence) separated by 1 week each. Rats in this cohort were exposed to the elevated plus maze on PND 75, the forced swim test on PNDs 82 – 83 and the open field test on PND 90. Experimenters blind to treatment condition assessed all animal behaviour by video.

4.2.3.1 Elevated plus maze

The elevated plus maze was employed in this study to assess the impact of adolescent AM-251 treatment on adult anxiety behaviour. It was constructed of painted black wood and consisted of 2 open arms (50 x 12.5 cm) and 2 enclosed arms (50 x 12.5 x 50 cm) that all extended from a common middle platform. The open, closed and middle platform were elevated 60 cm above the ground with 4 pedestals. Animals were placed in the middle platform and their behaviour recorded for 5 min by an overhead camera approximately 2 m above the apparatus. A 10% acetic acid solution was used to clean the apparatus between tests. Number of entries into open and closed arms, time spent in open and closed arms, head dips and stretch attends were assessed by video by experimenters blind to treatment condition.

4.2.3.2 Forced swim test

The forced swim test was used to assess the impact of adolescent AM-251 treatment on stress coping behaviour. Cylindrical glass containers (diameter 35 cm x height 66 cm) were filled with water to a height of approximately 40 cm and maintained at 24±1°C. After each animal completed a session, the water was replaced. Animals were subjected to two swim sessions on PND 82 and 83. The first swim session was a 15-min pre-exposure session, followed by a 5-min test session 24 hr later. During the second session, the duration of time spent engaging in passive (immobility) or active (swimming and struggling) coping behaviours was videotaped and scored by an experimenter blind to treatment conditions. Immobility was defined

as the animal remaining stationary with minimal movement of limbs to remain afloat. Swimming was defined as paddling movement of the animal's forelimbs and/or hind limbs in the water. Struggling was defined as quick, forceful movement of the forelimbs that broke the surface of the water.

4.2.3.3 Open field test

Animals were observed in an open field arena as an additional measure of anxiety behaviour and general locomotor activity. The open field arena used in this study was 120 x 120 x 30 cm. The arena was painted white and divided into 16 equal quadrants (30 x 30 cm) by black lines. For testing, animals were placed in the central quadrant and left to explore for 5 min. Animals were monitored and recorded by an overhead camera (Hitachi 2500A) approximately 2 m above the open field box. A 10% acetic acid solution was used to clean the apparatus between tests. The mean number of entries and time spent in peripheral and central quadrants as well as the mean speed and total distance travelled were measured.

4.2.4 HPA axis stress reactivity

All restraint stress sessions were conducted in a separate testing room, and occurred in the first third of the light cycle, during the daily nadir of HPA axis activity. During these sessions, rats were put into a polystyrene tube (diameter 6 cm, length 20 cm) with breathing holes for 10 consecutive days for 30 min each day, as described previously (Lee and Hill, 2013). On days 1 and 10 of the restraint stress paradigm, tail blood samples were taken at 0, 30, 60 and 90 min following restraint onset. Blood samples were collected in chilled EDTA- (3.75 mg/100 μL blood) and Aprotinin- (0.053 mg/100 μL blood) treated microcentrifuge tubes. All samples were centrifuged at 3000 x *g* for 15 min, after which plasma was removed and stored at -80 ºC until analyses.

4.2.4.1 Corticosterone (CORT) and adrenocorticotropic hormone (ACTH) radioimmunoassays

Total CORT (5 uL) and ACTH (50 uL) concentrations were measured using commercial ImmuChem RIA kits (MP Biomedicals Canada), using [^{125}I] as a tracer. For CORT, plasma samples were diluted 1:100 and 1:200 for basal and stress conditions, respectively, to render hormone detection within the linear part of the standard curve (3.125-1000 ng/mL). Plasma ACTH levels were determined according to manufacturer instructions, from a standard curve derived from 8 standards ranging from 0 – 1000 pg/mL. The inter- and intra-assay coefficients of variation were all under 8% for each assay.

4.2.5 Endocannabinoid system content and expression

4.2.5.1 CB_1 receptor radioligand binding assay

Membrane preparation and CB_1R radioligand binding assays were conducted as previously described (Lee and Hill, 2013). Membranes were collected from isolated brain regions by homogenization of frozen tissue in 20 volumes of TME buffer (50 mM Tris HCl, pH 7.4; 1 mM EDTA and 3 mM $MgCl_2$) and centrifuged at 18,000 *x g* for 20 min. The resulting pellet was re-suspended in 20 volumes of TME buffer. Protein concentrations were determined using a commercially available BCA kit (Pierce Biotechnology, Rockville, IL).

CB_1R agonist binding parameters were determined by radioligand binding using a Multiscreen Filtration System with Durapore 1.2-µM filters in 96 well filter plates (Millipore, Bedford, MA). 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 in triplicate to wells containing 0.1, 0.25, 0.5, 1.0, 1.5 or 2.5 nM [^{3}H]CP 55,940 (American Radiochemicals, St. Louis, MO), a cannabinoid CB_1R agonist, and incubated for 1 hr at room temperature. Ten µM AM-251 (Tocris Biosciences, Minneapolis, MN) was

used to determine non-specific binding. B_{max} (maximal binding site density) and K_D (binding affinity) values were determined by nonlinear curve fitting of specific binding data to the single site binding equation using GraphPad Prism (San Diego, CA).

4.2.5.2 Endocannabinoid extraction and analysis

Animals were sacrificed by rapid decapitation during the first third of the light cycle and brain tissue was collected for eCB content analysis. PFC, hippocampus, amygdala, and hypothalamus were dissected within 5 min, as previously described (Hill et al., 2010a; Lee et al., 2013), frozen on dry ice, and stored at -80° C until analysis. Brain regions underwent a lipid extraction process as previously described (Patel et al., 2003). Tissue samples were weighed and placed in borosilicate glass culture tubes containing 2 mL of acetonitrile with 84 pmol of [$^2H_8$] AEA and 186 pmol of [$^2H_8$] 2-AG for extraction. These samples were homogenized with a glass rod and sonicated for 30 min, incubated overnight at -20°C to precipitate proteins, then centrifuged at 1500 g for 5 min to remove particulates. Supernatants were removed to a new glass culture tube and evaporated to dryness under N_2 gas, re-suspended in 300 µL of methanol to recapture any lipids adhering to the tube and re-dried again under N_2 gas. The final lipid extracts were suspended in 20 µL of methanol and stored at -80°C until analysis. AEA and 2-AG contents within lipid extracts were determined using isotope-dilution, liquid chromatography-mass spectrometry as described earlier (Patel et al., 2005a).

4.2.6 Statistics

In cohort 1 and 2, all measurements were analyzed using independent t-tests. In cohort 3, HPA axis stress responsivity was analyzed using repeated measures analyses of variance (ANOVA) with blood sampling time point serving as the within-subjects variable and adolescent drug treatment as the between-subjects factor. To gauge relative CORT and ACTH responses to acute and repeated restraint as a function of adolescent drug treatment, total CORT and ACTH

responses (area under the curve) in the AM-251 group were calculated as a percentage of total CORT and ACTH response of the vehicle group for day 1 and 10 of restraint exposure. These values were analyzed using a repeated measures ANOVA with day of restraint (day 1 vs. 10) as the within subjects factor and adolescent drug treatment as the between subjects factor. Bonferroni corrections were used for all post-hoc comparisons. All statistical procedures were set at $\alpha = 0.05$.

4.3 Results

4.3.1 Adolescent AM-251 treatment increases stress coping behaviour and risk assessment behaviour

In the forced swim test, adolescent AM-251 treatment produced a significant decrease in time spent immobile ($t(19)=2.56$, $p=0.02$; Figure 4.2) coupled to an increase in time spent struggling ($t(19)=3.07$, $p=0.01$) relative to VEH. There was no significant effect of AM-251 treatment on swimming behaviour ($t(19)=1.22$, $p =0.24$).

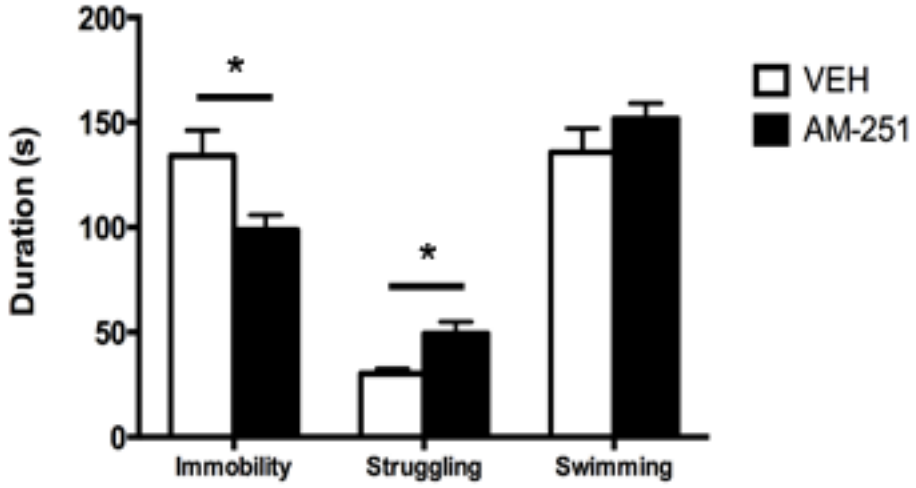

Figure 4.2. Adolescent AM-251 treatment increases stress-coping behaviour in the forced swim test in adult male rats. Mean ± SEM time (s) spent immobile, struggling and swimming in the forced swim test. * indicates statistically significant differences at $p<0.05$.

Independent t-test analyses revealed no significant effect of adolescent AM-251 treatment on time spent in the open ($t(19)=1.29$, $p=0.22$) and closed arms ($t(19)=1.23$, $p=0.24$) of the elevated plus maze; however, the AM-251 treated rats spent significantly less time in the middle zone than vehicle-treated rats ($t(19)=2.50$, $p=0.02$; Figure 4.3A). There was no significant effect of AM-251 treatment on the percentage of entries into the open arms ($t(19)=0.20$, $p=0.84$; Figure 4.3C), closed arms ($t(19)=1.88$, $p=0.07$) and middle zone ($t(19) = 0.08$, $p = 0.94$) of the elevated plus maze. Relative to VEH, AM-251 treatment induced significantly greater numbers of stretch attends ($t(19)=2.89$, $p=0.009$; Figure 4.3B) but not head dips ($t(19)=1.25$, $p=0.23$; Figure 4.3D).

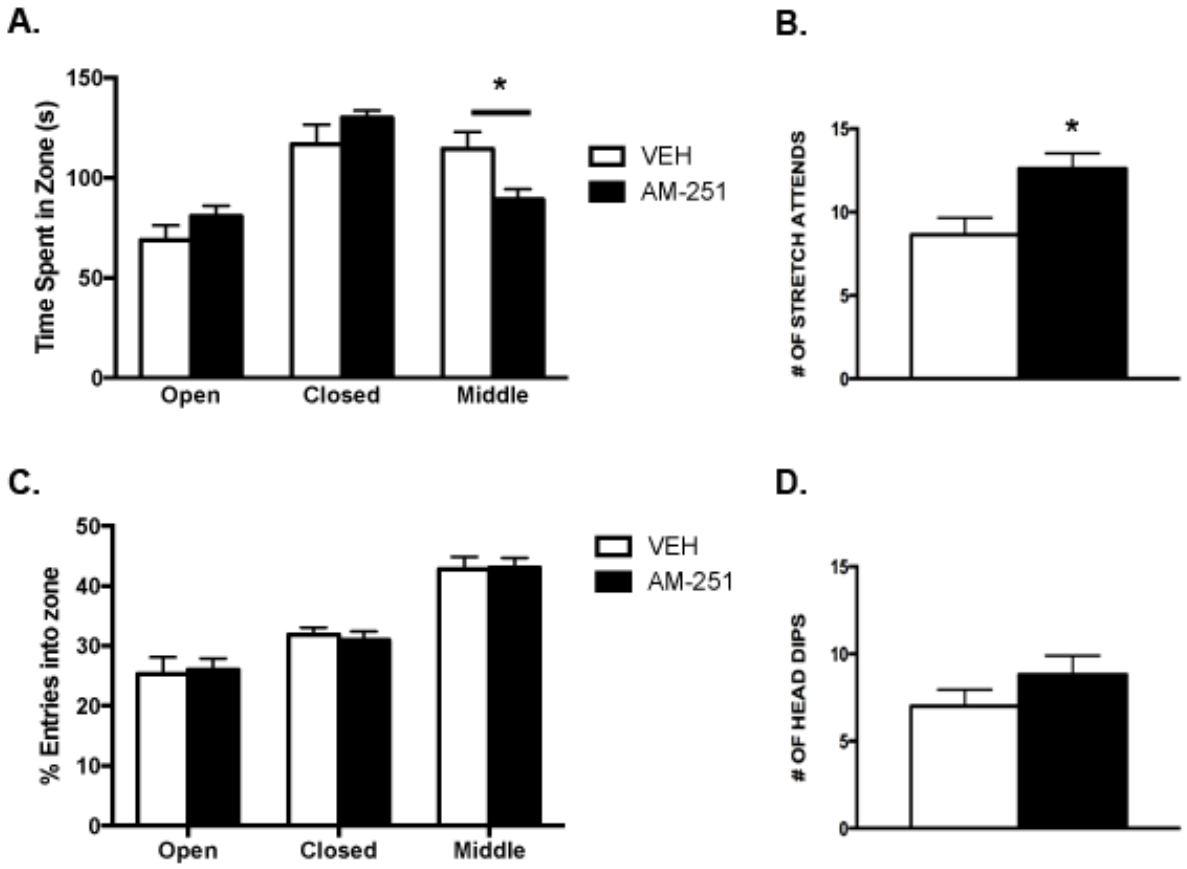

Figure 4.3. Adolescent AM-251 treatment increases risk assessment behaviour in adult male rats. (A) Mean ± SEM time (s) spent in the open, closed and middle zones of the elevated plus maze. (B) Mean ± SEM number of stretch attends. (C) Mean ± SEM percentage of entries into the open, closed and middle zones of the elevated plus maze. (D) Mean ± SEM number of head dips. * indicates statistically significant differences at $p < 0.05$ and t indicates a trend, $p < 0.07$.

No significant locomotor differences on mean speed ($t(19)=0.39$, $p=0.70$; Table 4.1) and total distance travelled ($t(19)=0.41$, $p=0.69$; Table 4.1) were detected between groups. There was also no significant effect of the AM-251 treatment on time spent in the centre ($t(19)= 0.01$, $p=0.99$; Table 4.1) and peripheral zones ($t(19)=0.12$, $p=0.91$; Table 4.1) of the open field.

Table 4.1. Mean speed and distance travelled in the open field test by adult male rats treated with AM-251 or vehicle (VEH) during adolescence.

	Vehicle (VEH)	AM-251
Speed (m/s)	0.09 ± 0.01	0.09 ± 0.01
Distance travelled (m)	26.3 ± 3.2	28.0 ± 2.8
Time spent in centre (s)	29.96 ± 6.86	29.90 ± 5.51
Time spent in periphery (s)	270.70 ± 7.14	269.6 ± 5.56

There was no significant effect of adolescent AM-251 treatment on any of the variables measured in the open field. For all treatment conditions, n = 10. Data are presented as means ± SEM

4.3.2 Adolescent AM-251 treatment accelerates adult HPA axis habituation

There was a significant interaction between time point following restraint onset and adolescent AM-251 treatment on adult ACTH levels ($F(9,78) = 5.33$, $p = 0.0001$; Figure **4.**4A, 4.4B). Post-hoc comparisons revealed no significant differences between groups at 0, 60 and 90 min following restraint onset on day 1 and 10; however, both AM-251 ($p = 0.001$) and VEH ($p = 0.0001$) treated groups exhibited lower ACTH levels 30 min following restraint onset on day 10 relative to day 1, indicating that habituation of the HPA axis had occurred. However, there were no significant differences in peak stress-induced ACTH levels (30 min) between VEH and AM-251 on day 1 ($p = 0.59$) and day 10 ($p = 0.24$) of restraint exposure. Lastly, there was no significant interaction between day of restraint and adolescent treatment (area under the curve; $F(1, 12) = 1.22$, $p = 0.29$; Figure 4C), nor any significant main effects of day ($F(1, 12) = 1.22$, $p = 0.29$) and treatment ($F(1, 12) = 1.01$, $p = 0.33$) on the magnitude of the ACTH response to restraint exposure.

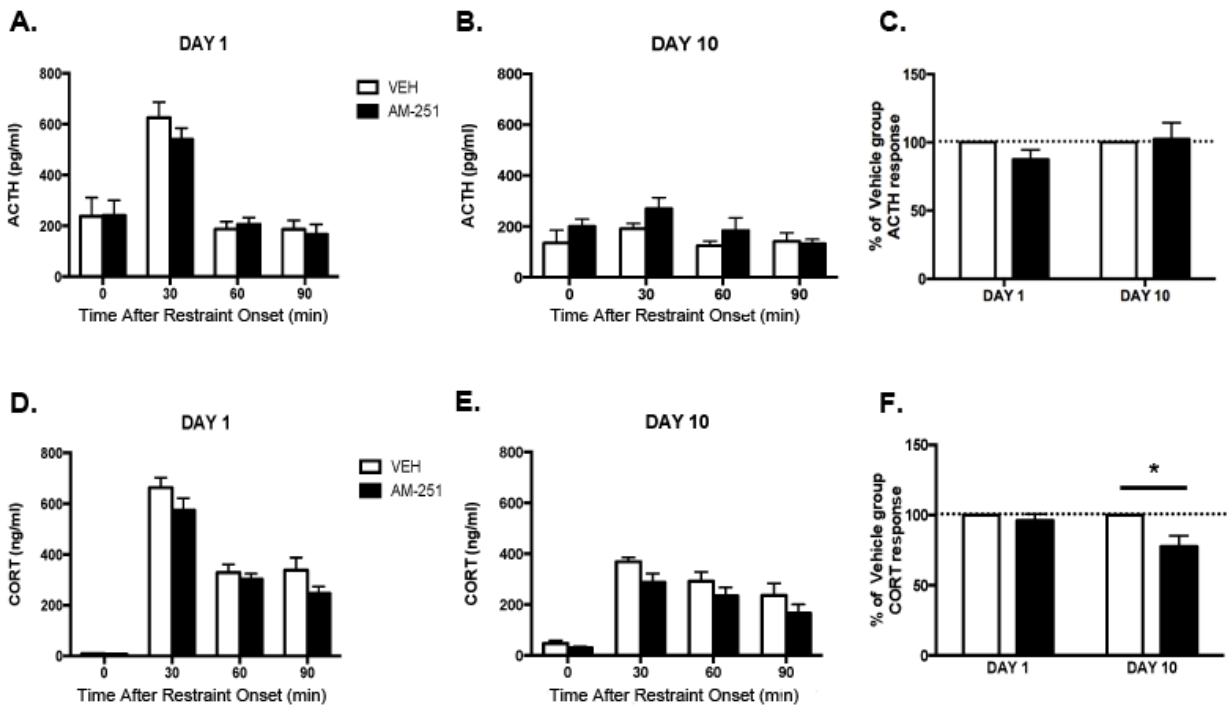

Figure 4.4. Adolescent AM-251 treatment accelerated corticosterone (CORT) stress habituation. (A) Mean ± SEM adrenocorticotropic hormone (ACTH) levels in response to repeated restraint stress exposure on Day 1. (B) Mean ± SEM ACTH levels in response to repeated restraint stress exposure on Day 10. (C) Relative adrenocorticotropic (ACTH) responses as percentage of the vehicle groups to acute and repeated restraint stress. (A) Mean ± SEM CORT levels in response to repeated restraint stress exposure on Day 1. (B) Mean ± SEM CORT levels in response to repeated restraint stress exposure on Day 10. (C) Relative CORT responses as percentage of the vehicle groups to acute and repeated restraint stress. * indicates statistically significant differences at $p < 0.05$.

There was also no significant interaction between time points following stress and adolescent AM-251 treatment on adult CORT levels ($F(9,81) = 1.61$, $p = 0.13$). However, there was a significant main effect of adolescent treatment ($F(3,27) = 3.62$, $p = 0.03$) such that AM-251 treated rats exhibited less of an increase in CORT levels in response to stress than exhibited in the VEH group (Figure 4.4D, 4.4E). There was also a significant main effect of time after restraint, indicating habituation of stress-induced CORT increases had occurred for both groups ($F(3, 81) = 141.4$, $p < 0.0001$). There was a significant day of restraint by adolescent treatment interaction on the magnitude of the CORT response ($F(1,14)=4.71$, $p=0.04$; Figure 4.4F) such that relative to the vehicle group, the AM-251-treated animals exhibited significantly lower CORT responses to restraint on day 10 ($p=0.005$), but not day 1 ($p>0.05$).

4.3.3 Adolescent AM-251 treatment modestly alters endocannabinoid system content and expression

Adolescent CB_1R antagonism produced a significant increase in adult prefrontal cortical CB_1R expression (B_{max}; $t(8) = 2.91$, $p = 0.01$; see Table 4.2 for all regions) with no significant effects in the amygdala ($t(7) = 0.51$, $p = 0.63$), hypothalamus ($t(8) = 1.06$, $p =0.31$) and hippocampus ($t(8) = 0.82$, $p =0.44$). Adolescent AM-251 treatment did not significantly affect binding affinity (K_D) within the amgydala ($t(8) = 1.19$, $p = 0.27$; see Table 1 for all regions), hippocampus ($t(8) = 1.78$, $p= 0.11$), hypothalamus ($t(8) = 0.09$, $p = 0.93$), or PFC ($t(8) = 1.19$, $p = 0.27$).

Table 4.2. Mean adult CB_1 receptor binding density (B_{max}) and affinity (K_D) within corticolimbic structures of adult male rats treated with AM-251 or vehicle (VEH) during adolescence.

	B_{max} (pmol/mg protein)		K_D (nM)	
	Vehicle (VEH)	AM-251	Vehicle (VEH)	AM-251
Amygdala	0.68 + 0.13	0.63 +0.06	0.54 + 0.09	0.64 + 0.07
Hippocampus	1.04 + 0.06	1.12 +0.07	0.89 + 0.05	1.03 +0.06
Hypothalamus	0.24 + 0.03	0.19 + 0.02	0.44 + 0.05	0.44 + 0.04
Prefrontal Cortex	0.81 + 0.05	1.00 + 0.05*	0.71 + 0.08	0.84 + 0.07

There was no significant effect of adolescent AM-251 treatment on CB1 receptor expression and binding affinity except in the prefrontal cortex. * denotes significant effect of AM-251 in the prefrontal cortex ($p \leq 0.05$). For all treatment conditions, n = 10. Data are presented as means $\pm$ SEM.

Independent t-test analyses revealed a significant increase in adult hypothalamic AEA levels ($t(8)=2.51$, $p=0.03$; see Figure 4.5 for all regions) and significant decrease in amygdalar AEA ($t(8)=2.72$, $p=0.03$). However, there was no effect of AM-251 treatment on adult AEA levels in the PFC ($t(8)=1.12$, $p=0.30$) nor the hippocampus ($t(8)=0.14$, $p=0.89$). Adolescent AM-251 treatment had no significant effect on adult 2-AG levels in the amygdala ($t(8)=0.35$, $p=0.74$; see Figure 4.5B for all regions), hypothalamus ($t(8)=0.97$, $p=0.36$), PFC ($t(8)=1.17$, $p=0.30$) and hippocampus ($t(8)=0.28$, $p=0.79$).

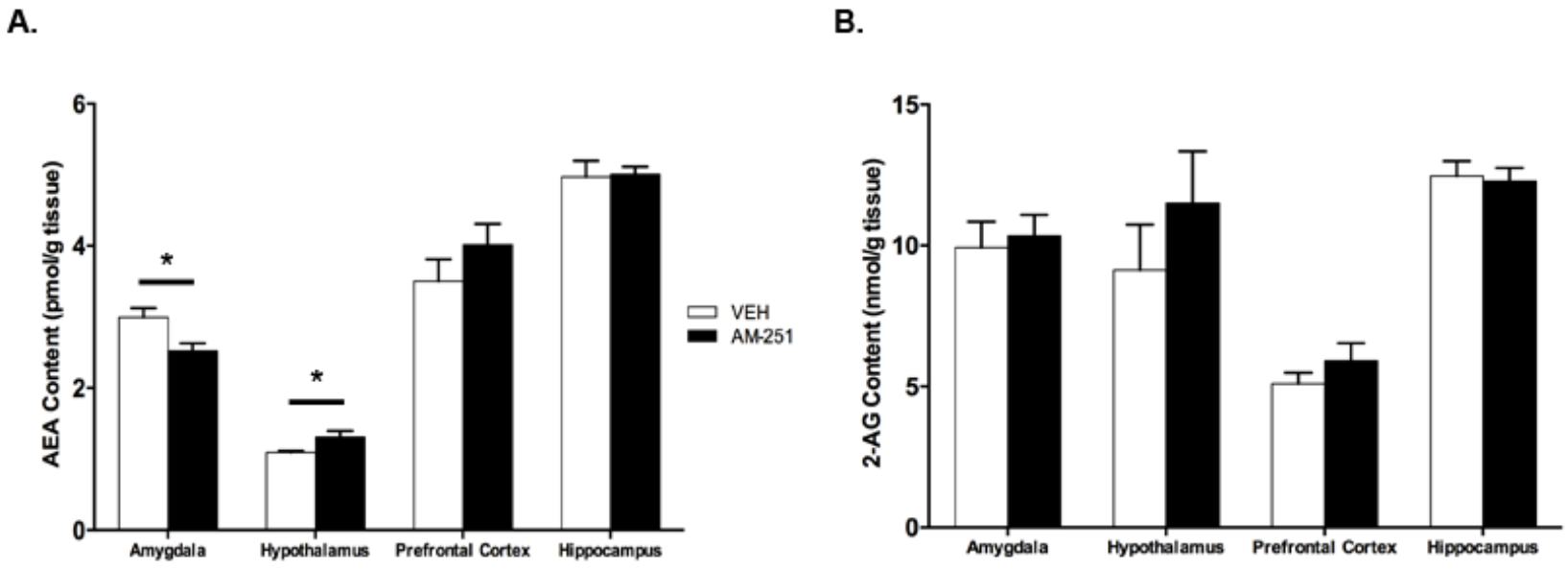

Figure 4.5. Adolescent AM-251 treatment alters anandamide (AEA) content in the amygdala and hypothalamus of adult male rats. (A) Mean ± SEM basal AEA and (B) 2-arachidonoylglycerol (2-AG) levels throughout adult corticolimbic brain structures following adolescent AM-251 treatment. * indicates statistically significant differences at $p<0.05$.

4.4 Discussion

Results of the current study were contrary to our hypothesis that disruption of adolescent eCB signaling would produce profound, negative long-term effects on emotional behaviour and stress responsivity. Instead, the current findings indicate adolescent CB_1R antagonism has moderate but significant organizational effects in altering the developmental trajectory of the adult corticolimbic eCB system, emotional behaviour, and stress responsivity. Sustained CB_1R blockade during adolescence increased active stress-coping behaviour in the forced swim test, while moderately increasing risk assessment behaviour in the elevated plus maze, but not the open field test, in adulthood. Additionally, the adolescent AM-251 treatment had no effect on the acute HPA axis stress response, but did accelerate habituation of CORT secretion in response to repeated restraint stress. Lastly, disruption of adolescent eCB signaling decreased amygdalar AEA, increased hypothalamic AEA content, and increased CB_1R expression in the PFC of adult rats.

Our findings were somewhat surprising in light of studies reporting that genetic or pharmacological deletion of CB_1Rs in adult male rodents generally results in a phenotype similar to that induced by chronic stress exposure. It is possible that corticolimbic eCB signaling during the proposed window of vulnerability to eCB-sensitive disruptions (P35-45) is more resilient to perturbations than hypothesized. However, numerous preclinical and clinical studies indicate that adolescent CB_1R agonist treatment in female rodents or heavy cannabis consumption in adolescent humans produces enduring impairments in emotional behaviour and psychotic-like symptoms (Bossong and Niesink, 2010; Rubino and Parolaro, 2008), whereas adult CB_1R agonism results in increased stress coping behaviour in male rats (McLaughlin et al., 2007) and adult cannabis use is often associated with stress reduction and elevated mood in humans (Hill and McEwen, 2009). In light of what appears to be age-dependent effects of CB_1R agonism, the

current findings are in line with age-dependent effects of CB_1R antagonism. There are also known sex differences in cannabinoid action with female rodents being more affected than males by cannabinoid exposure on behavioural measures of emotionality (Fattore and Fratta, 2010). This sex difference is consistent with studies investigating the long term consequences of escalating doses of THC exposure during adolescence, with females showing reductions in stress-coping behaviour and increases in anxiety behaviour whereas males exhibit greater cognitive deficits (Rubino et al., 2008b). Thus, it is feasible that adolescent AM-251 treatment may evoke more profound neural and emotional behaviour effects in female than male rats.

Nevertheless, sustained CB_1R blockade during adolescence increased active stress coping behaviour in the forced swim test in adulthood. These findings are consistent with previous work demonstrating that adolescent CB_1R agonist treatment decreases active coping behaviour in adult female rats (Rubino et al., 2008b), suggesting that pharmacological modulation of eCB signaling during adolescence affects stress coping behaviours in adulthood. Given that CB_1R signaling in the PFC has been shown to increase active stress coping in the forced swim test (Bambico et al., 2007), it is possible that the observed increase in PFC CB_1R expression could result in this behavioural alteration. Furthermore, the increase in active coping behaviour was specifically associated with an increase in struggling behaviour, suggesting a noradrenergic mechanism, given the importance of norepinephrine signaling in regulating struggling behaviour (Detke et al., 1995). Interestingly, CB_1R is co-localized with noradrenergic terminals in the PFC where CB_1R activation enhances norepinephrine release (Oropeza et al., 2007; Page et al., 2008). Given this relationship, the possibility exists that disruption of adolescent eCB signaling modulates the interaction between frontocortical eCB and noradrenergic systems to enhance active coping responses to stress in adulthood.

Unlike the effects on stress coping behaviour, disruption of adolescent eCB signaling did not alter gross measures of anxiety behaviour (i.e., entries or time spent in the open and closed

arms). AM-251-treated rats, however, did exhibit greater risk assessment behaviour as revealed by an increase in stretch attends to the open arms and less time spent in the middle zone relative to those in the VEH group. These findings are generally consistent with previously reported effects of adult male AM-251 treatment on anxiety behaviour, although chronic adult AM-251 treatment has been previously reported to produce a negative correlation between PFC CB_1R expression and time spent in the center of the elevated plus maze and stretch attend postures (Tambaro et al., 2013). Despite this, increased risk assessment following disruption of adolescent eCB signaling in the current study is reminiscent of the effects of stress during adolescence, which has been shown to increase risk assessment behaviour and corticolimbic noradrenergic activity in male rats (Bingham et al., 2011; Watt et al., 2009). One interpretation of these data is that disruption of adolescent eCB signaling could generate a state of stress that produces similar effects on the adult brain as exposure to stress itself during adolescence. Given that stress exposure during adolescent windows has been shown to impair the eCB system (Lee and Hill, 2013; Wamsteeker et al., 2010b) and the parallels between the effects of stress and disruption of eCB signaling during adolescence, it is possible that stress-induced impairments in eCB signaling during adolescence contribute to the sustained effects of stress on the adult brain.

With respect to adult regulation of anxiety, there is evidence that AEA signaling can reduce indices of stress and anxiety through signaling actions within the amygdala of male rodents (Bedse et al., 2014; Gray et al., in press; Hill et al., 2009). Given that the adolescent treatment did not dramatically modulate anxiety behaviour, it is possible the modest reduction in amygdalar AEA is contributing to moderately higher levels of anxiety behaviour observed in the AM-251 group. Furthermore, since the AM-251 group exhibited an increase in PFC CB_1R expression and lower amygdalar AEA content, it is possible adolescent CB_1R blockade induced dysregulation between PFC-amygdala connectivity that elicited greater risk-assessment

behaviour and a tendency towards more time spent in the closed arms of the elevated plus maze. However, further research assessing this possibility is required.

Analyses of basal and acute stress-induced induced increases in ACTH and CORT levels did not yield any significant treatment differences between AM-251 and VEH. However, AM-251 treated animals exhibited a lower CORT response with repeated restraint exposure, despite no treatment differences on the first day of restraint exposure. These findings indicate that adolescent CB_1R antagonism accelerated HPA axis stress habituation. Interestingly, the accelerated recovery from repeated restraint stress is somewhat reminiscent of the immature phenotype in which male prepubertal animals exhibit an accelerated return to non-stress levels when exposed to repeated restraint relative to adults (Romeo et al., 2006); however, the expression of HPA axis habituation remained intact in the adult AM-251 treated animals (in the current study), unlike prepubertal male rats. Furthermore, while corticolimbic eCB content was not measured during exposure to restraint stress, the increase in basal hypothalamic AEA following adolescent AM-251 treatment may promote greater negative feedback on HPA axis habituation, thus resulting in a lower CORT response to repeated restraint stress. This hypothesis is consistent with the role of CB_1R signaling in the PVN to facilitate negative feedback of the HPA axis (Evanson et al., 2010). Similarly, PFC CB_1R signaling has been shown to be important for feedback inhibition of stress-induced CORT secretion (Hill et al., 2011b), thus the increase in PFC CB_1R binding following adolescent AM-251 treatment may also be contributing to this effect.

Chronic administration of a CB_1R agonist during adolescence downregulates multiple components of the adult eCB system in male and female rats (Rubino et al., 2008b). Our data indicate adolescent blockade of CB_1Rs also results in alterations in the adult eCB system, suggesting that the establishment of steady-state eCB signaling in adulthood is sensitive to alterations in adolescent eCB signaling. These data parallel similar studies that have examined

the effects of sustained CB_1R blockade in adulthood. Chronic treatment with AM-251 in adult male mice induced region-dependent differences in CB_1R expression, with an upregulation in the PFC and striatum and downregulation in the hippocampus and midbrain (Tambaro et al., 2013). Together, the findings suggest that sustained CB_1R blockade in both adolescence and adulthood elicit an upregulation of PFC CB_1R expression.

Lastly, the current study revealed that adolescent AM-251 treatment significantly reduced and increased AEA in the adult amygdala and hypothalamus, respectively. In contrast, chronic low dose AM-251 treatment from late-adolescence to young adulthood (PND 56-77) failed to alter AEA and 2-AG content in corticolimbic structures of group housed adult male rats (Zamberletti et al., 2012a). This would suggest that there is differential sensitivity in the age through which alterations in adolescent eCB signaling alter expression of the adult eCB system. Importantly, the current results also suggest adolescent (PND 35-45) AM-251 treatment induced long lasting alterations in adult eCB signaling within the amygdala and PFC, which are key structures regulating emotionality and stress responsivity and are among the last neural structures to reach adult structural and functional maturity (Gogtay et al., 2004).

The present findings suggest that adolescent CB_1R activation plays a moderate, yet significant organizational role in the normative development of the corticolimbic eCB system, emotionality and stress responsivity. Results of the current study and previous work indicate adults may be more susceptible to the deleterious effects of sustained CB_1R blockade given that the neural and behavioural alterations reported in the current study are relatively modest, and are generally associated with what are typically viewed as more adaptive responses to stress (i.e., increased active stress coping behaviours and accelerated habituation to repeated stress exposure).

5. General Discussion

5.1 Overview

The overarching hypothesis of this collective body of work is that adolescent corticolimbic eCB signaling is an important mediator of age-dependent differences in HPA activity and contributes to the development of adult HPA axis stress responsivity and emotional behaviour. The results of the study described in chapter 2 indicate robust temporal-specific fluctuations in corticolimbic NAE content across the adolescent period, whereas 2-AG does not exhibit this pattern. However, in the stress-induced state outlined in chapter 3, age-dependent differences in both endogenous ligands emerge and are consistent with age-dependent differences in neuroendocrine data. Lastly, the studies described in chapter 4 suggest that adolescent CB_1R blockade has moderate, but significant effects on the development of adult emotional behaviour and stress responsivity.

5.2 Temporal-specific Changes in Corticolimbic Anandamide Content, but not 2-AG, Across the Adolescent Period

The eCB system critically regulates adult HPA axis stress responsivity (Hill and Tasker, 2012) and neural development (Maccarrone et al., 2014), making it a prime candidate as an important mediator of age-related differences in stress reactivity. However, there are relatively few reports outlining the developmental trajectory of eCB signaling throughout the adolescent period, particularly within limbic circuits regulating stress and reward processing. Therefore, the goal of the study in Chapter 2 was to describe the developmental trajectory of corticolimbic eCBs across the adolescent period. NAE (AEA, PEA and OEA) content was shown to exhibit the same general temporal specific pattern across all four corticolimbic structures, increasing from PND 25 to 35, then decreasing by PND 45 and finally increasing again to adult levels by PND 75. These changes were mirrored by corticolimbic FAAH activity, indicating that

fluctuations in NAE content were mediated by alterations in FAAH activity, although the possibility remains that differences in biosynthesis could also be contributing to this pattern. In stark contrast, 2-AG did not appear to exhibit any significant patterns within any of the corticolimbic structures, suggesting limited regulatory influence of 2-AG signaling on adolescent neurodevelopment.

Contrary to our hypothesis, corticolimbic AEA content fluctuated across adolescent development whereas statistically significant changes in 2-AG content were not detected. The current findings are surprising given that in the adult male rodent, AEA is known to exert a tonic signal that constrains HPA axis activity whereas 2-AG functions as a phasic signal in response to stress exposure that serves to terminate the stress response. While future research is necessary to determine the functional relevance of the fluctuating nature of adolescent corticolimbic AEA content and relatively stable 2-AG content, it is possible that a developmental shift in functionality of AEA and 2-AG may occur between adolescence and adulthood. Similar developmental shifts have been reported for some neurotransmitters. For example, a developmental shift from excitatory to inhibitory actions of GABA occurs in neonatal brain regions such as the hippocampus, neocortex and hypothalamus (Ben-Ari et al., 2007). Relatively high chloride concentrations in immature neurons of these brain regions result in excitatory actions of GABA; however, chloride concentrations progressively decrease with age (about PND 6-10), forcing a shift from excitatory to inhibitory actions of GABA (Ben-Ari et al., 2007). It has been suggested that this shift from excitatory to inhibitory actions allow the developing brain to equilibrate glutamatergic and GABAergic drives while avoiding excessive excitation or inhibition if one initially predominates over the other (Ben-Ari et al., 2007).

It is also possible that fluctuations in AEA/CB_1R signaling (e.g., on PND 35) underlie adolescent behaviours that are regulated by corticolimbic circuits, such as relatively low anxiety, high responsivity to rewards and reduced inhibitory control (Casey and Jones, 2010). Given that

adolescence represents a period when this behavioural profile is maximal, and is coincident with fluctuations in AEA/CB_1R activity, it is tempting to speculate that these temporal changes in FAAH activity and AEA content represent a neural substrate of the adolescent phenotype. Moreover, these age-dependent patterns of NAE content and FAAH activity could contribute to alterations in stress sensitivity, emotionality and executive function, which also vary during this developmental period.

The temporal specificity of corticolimbic FAAH activity and AEA content changes may also provide insight for a window within adolescence that is particularly sensitive to modulation of normative eCB signaling and eCB-sensitive disruptors such as stress or exogenous cannabinoid exposure (see Figure 5.1). Coincident with decreasing corticolimbic AEA content from PNDs 35-45, CB_1R expression is reported to decline to adult levels (de Fonseca et al., 1993; Heng et al., 2011), thus the expected net result would be an overall decrease in corticolimbic AEA-CB_1R signaling during this period. Several studies investigating the long-term impact of adolescent cannabinoid consumption, particularly studies conducted by the Parolaro laboratory, provide support for this idea given that THC administered between PNDs 35-47 has deleterious effects including reductions in stress-coping behaviour, increased anxiety behaviour, impaired cognition (Rubino et al., 2008b), reductions in hippocampal neuroplasticity (Rubino et al., 2009b), disruption of normative maturational processes within the PFC (Rubino et al., 2014), as well as permanent changes to adult eCB signaling in reward processing structures such as the nucleus accumbens and the corticolimbic stress circuit (Rubino et al., 2008b). The authors postulate that exogenous cannabinoid administration yields particularly long-lasting detrimental effects on cognition, behaviour and the brain by disrupting normative eCB signaling through a desensitization mechanism, which then disrupts eCB-regulated maturational processes, such as synaptic pruning (Rubino et al., 2014). Moreover, findings of a recently published study corroborate the idea that PNDs 35-45 represent a window of vulnerability by demonstrating that

cannabinoid treatment specifically during PNDs 35-40 and 40-45 but not in late adolescence (PND 50-55) or adulthood (PND 75-80), disrupts adult GABAergic transmission via a CB_1R-dependent mechanism in the PFC (Cass et al., 2014).

Furthermore, AEA-CB_1R signaling in the basolateral nucleus of the amygdala serves as a gatekeeper of basal and stress-induced HPA axis activity in the adult (Hill and Tasker, 2012). If the same is true of developing animals, this suggests that PNDs 35-45 represents a period in which AEA-CB_1R tone is relatively low (in relation to adults), leaving the adolescent more vulnerable to even slight perturbations in eCB tone and thus, contributing to greater vulnerability to external factors such as stress exposure; however, the functional consequences of this transient reduction in AEA-CB_1R tone on HPA axis activity remain to be determined. We attempted to gain some insight regarding this possibility in Experiment 2 in Chapter 3 and in Chapter 4.

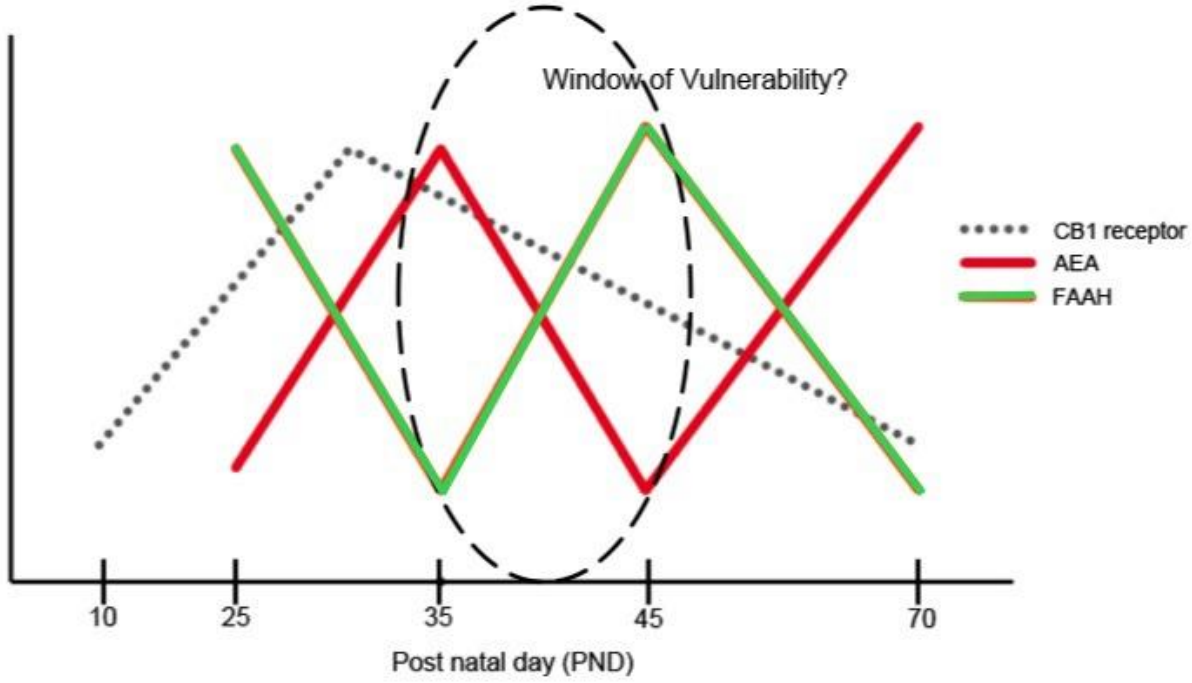

Figure 5.1. Schematic diagram outlining the developmental patterns of anandamide (AEA) and CB_1 receptor (CB_1R) expression. AEA-CB_1R signaling generally declines from post-natal days 35-45, potentially revealing a window of vulnerability to disruptions in eCB signaling or stress exposure.

5.3 Age-dependent Profiles of Corticolimbic Endocannabinoid Signaling and HPA Axis Stress Responsivity and the Long-term Effects of Repeated Stress Exposure

Age-dependent differences in HPA axis stress responsivity to acute and repeated restraint stress appear independent of the pubertal rise in gonadal hormones (Romeo et al., 2006; Romeo et al., 2004a; Romeo et al., 2004b) and corticolimbic GR mRNA and protein expression (Dziedzic et al., 2014; Romeo et al., 2008), leaving a gap in our understanding of the developmental mechanisms subserving these neuroendocrine differences. Moreover, repeated or chronic stress exposure during developmental periods such as adolescence appear to have longer lasting and often more detrimental consequences to cognition, behaviour and the brain than in adulthood (e.g., Koenig et al., 2011). Because the adolescent is undergoing numerous developmental changes, it is believed that stress exposure can disrupt the normative maturational trajectory of the brain to effect long-term behavioural and neural alterations that do not occur in fully mature adults (Andersen and Teicher, 2008; Lee et al., 2014a; McCormick et al., 2010).

Specific patterns of basal corticolimbic eCB system content and activity develop throughout the adolescent period as described in Chapter 2, suggesting that the eCB system acts as an integral mediator between development and HPA axis stress responsivity. Indeed, in Chapter 3, we replicate previous reports of age-dependent differences on the neuroendocrine stress response and extend this work by identifying several age-dependent differences in eCB signaling corresponding to these age-specific HPA axis stress responsivity profiles. Moreover, the adolescent rodent was challenged by restraint stress exposure, which revealed a common, perhaps basic feature of eCB regulation of the HPA axis stress response across the life span. Repeated restraint stress exposure provoked a general corticolimbic AEA decline in adolescent and adult rats (although not in the amygdala of the adolescent rats) within the current data and maternal separation stress elicits a similar AEA reduction in the neonatal hippocampus (Marco et

al., 2013). Collectively, this suggests that eCB signaling is a consistent regulator of the HPA axis and stress-induced reductions in AEA content in the hippocampus, hypothalamus and PFC may subserve a basic mechanism by which an organism engages the neuroendocrine stress response at any age, regardless of stressor-type.

In contrast, the adolescent amygdala, unlike the hippocampus, hypothalamus and PFC, did not exhibit a reduction in AEA content with exposure to acute and repeated stress. A reduction in corticolimbic AEA has been reliably shown to occur as an initiating step in launching the HPA axis stress response (Hill et al., 2010b; Hill et al., 2009; Hill et al., 2005b; Patel et al., 2003; Patel et al., 2004). As discussed earlier, there is evidence that AEA tone within the BLA is critical to maintaining basal stress hormone levels and gating activation of HPA axis activity (Hill and Tasker, 2012); however, the adolescent rats were capable of engaging the HPA axis in response to stress, indicating that amygdalar AEA regulation of HPA axis stress responsivity is more complex. To further complicate the clarity of these findings, we observed a significant age effect in which adolescent corticolimbic AEA content was reduced and FAAH activity was elevated compared to that observed in adult rats. These findings confirm the same observations documented in Chapter 2 and this is in line with the idea that AEA content and FAAH activity fluctuate throughout the adolescent period. It is equally possible that these fluctuations are indicative of a general instability in adolescent eCB signaling and therefore, immature eCB regulation of HPA axis stress responsivity.

The amygdala emerged as a particularly important neural locus that must undergo maturational development during the adolescent period in order for the HPA axis to express habituation to homotypic stress exposure. Adult rats display blunted HPA axis activity with repeated exposure to restraint stress and research from our laboratory has previously demonstrated that amygdalar 2-AG recruitment and CB_1R activation in the BLA is essential for the expression of HPA axis habituation (Hill et al., 2010b). In the current work, we found that

adolescent rats lack the ability to express blunted HPA axis activity following 10 days of 30 min restraint stress sessions and correspondingly, lack the ability to mobilize 2-AG in the amygdala. With repeated exposure to the same stressor in adulthood, 2-AG is recruited to suppress glutamatergic inputs to the BLA, thus reducing the outflow of the amygdala and resulting in a general dampening of activity in the PVN and other corticolimbic structures, concluding in a blunted HPA axis response to the previously stress-inducing stimulus (Hill et al., 2010b; Hill and Tasker, 2012). In this framework, the inability of the adolescent amygdala to mobilize 2-AG would prevent HPA axis stress habituation via basolateral input to corticolimbic structures.

Analysis of dorsal and ventral hippocampal MR and GR protein levels revealed no significant effects of age or stress exposure, suggesting that hippocampal MR and GR expression do not appear to contribute to any age-related differences in glucocorticoid negative feedback processes. However, stress exposure also failed to affect receptor protein levels in adults, which prevented further insight into the existing conflicting literature. It is likely that some of these conflicting reports are a result of varying stressor types, procedures (e.g., whole cell versus translocation) and the time at which tissue is harvested following stress exposure, indicating a need for a more comprehensive and systematic study of the effects of stress exposure on central MR and GR expression.

In Chapter 2, it was proposed that the fluctuating nature of AEA and FAAH indicates a potential window of vulnerability to perturbation such as stress exposure (i.e., PNDs 35-45). Therefore, in the studies described in Chapter 3, adolescent stress exposure was aimed at this specific period and the recovery of the corticolimbic eCB system (CB_1R binding density) to this stress exposure was assessed both immediately (24 hrs) and in the long-term (40 days). The results of Experiment 2 indicate that some effects of the repeated stress paradigm (immediate upregulation of CB_1 receptors in the PFC and enduring increase in CB_1 receptors in the

hippocampus following recovery) occurred regardless of age of exposure, while other effects were age-specific.

One striking age-specific effect was observed in the PFC. Consistent with the existing literature (see review, McLaughlin et al., 2014), stress exposure immediately upregulated adolescent and adult CB_1Rs in the PFC; however, in the long-term, adult PFC CB_1Rs recovered to normative adult levels whereas the adolescent (now adult) rats exhibited a downregulation in CB_1Rs. Compromised PFC CB_1R signaling is associated with shorter, less complex dendritic morphology of PFC pyramidal neurons (Hill et al., 2011a), suggesting that downregulation of PFC CB_1Rs induced by adolescent stress exposure could also trigger corresponding long-term alterations to neuronal architecture and eventually act upon PFC-regulated cognitive and behavioural processes. These alterations may be especially relevant to the modulation of adult mood and anxiety given that preclinical studies have shown that CB_1R signaling in the PFC regulates emotional behaviour (Bambico et al., 2007; Hill et al., 2011b; Lafourcade et al., 2011; McLaughlin et al., 2012; Rubino et al., 2008a; Rubino et al., 2008b). Together, the current findings and previous research suggest a possible neural substrate by which stress exposure in adolescence can exert more long lasting consequences than in adulthood.

5.4 Adolescent CB_1 Receptor Disruption Moderately Alters Adult Emotional Behaviour, Stress Responsivity and Endocannabinoid Signaling

Building on the idea that early to mid-adolescence represents a window of vulnerability to perturbations (such as stress exposure or exogenous cannabinoid treatment) and preclinical research indicating that adult pharmacological antagonism and genetic deletion of CB_1Rs emulate a phenotype similar to that of chronic stress exposure in adults, including heightened emotionality (Haller et al., 2002), hypothalamic-pituitary-adrenal (HPA) axis dysregulation (Barna et al., 2004; Hill et al., 2011a), and shorter, less complex pyramidal neurons in the medial

PFC (Hill et al., 2011a; Lee et al., 2014c), the studies in Chapter 4 examined the long-term effects of CB_1R blockade during PNDs 35-45 on the development of adult emotional behaviour, HPA axis stress responsivity and corticolimbic eCB system content and expression. We hypothesized that adolescent CB_1R signaling blockade would elicit more permanent and deleterious consequences than those previously reported in adults. However, adolescent CB_1R antagonism produced relatively moderate long-term effects on emotional behaviour, stress responsivity and corticolimbic eCB signaling. These findings indicate that the proposed window of vulnerability is more resistant to adolescent AM-251 treatment than originally theorized. However, separate pharmacological studies of adolescent and adult CB_1R agonism suggest that there are some age- and sex-dependent effects of this treatment on emotional behaviour and stress responsivity. Thus, it is reasonable to suggest that sustained CB_1R disruption would also result in age- and sex-specific effects on adult eCB signaling, stress responsivity and emotional behaviour.

Behaviourally, adolescent CB_1R disruption resulted in increased stress coping behaviour in the forced swim test and was associated with a basal upregulation of PFC CB_1Rs, which is consistent with previous work in the adult demonstrating that CB_1R signaling in the PFC increases active stress coping in the forced swim test (Bambico et al., 2007). Moreover, this treatment regime accelerated HPA axis stress habituation in adulthood, which was supported by observations of a small but significant increase in basal hypothalamic AEA and upregulation of CB_1Rs in the PFC. As CB_1R signaling in the PVN facilitates negative feedback of the HPA axis (Evanson et al., 2010) and PFC CB_1R signaling is central for feedback inhibition of stress-induced CORT secretion (Hill et al., 2011b), these corticolimbic eCB alterations following adolescent AM-251 treatment could promote greater negative feedback on HPA axis habituation. Interestingly, the accelerated nature of the HPA axis stress response on the final day of repeated restraint stress exposure is reminiscent of the adolescent stress response to repeated restraint

(Romeo et al., 2006), although adolescents did not exhibit the ability to habituate (Romeo et al., 2006) whereas the animals in the current study did display HPA axis habituation.

In sum, the results indicate that adults may be more susceptible to the deleterious effects of sustained CB_1R blockade given that the neural and behavioural alterations reported in the current study are relatively modest, and are generally associated with what are typically viewed as more adaptive responses to stress (i.e., increased active stress coping behaviours and accelerated habituation to repeated stress exposure). While future research examining the mechanisms of action is necessary, it is possible that the adaptive aspects of this treatment could have some therapeutic value. For example, pharmacological CB_1R antagonism in male rats reverses isolation rearing-induced deficits in cognition and sensorimotor gating and normalizes eCB, dopaminergic and glutamatergic imbalances associated with this model of psychotic-like symptoms in male and female rats (Guidali et al., 2011; Zamberletti et al., 2012a; Zamberletti et al., 2012b).

5.5 Strengths & Limitations

A major strength of the current body of work is that it provides a foundational biochemical characterization of adolescent corticolimbic eCB signaling, both under basal (Chapter 2) and stress-induced (Chapter 3) conditions. Building on these findings, research in this dissertation examined adolescent eCB signaling following exposure to stress or cannabinoid exposure, and assessed the long-term impact of these perturbations on the adult eCB system, emotionality and stress responsivity (Chapters 3 and 4). Based on this series of research studies, one can conclude that adolescent corticolimbic eCB signaling is an important mediator between development and HPA axis stress responsivity. Therefore, it is reasonable to suggest that disruptions to normative adolescent eCB signaling stimulate alterations to neuronal architecture or other neurotransmitter systems, providing a neural mechanism by which perturbations such as

stress exposure can exert their long-term, often deleterious effects. Lastly, the data presented herein provide preliminary evidence that in the male rodent, the early- to mid-adolescent period (PNDs 35-45) could represent a window of particular vulnerability to the enduring consequences of stress exposure or alterations in eCB signaling. On a broader health level, by understanding how corticolimbic endocannabinoid signaling regulates both development and the HPA axis, we gain a more comprehensive understanding of the risks associated with cannabis consumption and stress exposure, and identify possible mechanisms by which these factors could disturb and produce their long-lasting effects.

However, there are some limitations to this work that deserve discussion. As a whole, the majority of the studies outlined in this dissertation are descriptive in nature, leaving the ability to investigate the functional role of the observed changes somewhat restricted. Our current understanding of how eCBs regulate adolescent development is still in its infancy and while these characterization studies reveal several common effects of stress exposure in adolescents and adults, other age-specific differences do emerge and indicate possible areas for future study. Furthermore, it is well known that CB_1Rs can be found on a number of different neuronal subtypes and neurotransmitters (Haring et al., 2012), which promotes greater diversity and complexity by which eCBs can regulate a variety of processes such as HPA axis stress responsivity. The biochemical profiles we have characterized preclude the ability to determine whether adolescent AM-251 treatment produced differences in CB_1R expression on specific neuronal populations within corticolimbic neural structures. For example, while adolescent AM-251 treatment enhanced adult struggling behaviour in the forced swim test, which is associated with noradrenergic activity (Detke et al., 1995) and CB_1R is co-localized with noradrenergic terminals in the PFC to enhance norepinephrine release (Oropeza et al., 2007; Page et al., 2008), the current studies did not directly measure whether adolescent treatment specifically altered noradrenergic neurotransmission.

Secondly, all the animals used in the current studies were obtained from a breeding facility in Charles River, QC, Canada. Shipping to our laboratory occurred on PND 21 in all cases; however, previous work does indicate that adolescent shipping stress (i.e., at 42 days of age) reduces behavioural responses to gonadal hormone treatment relative to mice shipped in adulthood. Furthermore, activated CORT levels were lower in male mice shipped during adolescence relative to mice shipped during adulthood (Laroche et al., 2009). Therefore, it is possible that the stress of the shipping could have had some impact on the neural and behavioural development of all the animals prior to the experiment. Future research should endeavor to use animals obtained by on-site breeding to avoid the likelihood of alterations induced by shipping stress exposure.

A third major limitation of this work is that all of the studies were conducted in male rats. It is currently unknown whether these findings are generalizable to females. A major sex difference in male and female adolescent rodent development is that pubertal onset in females occurs approximately on PND 30 whereas pubertal onset in males is generally considered around PND 40 (Spear, 2000). Therefore, the pubertal rise in gonadal hormones occurs at different ages, as does the influence of gonadal hormones on the developing brain (Sisk and Zehr, 2005). Furthermore, eCBs modulate the release of gonadal hormones (Gorzalka et al., 2010) and there are significant region-dependent sex differences in CB_1R density throughout adult corticolimbic structures (Castelli et al.; Riebe et al., 2010). These sex differences are further augmented by relatively recent work demonstrating that estradiol can suppress synaptic inhibition in hippocampal slices via an eCB-mediated mechanism involving a mobilization of AEA in female tissue, whereas this mechanism is absent in male tissue (Huang and Woolley, 2012). Lastly, there are several sex differences in the physiological and behavioural aspects of cannabinoid action with males exhibiting greater effects in food intake and energy homeostasis whereas cannabinoid action in females appear to produce greater effects on measures of emotionality,

analgesia, and motor activity (Fattore and Fratta, 2010). Sex differences in HPA axis stress responsivity likely reflect, at least in part, sex-specific differences in corticolimbic eCB signaling.

Previous reports indicate a rise in hypothalamic AEA approximately on PND 30 (Wenger et al., 2002) and fluctuating AEA content during later adolescence in the PFC of female rats (Rubino et al., 2014), which fits with the findings presented here. It is likely that female corticolimbic eCB signaling in response to stress exposure would be distinctive from the male profile outlined in Chapter 3 given that adolescent HPA axis stress responsivity does differ by sex. Romeo and colleagues (Romeo et al., 2004a; Romeo et al., 2004b) demonstrate that in response to acute restraint stress exposure, both male and female prepubertal rats exhibit a protracted ACTH and CORT response compared to adults; however, female rats, regardless of age, exhibit a higher peak CORT response (30 min following restraint onset) and faster recovery to basal levels than males (McCormick et al., 2010; Romeo, 2010b; Romeo et al., 2006). With repeated restraint stress exposure, adolescent male rats fail to exhibit a blunted CORT response (on the final day of restraint) relative to adult males (Doremus-Fitzwater et al., 2009; Romeo, 2010b; Romeo et al., 2004a). In contrast, adolescent female rats exhibit a more habituation-like CORT response following repeated exposure to a stressor (Doremus-Fitzwater et al., 2009; Romeo, 2010b). Therefore, given these sex- and age- specific differences in HPA axis stress responsivity, future research should endeavor to determine how corticolimbic eCB signaling varies in females.

5.6 Future Directions

Based on the limitations discussed in this body of work, future research should aim to determine the functional role of adolescent eCB system activity in males and females. Moreover, future research might consider whether there is a function for the unified, but temporal-specific

nature of AEA/FAAH fluctuations throughout the corticolimbic stress circuit during adolescence, or are they simply an indication of instability or immaturity of the circuit? Further research is also necessary to investigate whether the period of PNDs 35-45 are truly a window of vulnerability to stress exposure or eCB modulation. While we have proposed some reasons for the transient decline in AEA-CB_1R signaling during this period and have found some evidence for this in the current data and existing literature, these possibilities should be more rigorously tested.

Further studies should also aim to determine other mechanisms of action on HPA axis stress responsivity and emotionality by the eCB system. It is likely that the eCB system exerts at least some of its neural and behavioural effects via other neurotransmitter systems as the eCB system can inhibit glutamate, GABA, acetylcholine, dopamine, serotonin and norepinephrine release (Freund et al., 2003; Schlicker and Kathmann, 2001). For example, chronic administration of the highly potent cannabinoid agonist, HU-210, increases serotonin 5HT1A receptor binding and mRNA expression in the hippocampus of adult but not adolescent male rats, suggesting differential age-dependent effects on serotonergic activity (Zavitsanou et al., 2010), a neurotransmitter system also known to influence HPA axis function (e.g. Belay et al., 2011). Furthermore, chronic administration of cannabinoid agonists during adolescence has been shown to increase adult stress-coping behaviour, effects consistent with a dampening of serotonergic and enhancing of noradrenergic activity (Bambico et al., 2010).

The present body of work suggests a convincing regulatory role for the eCB system as a mediator between maturational stage and HPA axis stress responsivity, but also reveals several gaps in our understanding of adolescent development. Corticolimbic eCB signaling is an integral regulator of the adult HPA axis and the data presented in Chapters 2 and 3 provide evidence that corticolimbic eCB signaling also plays a role in regulating development of HPA axis stress responsivity and emotional behaviour throughout the rodent adolescent period. The data

presented in Chapters 3 and 4 indicate that, because of this ability to mediate developmental influences on the HPA axis, the developing eCB system, itself, can become a neural substrate of vulnerability to perturbations such as stress exposure. While the developing eCB system is capable of participation in the regulation of HPA axis functioning in the neonate (D'asti et al., 2010; Marco et al., 2013), it appears that full eCB regulation of the HPA axis stress response is not complete in adolescence. In support of this, the adolescent rat cannot launch a habituated neuroendocrine response to repeated restraint stress (Doremus-Fitzwater et al., 2009; Romeo, 2010b; Romeo et al., 2006; Romeo et al., 2004a; Romeo et al., 2004b) and this coincides with reduced eCB recruitment of the HPA axis (data presented in Chapter 3; Wamsteeker et al., 2010b). Further investigation of this relationship and mechanisms of action is warranted, particularly in the face of growing long-term mental health concerns over the risks associated with cannabis use or abuse during developmental windows of vulnerability and the increasing emergence of adolescent affective disorders.

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