ENDOCANNABINOID REGULATION OF ADOLESCENT DEVELOPMENT IN

MALE AND FEMALE RATS

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Abstract

The present thesis investigated the contributions of adolescent endocannabinoid signalling to brain and behaviour development in male and female rats. In chapter 2, daily administration of the CB1 antagonist AM251, alone or in tandem with a psychological stressor, increased social interactions, reduced dorsal hippocampal CB1 expression, and increased mPFC GAD67 expression in female rats 24-48 h after treatment, with no effects in males. In chapter 3, adolescent CB1 antagonism reduced anxiety in adult males, with no effects in females. Conversely, adolescent AM251 increased contextual fear in adult females, with no effects in males. In chapter 4, AM251 females spent more time initiating social interactions after a 5-day drug washout period than vehicle females, with no effects in males. To identify brain regions underlying the effects of AM251 on social behaviours, I repeated social interaction testing in vehicle and AM251 females and collected brains for immunohistochemical labelling of EGR-1 as a marker of neural activation in the CA1, CA2, and CA3 subfields of the dorsal hippocampus and the shell and core divisions of the nucleus accumbens (NAc). Consistent with my previous findings, AM251 females spent more time initiating social interactions and had greater EGR-1 cell counts in the NAc shell than vehicle females, with no group differences in the NAc core or in any of the hippocampal subfields investigated. EGR-1 cell counts in the dCA2 were negatively correlated with social interactions in vehicle and AM251 females. A positive correlation between NAc shell EGR-1 cell counts and social interactions was observed only in AM251 females. Regression analysis using drug treatment and EGR-1 cell counts in dCA2 and NAc shell resulted in a model with an adjusted $R^2$ of 0.90. Both drug treatment and EGR-1 cell counts in the dorsal CA2 emerged as unique predictors of
individual differences in social interaction, and drug and NAc shell EGR-1 cell counts interacted to significantly predict social interactions in AM251 females only. Together, these studies provide support for sex-specific contributions of endocannabinoid signalling to the development of brain and behaviour in adolescence in male and female rats.

**Keywords:** adolescent; endocannabinoids; CB1 receptor; stress; anxiety; social behaviours
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Abbreviations

2-AG, 2-arachidonylglycerol

ACS, adolescent confinement stress

AEA, N-arachidonylethanolamide

Akt, protein kinase B

AM251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide

ANOVA, analysis of variance

BSA, bovine serum albumin

BL, baseline

cAMP, cyclic adenosine monophosphate

CA1, Cornu Ammonus area 1

CA2, Cornu Ammonus area 2

CA3, Cornu Ammonus area 2

CB1, cannabinoid receptor type-1

CB2, cannabinoid receptor type-2

CORT, corticosterone

CP55,940, (-)-cis-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol

CYP2C6, cytochrome P450 subfamily 2 polypeptide 6

CYP2C11, cytochrome P450 subfamily 2 polypeptide 11

D1, dopamine receptor 1

D2, dopamine receptor 2
D4, dopamine receptor 4
DAB, 3,3’-diaminobenzidine
DAG, diacylglycerol
DAGL, diacylglycerol lipase
DH, dorsal hippocampus
DMSO, dimethyl sulfoxide
DSE, depolarization-induced suppression of excitation
DSI, depolarization-induced suppression of inhibition
E, embryonic day
eCB, endogenous cannabinoid
ECS, endogenous cannabinoid system
EDTA, ethylenediaminetetraacetic acid
EGR-1, early growth response protein 1
ELISA, enzyme-linked immunosorbent assay
EPM, elevated plus maze
ERK1/2, extracellular signal-regulated protein kinase-1 and-2.
FAAH, fatty acid amide hydrolase
GABA, gamma-aminobutyric acid
GAD67, glutamic acid decarboxylase isoform 67
GIRK, G protein-activated inwardly-rectifying potassium channel
GluA1, glutamate ionotropic receptor AMPA type subunit 1
GluN2A, glutamate ionotropic receptor NMDA type subunit 2A
GnRH, gonadotropin releasing hormone
GPCR, G protein-coupled receptor
GR, glucocorticoid receptor
H\textsubscript{2}O\textsubscript{2}, hydrogen peroxide
IgG, immunoglobulin G
ir, immunoreactive
LH, luteinizing hormone
LSD, least significant difference test
mAb, monoclonal antibody
MAGL, monoacylglycerol lipase
MANOVA, multivariate analysis of variance
mPFC, medial prefrontal cortex
mRNA, messenger ribonucleic acid
mTOR, mammalian target of rapamycin
NAc, nucleus accumbens
NAPE, N-acylphosphatidylethanolamine
NAPE-PLD, N-acylphosphatidylethanolamine-dependent phospholipase D
NMDA, N-methyl-D-aspartate receptor
noACS, no adolescent confinement stress
NoINJ, no injection
OFT, open field test
PBS, phosphate-buffered saline
PBS-X, phosphate buffered saline with Triton-X
PFC, prefrontal cortex
PI3K, phosphatidylinositol-3-kinase

PKA, protein kinase A

PL-PFC, prelimbic division of the prefrontal cortex

PND, postnatal day

PSD95, post-synaptic density protein 95

PVDF, polyvinylidene fluoride

S.D., standard deviation

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

S.E.M., standard error of the mean

SPINO, spinophilin

T0, immediately after cessation of stress

T45, 45 min after cessation of stress

T60, 60 min after cessation of stress

T90, 90 min after cessation of stress

TBS, tris-buffered saline

TBS-T, tris-buffered saline with Tween-20

THC, Δ⁹-tetrahydrocannabinol

TNFR, tumor necrosis factor receptor

TRPV1, transient receptor potential vanilloid 1 channel

UV, ultraviolet

VEH, vehicle

VGCC, voltage-gated calcium channel

VH, ventral hippocampus
WIN 55,212-2, (R)-(+)\-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone

Zif268, zinc finger protein 268

\(\eta^2_p\), partial eta squared
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Chapter 1: General Introduction
Introduction

The endogenous cannabinoid system (endocannabinoid system; ECS) is a lipid-based neuromodulatory system best known as the neural substrate mediating the effects of Δ⁹-tetrahydrocannabinol (THC), the main psychoactive constituent of cannabis (French, 1997; Huestis et al., 2001; Jarbe et al., 2002; Ledent et al., 1999; Onaivi et al., 1995). Cannabis is one of the most widely used psychoactive substances globally (Gowing et al., 2015), and its stress- and anxiety-reducing effects are the most commonly reported reasons for its consumption (Hyman and Sinha, 2009). In accordance with the effects of cannabis on stress and anxiety, studies in humans (Chakrabarti and Baron-Cohen, 2011; Chakrabarti et al., 2006; Domschke et al., 2008; Lu et al., 2008; Phan et al., 2008) and in rodents (Crowe et al., 2014; Draycott et al., 2014; Finn et al., 2012; Hill and McEwen, 2010; Hill et al., 2010; Hill and Patel, 2013; Laviolette and Grace, 2006; Litvin et al., 2013; Martin et al., 2002; McLaughlin et al., 2013; Patel et al., 2005; Patel et al., 2004; Roberts et al., 2014) have demonstrated a role of endocannabinoid signalling in the acute regulation of anxiety-like behaviour and neuroendocrine stress responses. Further, studies in rodents have demonstrated long-term effects of adolescent cannabinoid exposure on emotion and cognition (O'Shea et al., 2006; O'Shea et al., 2004; Renard et al., 2013; Rubino et al., 2009a; Rubino et al., 2008b; Schneider and Koch, 2003; Schneider et al., 2008), as well as on neural development and maturation (Renard et al., 2017; Renard et al., 2016b; Rubino et al., 2015; Rubino et al., 2009b; Zamberletti et al., 2014), that suggest a role of endocannabinoid signalling in normative adolescent development. Consistent with the hypothesis that adolescence is a sensitive period of development, repeated CB1 receptor agonism produced lasting behavioural changes in
adolescent, but not adult, rats (Aboussi et al., 2014; Bambico et al., 2010; O'Shea et al.,
2004; Schneider and Koch, 2003). The evidence that exposure to cannabinoids in
adolescence can alter brain and behaviour long-after cessation of treatment suggests that
cannabinoid drugs, via disruptions to normative endocannabinoid signalling, alter the
developmental trajectory of the adolescent brain. Though studies with agonists have
identified CB1 receptor signalling as a likely contributor to normative adolescent brain
and behaviour development, little is known about the role of endogenous cannabinoid
signalling in this period.

In the following sections, I will describe the endocannabinoid system with a focus
on its development across the lifespan. I will then describe adolescence as a sensitive
period of neurodevelopment, highlighting research into the effects of CB1 receptor
agonism in adolescence on brain and behaviour development. Next, I will describe what
is known about endogenous cannabinoid signalling in adolescence from studies with CB1
receptor antagonists, highlighting prominent gaps in knowledge that I have addressed
with my thesis research. Lastly, I will summarize the main goals of my thesis research,
and the overarching hypotheses that I have tested.

The endocannabinoid system

The endocannabinoid system is comprised of two main receptor subtypes, the
cannabinoid type-1 (CB1) and type-2 (CB2) receptors, both of which are members of the
inhibitory G-protein coupled receptor (GPCR) family (Howlett, 2002). CB2 receptors are
expressed primarily in immune cells of the periphery, with greatest expression in the
spleen and lymphatic system (Svizenska et al., 2008). Within the brain, CB2 receptors are
most commonly expressed on microglia (Cabral et al., 2008; Maresz et al., 2005; Nunez
et al., 2004) and are thought to be involved in the regulation of cytokine release. Neuronal CB2 receptor expression has been reported (Gong et al., 2006; Onaivi et al., 2006), however, the contribution of CB2 receptors to neuronal signalling and behavioural regulation is largely unknown (Malfitano et al., 2014). CB1 receptors, on the other hand, show greatest expression in the central nervous system, with densest neuronal expression in the cerebellum and in corticolimbic regions involved in emotion and cognition, such as the medial prefrontal cortex (mPFC), the hippocampus, and the amygdala (Egertova and Elphick, 2000; Herkenham et al., 1991; Hohmann and Herkenham, 1999; Tsou et al., 1998; Tsou et al., 1999). Research to date has identified CB1 receptor signalling as the predominant cannabinoid receptor involved in the regulation of emotional behaviour and physiological stress responses (Busquets Garcia et al., 2016; Griebel et al., 2005; Hill and Gorzalka, 2004, 2005; Rey et al., 2012; Simone et al., 2015a; Simone et al., 2015b), consistent with its expression throughout the brain’s emotional centres. Thus, the remainder of this chapter will focus on CB1 receptor signalling.

Within the brain, CB1 receptors are predominantly expressed pre-synaptically (Dudok et al., 2015; Hebert-Chatelain et al., 2014a; Katona et al., 1999; Katona et al., 2006), though there are reports of post-synaptic (Salio et al., 2001; Salio et al., 2002) and mitochondrial (Benard et al., 2012; Hebert-Chatelain et al., 2014b) CB1 receptor expression. Pre-synaptic CB1 receptors act to regulate the release of neurotransmitters from the post-synaptic cell through inhibition of voltage-gated calcium channels (VGCCs) (Daniel et al., 2004; Gebremedhin et al., 1999; Mato et al., 2009; Poling et al., 1996; Shen and Thayer, 1998) and activation of G protein-coupled inwardly rectifying potassium (GIRK) channels (Guo and Ikeda, 2004; Ho et al., 1999; Pertwee, 1997), thus
preventing depolarization-induced increases in presynaptic calcium influx. Because presynaptic depolarization and calcium influx are requirements for vesicle docking and fusion to the pre-synaptic membrane (Kelly, 1993; Schneggenburger and Neher, 2000; Smith and Augustine, 1988), CB1 receptor activation of GIRKs and inhibition of VGCCs acts to reduce the probability of neurotransmitter release from the pre-synaptic terminal.

Although CB1 receptors are known to influence the release of most major neurotransmitters, their actions have been best characterized on glutamatergic and GABAergic terminals where they mediate the processes of depolarization-induced suppression of excitation (DSE) and inhibition (DSI), respectively (Diana and Marty, 2004; Maejima et al., 2001; Piomelli, 2003). Thus, the functional output of CB1 receptor signalling is both region- and cell type-dependent.

In addition to regulating intercellular synapto-plastic changes via the induction of DSE and DSI, CB1 receptor activation can also modulate intracellular plasticity via direct G\textsubscript{a} inhibition of membrane bound adenylyl cyclase (Bayewitch et al., 1995; Rhee et al., 1998; Slipetz et al., 1995), reducing the production of cyclic adenosine monophosphate (cAMP) (Pertwee, 1997) and modulating the activity of several downstream signalling pathways, most notably the cAMP-dependent Protein Kinase A, phosphoinositide 3-kinase / Protein Kinase B, and extracellular-regulated protein kinase-1 and -2 (ERK1/2) pathways (Dalton and Howlett, 2012; Dalton et al., 2009; Davis et al., 2003; del Pulgar et al., 2000; Ellert-Miklaszewski et al., 2005; Gomez del Pulgar et al., 2000; Ozaita et al., 2007). Thus, the effects of CB1 receptor activation on neurotransmission involve both inter- and intra-cellular effects, leading to alterations in both short- and long-term neural plasticity and organization.
**AEA and 2-AG signalling**

Under normative physiological conditions, CB1 receptor activation is induced via the binding of endogenous cannabinoids, or endocannabinoids (Wilson and Nicoll, 2002). Canonical retrograde endocannabinoid signalling is activity-dependent and occurs in response to pre-synaptic release of neurotransmitter onto a post-synaptic cell (Wilson and Nicoll, 2002). The binding of neurotransmitters to their cognate receptors triggers the activation of enzymatic pathways that convert phospholipid precursors in the post-synaptic membrane into active endocannabinoids (Di Marzo, 2011). Once synthesized, endocannabinoids are released into the synapse by an unidentified endocannabinoid membrane transport protein (Fu et al., 2011) where they then bind to and activate pre-synaptic CB1 receptors (Piomelli, 2003; Wilson and Nicoll, 2002). After binding to CB1 receptors, endocannabinoids are taken back into the cell and shuttled to their catabolic enzymes where they are degraded into their respective metabolites (Giuffrida et al., 2001). The two most well characterized endocannabinoids are \(N\)-arachidonoylethanolamide (AEA) and 2-arachidonylglycerol (2-AG) (Di Marzo et al., 1998; Pertwee, 2015). AEA and 2-AG are co-localized throughout much of the central nervous system, and though both are cannabinoid receptor agonists (Di Marzo and Maccarrone, 2008), evidence to date suggests a certain degree of independence in AEA and 2-AG signalling properties. For instance, relative to 2-AG, AEA has a higher binding affinity to CB1 and CB2 receptors (Hillard, 2000; Pertwee, 2010). Nevertheless, whereas AEA acts as only a partial cannabinoid receptor agonist, 2-AG possesses full agonist properties (Hillard, 2000). Additionally, AEA also acts as partial agonist at the post-synaptic transient receptor potential vanilloid type-1 (TRPV1) receptor (Ross, 2003),
eliciting neuro-excitatory effects opposite to those of CB1 receptor activation (Casarotto et al., 2012). In addition to the different pharmacodynamic properties of AEA and 2-AG, their relative abundancies within the brain further suggest a degree of independence in their functional roles, with neural concentrations of 2-AG being approximately 200-fold greater than that of AEA (Stella et al., 1997; Sugiura et al., 1995).

The metabolic pathways leading to AEA and 2-AG synthesis and degradation also maintain a certain degree of independence and provide further insight into their differential functions within the brain. The most commonly described pathway to 2-AG synthesis involves activation of the post-synaptic metabotropic glutamate receptor 5 (mGluR5), triggering the sequential activation of phospholipase C (PLC) and diacylglycerol lipase (DAGL), and the DAGL-mediated conversion of diacylglycerol (DAG) to 2-AG (Di Marzo, 2011; Hillard, 2000; Mechoulam and Parker, 2013). AEA synthesis, on the other hand, is preceded by increases in presynaptic intracellular Ca\textsuperscript{2+} concentrations and involves the conversion of N-acyl phosphatidylethanolamine (NAPE) into AEA via the enzyme NAPE-dependent phospholipase-D (NAPE-PLD) (de Fonseca et al., 2005; Di Marzo, 2011; Hillard, 2000; Mechoulam and Parker, 2013). The anabolic machinery for both 2-AG and AEA are localized to the post-synaptic membrane (de Fonseca et al., 2005), indicating that both endocannabinoids originate in the post-synapse. However, whereas 2-AG is hydrolyzed into arachidonic acid and glycerol by the presynaptic enzyme monoacylglycerol lipase (MAGL) (Blankman et al., 2007; Dinh et al., 2002), AEA degradation is mediated by the post-synaptic enzyme fatty acid amide hydrolase (FAAH) (Gulyas et al., 2004) and results in the catabolism of AEA into arachidonic acid and ethanolamine (Cravatt et al., 1996; Cravatt and Lichtman, 2003).
Thus, that 2-AG is synthesized in the post-synapse and degraded in the pre-
synapse and has a greater neural abundance and efficacy at CB1 receptors than AEA,
suggests that 2-AG is the predominant neural endocannabinoid involved in classical
retrograde CB1 receptor signalling. Conversely, given that AEA synthesis and
degradation is localized predominantly to the post-synapse and that AEA signalling
involves mechanisms independent of cannabinoid receptors (e.g., TRPV1 activation), it is
likely that AEA is involved in tonic regulation of synaptic activity via post-synaptic CB1
and / or TRPV1 receptor activation (Casarotto et al., 2012; Ross, 2003; van der Stelt et
al., 2005).

**Developmental patterns of the endocannabinoid system in pre- and post-natal
periods**

There are functional contributions of endocannabinoid signalling early on in
neurodevelopment involving influences over processes such as cell proliferation and fate
determination (Aguado et al., 2006; Guzmán et al., 2002; Mulder et al., 2008; Rueda et
al., 2002), axonal migration and pathfinding (Berghuis et al., 2005; Berghuis et al., 2007;
Gómez et al., 2008; Harkany et al., 2008; Mulder et al., 2008; Saez et al., 2014; Watson
et al., 2008), and the establishment and induction of appropriate synaptic connections
(Berghuis et al., 2007; Bernard et al., 2005). In support of ECS influences on early
neurodevelopment, CB1 receptor mRNA expression has been observed as early as
embryonic day (E) 11 in rodent fetal brain tissue (Buckley et al., 1997; Mulder et al.,
2008; Rodríguez de Fonseca et al., 1993), and gestational week 9 in human cortical
embryonic tissue (Zurolo et al., 2010), with expression increasing with fetal age
(Berrrendero et al., 1998; Berrendero et al., 1999). In rodents, gestational CB1 receptor
expression peaks in cortical excitatory pyramidal cells at E 14-15, whereas expression in inhibitory interneurons remains relatively low until late gestation / birth (Mulder et al., 2008), suggestive of a cell-type specific role of CB1 receptor signalling in neuronal development.

Although the current understanding of CB1 receptor expression patterns in the postnatal brain is far from complete, the available evidence suggests that the ECS continues to develop into adolescence and adulthood (Fride, 2008; Harkany et al., 2008; Lee and Gorzalka, 2015). In both male and female rats, CB1 receptor expression in midbrain and limbic regions increased from PND 10 to peak expression in early-adolescence (PND 30-40), then gradually decreased to stable adult-typical levels by PND 70 (de Fonseca et al., 1993). Although the pattern of CB1 receptor expression overall was similar for the sexes, females had greater mid-brain CB1 receptor expression than did males at PND 10, and males had greater expression from PND 15 onwards (de Fonseca et al., 1993). In the prefrontal cortex of female rats, the expression of CB1 receptors declined from mid-adolescence (PND 46) to adulthood (PND 70) (Rubino et al., 2015), providing further support for an inverted-U shaped pattern of post-natal CB1 receptor expression in the brain. Although the reduction from adolescent to adult levels of CB1 receptor expression seems consistent throughout the brain, the rate of change of expression was found to vary by region, with gradual reductions occurring across the adolescent period in limbic regions, and more abrupt declines in sensory-motor regions occurring in mid-late adolescence (Heng et al., 2011).

Consistent with fluctuations in CB1 receptor expression across early development, concentrations of the endocannabinoids AEA and 2-AG also vary
according to developmental stage. In rodent brain tissue, AEA concentrations remain low in early to mid-gestation, and increase gradually throughout the late-gestational and early postnatal periods to peak concentrations that remain stable into adulthood (Berrendero et al., 1999; Fride, 2004). Conversely, concentrations of 2-AG remain relatively stable across the lifespan, with peak concentrations occurring at postnatal day (PND) 2 in rats, and adult-typical concentrations observed by PND 5 (Berrendero et al., 1999; Fernández-Ruiz et al., 2000).

Despite postnatal concentrations of 2-AG being relatively stable compared with those of AEA, neural concentrations of both endocannabinoids were reported to fluctuate across the adolescent period in a region-dependent manner (Ellgren et al., 2008). In the nucleus accumbens, adolescent AEA concentrations peaked in mid-adolescence, with no differences between early- and late-adolescent periods, whereas 2-AG concentrations decreased from early- to mid-adolescence, remaining stable into late-adolescence (Ellgren et al., 2008). In the PFC, AEA concentrations steadily increased across adolescence, whereas 2-AG concentrations peaked in early-adolescence, then declined into mid-adolescence, before gradually increasing to sub-maximal concentrations in late-adolescence (Ellgren et al., 2008). Thus, the main components of the endocannabinoid system continue to develop through early pre- and post-natal periods, into adolescence and adulthood.

The pattern of CB1 receptor and endocannabinoid expression during neurodevelopment provides some insight into the functional role of endocannabinoid signalling during different developmental periods. The highly-specific expression of CB1 receptors in excitatory cells during early gestation (Mulder et al., 2008) is mirrored by
atypically high expression levels in white matter fibre tracks of the gestational brain (Romero et al., 1997), suggesting a role of CB1 receptor signalling in axonal migration and pathfinding of excitatory cells. Conversely, the increase in inhibitory interneuron CB1 receptor expression in late-gestation / early-perinatal periods (Mulder et al., 2008) suggests an increase in the influence of endocannabinoid signalling in localized circuit development and refinement. Further, the highly localized expression of CB1 receptors to the amygdala and hippocampus of the rat neonate (Rodríguez de Fonseca et al., 1993) suggests endocannabinoid involvement in early development of the emotional brain, whereas the near ubiquitous expression in the more developed adult-typical brain (Egertova and Elphick, 2000; Moldrich and Wenger, 2000; Tsou et al., 1998; Tsou et al., 1999) highlights the widespread neuromodulatory role of the ECS in adulthood. Relative to perinatal and adult periods, however, little is known about endocannabinoid signalling in adolescence. Nevertheless, the dynamic expression of CB1 receptors and their endogenous ligands in adolescence (de Fonseca et al., 1993; Ellgren et al., 2008; Rubino et al., 2015) suggests that it is a unique period for endocannabinoid development and developmental contributions.

**Adolescence as a sensitive period of development**

Adolescence is a transitional period of development between childhood and adulthood that involves maturation and refinement of corticolimbic brain regions involved in emotion, cognition, and physiological stress responses (McCormick and Mathews, 2010). Adolescence is also associated with increases in social behaviours, risk taking, and novelty- and reward- seeking, which are thought to help prepare the organism for the challenges and independence of adulthood (Burke et al., 2017; Spear, 2000). Until
recently, research into adolescent development was largely underrepresented (Grant et al., 2003), partially driven by the concept that adolescence was a period of development exclusive to humans, with non-human animals transitioning directly from a juvenile to adult stage (Bogin and Smith, 1996). In the past two decades, adolescence has come to be accepted as a bona fide period of development in not only humans, but also in non-human mammals and birds (Brown and Spencer, 2013). Consequently, there has been a surge in animal models of adolescent development, particularly rodent models, that has led to marked advancements in the current understanding of neural development across the lifespan (Spear, 2004; Stevens and Vaccarino, 2015).

There are many parallels that can be drawn between adolescence in rodents and adolescence in humans. In both humans and rats, there are no clear markers for the onset and offset of adolescence, though puberty is a physiological hallmark of adolescence in both species (McCormick et al., 2017; Sisk and Foster, 2004). In rats, adolescence is generally defined as being between postnatal days (PND) 28-59 (Tirelli et al., 2003), with the average age of pubertal onset at PND 40 in males (assessed by balanopreputial separation) and PND 35 in females (assessed by vaginal opening (McCormick and Mathews, 2007)). In humans, adolescence has been defined by the World Health Organization as spanning the ages of 10 to 19 years, with the average age of pubertal onset occurring at 8-10 years of age, though males generally reach puberty 1 year later than females (Cortés et al., 2015).

In addition to similarities in physiological markers of adolescence, the behavioural repertoire of adolescent rats and humans share considerable overlap, with both species demonstrating increased exploratory and novelty-seeking behaviours,
increased impulsivity, and increased reward sensitivity (Viveros et al., 2011). The behavioural shifts that occur in adolescent mammals are paralleled by pronounced shifts in neuronal structure and function, and network connectivity, across corticolimbic brain regions (Crews et al., 2007; Rice and Barone Jr., 2000). In both rodents and humans, there is a biphasic pattern of adolescent neurodevelopment whereby brain regions involved in more fundamental processes (e.g., primary sensorimotor cortices) mature earlier in adolescence, and those involved in higher-order information processing and integration (e.g., medial temporal lobes, frontal cortex) undergo a more protracted development in later adolescent periods (Giedd et al., 1999; Gogtay et al., 2004). Thus, adolescence represents a period of transition from affective, limbic-driven behaviours, to a period of more cortically-regulated behaviours that involve the complex integration and consideration of environmental and social cues.

The refinement and maturation of corticolimbic networks in the adolescent brain involves both progressive and regressive developmental processes. In rats, there is an increase in hippocampal and amygdala volume, as well as increases in the volume of ventral medial prefrontal cortical white matter tracts across adolescence. Likewise, there are increases in glutamatergic and dopaminergic innervation of the PFC from the basolateral amygdala and nucleus accumbens, respectively (Benes et al., 2000; Berger et al., 1985; Cunningham et al., 2002, 2008; Kalsbeek et al., 1988). Serotonin transport also steadily increases throughout adolescence, with peak transport protein expression in late-adolescence in the striatum, and in adulthood in the PFC (Moll et al., 2000). Conversely, adolescence is a period of marked decline in the number of cortical and hippocampal synapses and dendrites (Andersen and Teicher, 2004; Andersen et al., 2000), in cortical
grey matter volume (Giedd et al., 1999; Gogtay et al., 2004), and in total prefrontal cortical volume (van Eden et al., 1991). Likewise, the expression of the GABA_A receptor subunit decreases from peak expression in early-adolescence to adult-typical levels (Yu et al., 2006) and corticolimbic glutamatergic N-methyl-D-aspartate (NMDA) receptor expression (Guilarte, 1998; Insel et al., 1990) and dopamine receptor density (Tarazi et al., 1998) are reduced by as much as 1/3 from mid-adolescence to adulthood. Of note, the reduction of dopamine receptors in adolescence is region and receptor-type specific, with reductions in dopamine D2 and D4 receptors occurring in reward centres of the brain such as the nucleus accumbens and striatum (Tarazi and Baldessarini, 2000; Teicher et al., 1995), and steady increases in frontal-cortical and hippocampal D1, D2, and D4 dopamine receptors occurring into mid-adolescence before reaching a plateau in adulthood (Tarazi and Baldessarini, 2000). Thus, adolescence involves both progressive and regressive neurodevelopmental alterations that ultimately lead to the maturation of the brain and the ability of the organism to face the challenges of a new period of life.

Nevertheless, while providing the plasticity required for appropriate learning and adaptation to the changing environment, the increased malleability of the developing adolescent brain renders it vulnerable to effects of external influences, such as exposure to stressors or drugs of abuse (e.g., cannabis) (Crews et al., 2007).

**Long-term effects of CB1 receptor agonism in adolescence**

Most of what is known regarding the developmental influences of endocannabinoid signalling in adolescence comes from studies with CB1 receptor agonists, given their use in preclinical animal models of adolescent cannabis exposure (Lee and Gorzalka, 2015; Rubino and Parolaro, 2016). Consistent with the hypothesis
that adolescence is a sensitive period for environmental influences (Crews et al., 2007), exposure to cannabinoid agonists in this period have been found to result in long-term alterations to emotional and cognitive behaviours and their underlying neural circuits that are absent when exposure occurs in adulthood (Aboussi et al., 2014; Bambico et al., 2010; O'Shea et al., 2004; Schneider and Koch, 2003), implicating CB1 receptor signalling in adolescent brain development.

Corticolimbic brain regions that continue to mature in adolescence (e.g., prefrontal cortex, hippocampus, nucleus accumbens) also demonstrate high levels of CB1 receptor expression (Egertova and Elphick, 2000; Tsou et al., 1998; Tsou et al., 1999). Thus, it is not surprising that exposure to CB1 receptor agonists in adolescence produces long-term developmental disruptions to emotional and cognitive behaviours that are evident long after exposure. For example, adolescent exposure to the CB1 receptor agonists WIN55,212-2 (WIN) and CP55,940 produced lasting impairments in object recognition memory (Abush and Akirav, 2012; O'Shea et al., 2006; O'Shea et al., 2004; Renard et al., 2013; Schneider et al., 2008) and spatial location memory (Aboussi et al., 2014; Renard et al., 2013) in rats; when allowed to self-administer the CB1 receptor agonist, improvements in spatial memory were observed in adult female rats (Kirschmann et al., 2017). Repeated adolescent CB1 receptor agonism (THC, WIN, CP55,940) also produced long-term impairments in social behaviours in rats, with notable reductions observed in social interactions (O'Shea et al., 2006; O'Shea et al., 2004), social recognition (Renard et al., 2017; Schneider et al., 2008), and social motivation (Renard et al., 2017). Adolescent cannabinoid exposure was found to alter anxiety-like behaviours in adulthood, though the direction of effects is inconsistent;
adolescent exposure to CP55,940 led to modest reductions in anxiety-like behaviour in adult rats (O'Shea et al., 2006), whereas exposure to THC (Renard et al., 2017) or WIN (Bambico et al., 2010) increased anxiety-like behaviours in adulthood.

In accordance with effects on behaviour, adolescent exposure to cannabinoid agonists is associated with long-term alterations to brain structure and function that are evident long after exposure and into adulthood (Renard et al., 2017; Renard et al., 2016b; Rubino et al., 2015). In male rats, repeated exposure to THC in adolescence induced a state of dopaminergic hyperactivity in the mesocorticolimbic system, as well as reduced the activity of the Akt/mTOR signalling pathway in the PFC when measured in adulthood (Renard et al., 2017). PFC GABAergic neurotransmission is also sensitive to adolescent CB1 receptor agonism, as exposure to WIN elicited a state of prefrontal disinhibition and downregulation of GABAergic signalling (Cass et al., 2014). Further, the effect of WIN on GABA transmission was specific to the early- and mid-adolescent periods, with no effects observed when treatment occurred in late-adolescence and adulthood (Cass et al., 2014).

Effects of adolescent CB1 receptor agonism on regional- and network-level neurotransmission are likely explained by effects at the cellular level. Male rats exposed to the synthetic CB1 receptor agonist CP55,940 in adolescence had reduced PFC dendritic complexity, reduced hippocampal-induced PFC plasticity, and reductions in the glutamatergic synaptic marker PSD95 in the PFC compared to vehicle-treated rats (Renard et al., 2016b). In female rats repeatedly exposed to THC during adolescence there was a similar reduction in dendritic complexity within the PFC, however, the effects on markers of glutamatergic synapses were opposite to those previously reported in
males, with THC treated females demonstrating increased expression relative to vehicle treated females (Rubino et al., 2015). Further, both male and female rats exposed to THC in adolescence had greater CB1 receptor desensitization in the PFC and hippocampus relative to vehicle-treated rats (Burston et al., 2008), and male rats exposed to WIN in adolescence had reduced dorsal hippocampus neurogenesis compared with non-treated controls. Thus, studies with CB1 receptor agonists collectively demonstrate that disruptions to normative adolescent endocannabinoid signalling via pharmacological activation of CB1 receptors can induce lasting neural and behavioural changes and have identified the endocannabinoid system as crucial for normative adolescent development. Although studies with agonists provide insight into the consequences of exposure to cannabinoid drugs, as well as into the potential of endocannabinoid signalling to influence adolescent development, studies with antagonists provide the opportunity to investigate the physiological role of endogenous cannabinoid signalling to these processes.

**Long-term effects of CB1 receptor antagonism in adolescence**

Little is known about the developmental role of endogenous cannabinoid signalling in adolescence. Until the time of this thesis in mid 2018, only two studies had investigated the role of adolescent-specific endocannabinoid signalling on brain and behaviour development, the first in females (Rubino et al., 2015) and the second in males (Lee et al., 2015). In female rats, repeated exposure to the highly-selective CB1 receptor antagonist / inverse agonist AM251 during adolescence (0.5 mg / kg, PND 35-45) prevented normative developmental decreases in glutamatergic markers within the PFC, with effects evident into adulthood (Rubino et al., 2015). In male rats, adolescent AM251
exposure (5 mg / kg, PND 35-45) resulted in increased risk-assessment and stress-coping behaviours, as well as increased PFC CB1 receptor binding, when testing occurred several weeks after the final drug exposure (Lee et al., 2015). Together, the findings from Rubino et al., (2015) and Lee et al., (2015) confirm a role of endogenous CB1 receptor signalling in normative adolescent development.

**Summary and main goals of thesis**

In summary, rodent studies involving CB1 receptor agonists and antagonists have highlighted the endocannabinoid system as a potential regulator of adolescent brain and behaviour development. Nevertheless, the extent to which effects of adolescent CB1 receptor antagonism on brain and behaviour are evident soon after treatment, or require a period of incubation to manifest, are unclear. Additionally, the extent to which adolescent endocannabinoid signalling regulates development differently between the sexes is not clear, though sex-differences in adolescent brain development and in the effects of CB1 receptor agonism suggest a certain degree of sex-specificity. Further, classical CB1 receptor signalling requires on-demand activation (i.e., activation in response to a specific stimulus). Previous research into the effects of adolescent CB1 receptor antagonism have investigated CB1 receptor contributions to development only under basal conditions. Thus, whether effects of CB1 receptor antagonism would be greater under on-demand conditions is not known. To address these gaps in knowledge, my thesis research investigated the contributions of adolescent CB1 receptor signalling to the development of brain and behaviour in both male and female rats under basal and on-demand conditions. I hypothesized that (1) endogenous CB1 receptor signalling regulates normative development of corticolimbic structures in the adolescent brain and their
associated affective and cognitive behaviours, (2) downregulation of endogenous CB1 receptor signalling in adolescence would have greater developmental effects under on-demand versus basal conditions, and (3) the contributions of endogenous CB1 receptor signaling to normative adolescent brain and behaviour development are moderated by sex. To address these hypotheses, I performed a series of experiments involving repeated administration of the highly selective CB1 receptor antagonist / inverse agonist AM251 in adolescence and collected a variety of measures of affective and cognitive behaviour, neural development, and neuroendocrine stress responses soon (24–48 h), and long (4+ weeks), after cessation of treatment. Together, the findings from my thesis research identify the endocannabinoid system as a regulator of normative adolescent development in both sexes and demonstrate greater contributions of adolescent CB1 receptor signalling to the development of anxiety-like behaviours in males, and social and cognitive behaviours in females.
Chapter 2: Effects of CB1 receptor antagonism and stress exposures in adolescence on socioemotional behaviours, neuroendocrine stress responses, and expression of relevant proteins in the hippocampus and prefrontal cortex in rats.
The published version of this chapter is:


Author contribution: I was the primary investigator of this work, responsible for the majority of study design, data collection, data analysis, and writing of the manuscript. I performed the injections and stress procedures, as well as the behavioural testing and scoring, hormone extractions and CORT assays, and protein extractions and Western blot experiments.

Jennet Baumbach assisted with the behavioural testing and scoring of elevated plus maze videos. Dr. Cheryl McCormick assisted in designing the experiments, analyzing the data, and editing the manuscript.
Introduction

The endogenous cannabinoid system (ECS) is involved in the regulation of a variety of behavioural and physiological processes, including emotional regulation (Lutz, 2009; Ruehle et al., 2012), stress responsivity (Hill and Tasker, 2012; Steiner and Wotjak, 2008), and synaptic plasticity (Castillo et al., 2012; Viveros et al., 2007). The ECS is comprised of two main receptors, the cannabinoid receptor type-1 (CB1) and type-2 (CB2), the endogenous ligands for these receptors, namely N-arachidonylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG), and the enzymes involved in the metabolism of these ligands, most notably fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) (Di Marzo, 2011). Whereas CB2 receptors are most predominantly localized to immune tissues of the periphery, CB1 receptor expression is most dense in the central nervous system (Mechoulam and Parker, 2013), particularly in corticolimbic regions involved in the regulation of emotional behaviours and neuroendocrine stress responses (Herkenham et al., 1991).

Many of the behavioural and neurophysiological effects of cannabinoid exposure in adult rats are sex-specific, and greater sensitivity to cannabinoid drugs is often observed in females compared with males (Craft et al., 2012; Tseng and Craft, 2001; Tseng et al., 2004). Fewer studies have focused on the immediate effects of such exposures in adolescence in either sex. In male rats, repeated exposure to Δ⁹-tetrahydrocannabinol (THC; a CB1 receptor agonist) during adolescence reduced social interactions when testing occurred in the adolescent period (Klein et al., 2011), with similar impairments in social behaviours found when rats were administered the CB1 receptor agonist CP-55,940 (O'Shea et al., 2006), or WIN55,212-2 (Leweke and
Schneider, 2011; Schneider et al., 2008) and tested in adolescence. Consistent with the cannabinoid-induced impairments in social behaviours observed in adolescent male rats, female rats that were repeatedly exposed to the CB1 receptor agonist CP-55,940 during adolescence also had reduced social interactions relative to vehicle-treated females when testing occurred in adolescence (O'Shea et al., 2004). Further, repeated agonism of CB1 receptors during adolescence has been linked to immediate functional and morphological changes in brain regions involved in social function. For instance, adolescent THC exposure increased AEA concentrations in the nucleus accumbens (Ellgren et al., 2008), and decreased GABAergic transmission in the prefrontal cortex (Cass et al., 2014) of adolescent male rats. Likewise, repeated exposure to the CB1 receptor agonist WIN55,212-2 during adolescence impaired the induction of long-term potentiation (LTP; functional marker of synaptic plasticity) along the ventral subiculum-nucleus accumbens pathway in adolescent male rats (Abush and Akirav, 2012). In adolescent female rats, repeated exposure to cannabinoids during the adolescent period reduced AEA concentrations and CB1 receptor binding, reduced the number of distal basal dendrites, and increased the expression of markers of glutamatergic signalling in the prefrontal cortex (Rubino et al., 2015). In sum, results from experiments involving CB1 receptor agonists suggests a role of endocannabinoid signalling in the development of the adolescent brain.

Only two studies have investigated the effects of repeated antagonism of CB1 receptors during the adolescent period on subsequent function. In male rats, repeated administration of the CB1 receptor-selective antagonist/inverse agonist AM251 from postnatal days 35 to 45 demonstrated increased active stress-coping behaviour in the
forced swim test, increased risk-assessment behaviour in the elevated plus maze, and greater habituation to repeated restraint stress in adulthood (Lee et al., 2015). Further, AM251 treated rats had increased CB1 receptor expression in the prefrontal cortex (PFC), increased AEA concentrations in the hypothalamus, and decreased AEA concentrations in the amygdala in adulthood compared with vehicle-treated rats (Lee et al., 2015). Although the immediate effects of adolescent CB1 receptor blockade in male rats were not investigated, evidence in females has demonstrated both immediate and long-lasting effects on the development of the PFC (Rubino et al., 2015). Daily administration of AM251 (PND 35-45) increased the expression of glutamatergic markers in the PFC of female rats on PND 46, with the increases still evident at PND 60 and PND 75 (early adulthood) (Rubino et al., 2015). The results from these two studies suggest an important role for basal endocannabinoid signalling in the adolescent period on the neural mechanisms subserving socioemotional function.

Many endocannabinoid mediated-processes involve on-demand signalling (i.e., signalling in response to specific stimuli) (Zanettini et al., 2011); thus, developmental effects of manipulation of the endocannabinoid system may be greater when conducted under “on demand” conditions, such as those shown for both the activation and inhibition of neuroendocrine stress responses in adult animals (Hill and Tasker, 2012). How activation of stress systems interacts with endocannabinoid signalling during adolescence, however, is unknown. Nevertheless, high, stress-induced exposures to glucocorticoids in adolescence are associated with altered neural and behavioural function, such as increased anxiety-like behaviour (McCormick and Green, 2013).
Here, we tested the hypothesis that reducing endocannabinoid signalling when confronting stressors in adolescence will have greater residual / developmental effects (i.e., effects that are observable in a drug-free state) on socioemotional behaviours, neuroendocrine stress responses, and expression of associated proteins in the hippocampus and PFC than would reducing endocannabinoid signalling under baseline conditions. The experimental design involved two main factors, Drug Groups (AM251, vehicle [VEH], no injection [NoINJ]) and Stress Groups (adolescent confinement stress [ACS], no adolescent confinement stress [noACS]); adolescents are particularly vulnerable to stress of injection effects (Keeley et al., 2015a; McCormick et al., 2010), thus it was important to include a no injection control group. We first tested the hypothesis in males with several endpoints for behavioural and endocrine function, and protein expression relevant for cannabinergic, glutamatergic, and GABAergic function in key neural regions (hippocampus and medial PFC). We then tested the hypothesis in a second experiment in females with the same design and measures, and some minor modifications and additional measures (described in methods).

Methods
See Fig. 1 for the experimental design and timeline. Testing procedures were identical for experiments 1 (males) and 2 (females) unless otherwise stated.

Animals
144 male (experiment 1) and 158 female (experiment 2) Long-Evans rats were shipped from Charles River (Kingston, New York, USA) on postnatal day (PND) 23, and arrived at the Brock University Comparative Bioscience Facility on PND 25. Upon arrival, rats were housed in same-sex pairs and allowed to acclimate to the facility for four days before the start of the experimental procedures. Rats were kept on a 12-hour
light-dark cycle (lights on at 8:00 h) and were provided access to food and water *ad libitum*. All procedures were approved by the Brock University Institutional Animal Care Committee and were in accordance with the Canadian Council on Animal Care and National Institutes of Health guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1996). All efforts were made to minimize the number of animals used and their suffering.

**Weights**

All animals were weighed daily soon after lights-on (8:00 h) for the 15 days of treatment (PND 30-44). To minimize the effects of handling on the experimental procedures, all animals were left undisturbed for a minimum of 1 h after weighing and before the onset of testing.

**Adolescent confinement stress**

Adolescence in rats has been defined liberally as spanning from PND 21-59 (Tirelli et al., 2003). The average age of pubertal onset in males (assessed by balanopreputial separation) is PND 40, and in females (assessed by vaginal opening) is PND 35 (McCormick and Mathews, 2007). For the present experiment, we chose a peri-pubertal range of PND 30-45 that corresponds with the period of mid-late adolescence and is known to be a sensitive period for the effects of stressors (Burke et al., 2017). Animals were assigned to either the Adolescent Confinement Stress (ACS) protocol or to the No Adolescent Confinement Stress (noACS) protocol daily for 15 days (PND 30-44). ACS rats were removed from their homecage and placed individually into small, ventilated plastic containers (14 cm in height, 10 cm in diameter) in a separate room from the housing colony for 1 h, a procedure known to result in high concentrations of plasma
corticosterone (Hodges and McCormick, 2015). After 1 h of confinement stress, rats were returned to their homecage. The stress protocol was performed at a different time each day (09:00-20:00 h) during the light cycle to minimize any anticipatory or habituation effects. Rats in the noACS group remained undisturbed in their homecages during the confinement stress procedure.

**Drugs and injections**

The CB1 receptor antagonist / inverse agonist AM251 (Cayman Chemical, USA) was dissolved in a 1:1:18 dilution of DMSO, Tween-80, and 0.9% saline and administered i.p. at a concentration of 1.0 mg / kg. The vehicle was a 1:1:18 mixture of DMSO, Tween-80, and 0.9% saline in the absence of AM251. Effects of AM251 have been reported across a wide range of doses (Lee et al., 2015; Rubino et al., 2015; Simone et al., 2015a; Sink et al., 2010a). Effects of repeated AM251 administration during adolescence were reported in male rats (5 mg / kg) (Lee et al., 2015) and female rats (0.5 mg / kg) (Rubino et al., 2015) when administered daily for 10 consecutive days (PND 35-45). Given the greater duration of treatment in the present study (PND 30-45), we chose an intermediate dose of 1 mg / kg AM251; we have previously reported acute effects of 1 mg / kg AM251 in adult female rats (Simone et al., 2015b). All injections were administered at a volume of 1.0 mL / kg. Vehicle and AM251 injected rats in the noACS groups were returned directly to their homecages after injection. Vehicle and AM251 injected rats in the ACS groups were injected immediately before the confinement stress procedure each day.

**Observations of behaviour in the homecage**
Behaviour in the homecage was recorded for the first three days (PND 30-32) and the last three days (PND 42-44) of the stress and injection protocol. Immediately after injection (noACS), or immediately after the 1 h confinement (ACS), rats were returned to their homecage, and behavioural observations were conducted. noACS rats that did not receive either vehicle or AM251 injection were not disturbed before the recording of behavioural observations, and thus they represent the baseline measure of homecage behaviour. A total of sixteen behaviours were observed and subsequently classified into the following categories: Social Active (playing, allogrooming, sniffing cage partner), Social Inactive (immobile while in physical contact with cage partner), Non-Social Active (walking, rearing, eating, drinking), Non-Social Inactive (immobile and not in contact with cage partner), Digging (digging, burying), Self-directed (scratching, self-grooming), and Atypical (shaking, stretching on belly) (adapted (Cirulli et al., 1996; McCormick et al., 2007)). An observation was made for each cage once per minute for 10 minutes, with each animal in the cage being assigned one behaviour category during each observation for a total of 20 observations per cage (10 observations per rat X 2 rats per cage) per day. The total number of observations per cage per day for each behavioural category was averaged across the initial three days of treatment (PND 30-32) and the final three days of treatment (PND 42-44). To allow a degree of freedom in statistical analyses, scores for Non-Social Inactive were excluded from all analyses. Behaviours were scored by an observer blind to experimental conditions.

*Elevated plus maze*

The elevated plus maze is a test of unconditioned avoidance behaviour that has been well-validated as a measure of anxiety in rodents (Wall and Messier, 2001). The
apparatus consisted of two open arms and two closed arms extended from a common central platform 80 cm in height. The maze was constructed of grey plastic and was situated in the centre of the testing room. Rats were tested in the elevated plus maze on PND 45 (see Fig. 1 for experimental timeline). Rats were individually transported from the housing room to the testing room in an empty cage lined with paper towel, placed onto a closed arm of the maze, and left to explore the maze for 5 min. Testing took place under low illumination as we have previously reported anxiogenic effects of acute AM251 exposure in adult rats under these conditions. Testing occurred between 8:00 h and 11:00 h, and behaviour was recorded by an overhead camera. Behaviours scored by an observer blind to experimental condition were the time spent on the open arms, time spent on the closed arms, number of entries into an open arm, and number of entries into a closed arm. An arm entry was recorded when the two front paws of the animal were in the arm. The time spent on the open arms of the maze and the number of entries onto an open arm are the standard measures of anxiety-like behaviour in this test (Wall and Messier, 2001). The number of entries onto the closed arms of the maze is used as a measure of locomotor activity (Cruz et al., 1994; Rodgers and Dalvi, 1997). The maze was cleaned with Virox disinfectant between each test session. To minimize effects of separation of cage partners on performance, cage partners were tested 2 h apart to allow recovery from any stress attributable to the cage partner’s absence of approximately 7 min.

**Blood collection and plasma hormone analysis on PND 45**

On PND 45 (24 h after the final drug treatment), blood was collected from rats either directly from the homecage (baseline; BL), immediately after (T0), or 60 min after
(T60), 1 h of confinement stress (for rats in the ACS group, this was their 16\textsuperscript{th} stress exposure, for rats in the noACS group, it was their first exposure, which allowed a comparison to test for habituation of the stress response). Trunk blood was collected for the BL and T0 timepoints in chilled borosilicate glass tubes containing 75 \( \mu \)L of EDTA-saturated \( \text{H}_2\text{O} \). Blood samples were centrifuged at 1900 x g for 15 min and plasma (supernatant) was collected and stored at -20\(^{\circ}\text{C}\) until the time of assay. Blood was collected from T60 animals via tail nick into chilled microcentrifuge tubes containing EDTA powder (Sarstedt) (these rats were used for subsequent behavioural measures, hence the different method of collection). Tail blood samples were centrifuged at 1730 x g for 10 min and plasma (supernatant) was collected and stored at -20\(^{\circ}\text{C}\) until the time of assay.

\textit{Enzyme-linked immunosorbent assays}

Steroid hormones were extracted from previously prepared plasma samples using diethyl ether, and the samples were reconstituted in buffer provided in the enzyme-linked immunosorbent assay (ELISA) kits (Neogen, Lansing, MI, USA) for the measurement of corticosterone. The assays were conducted according to kit instructions and using a BioTek Synergy plate reader. Assay sensitivity was 0.05 ng / mL for corticosterone.

\textit{Brain collection, tissue processing and protein extraction}

The brains were taken from rats in the baseline (BL) trunk blood collection group at the time of decapitation. Brains were immediately cut down the midline on ice with a razor blade, rapidly frozen in isopentane, and each hemisphere was stored at -80\(^{\circ}\text{C}\) until processing. The left hemisphere of each animal was used for Western blot experiments. The dorsal hippocampus (-3.12 mm from bregma), ventral hippocampus (-4.56 mm from
bregma), and medial prefrontal cortex (+3.24 mm from bregma) were dissected out from the left hemisphere of each sample, and each dissected brain region was placed into a separate microcentrifuge tube on dry ice. Glass beads (0.5 mm diameter) and ice-cold whole-cell lysis buffer (1 M Tris, 150 mM NaCl, 0.1% Triton-X) with protease and phosphatase inhibitors (Roche, USA) were added into each tube containing tissue, and samples were homogenized using a BulletBlender (NextAdvance, USA) at 4°C for 3 min at a power setting of 7. After homogenization, samples were incubated on ice for 10 min with gentle rocking. After incubation, samples were centrifuged at 4°C at 16 000 X g for 10 min. After centrifugation, supernatant was collected as whole-cell soluble protein.

Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, USA) and a BioTek Synergy plate reader. Samples were then diluted to equal concentrations using whole-cell lysis buffer, and heated 2X Laemmli buffer was added to each sample at a 1:1 ratio. Samples were then heated for 5 min at 70°C, centrifuged at 4°C at 16 000 X g for 10 min, and stored at -20°C until use in Western blot experiments.

**Western blots**

For Western blot experiments, processed samples were heated for 5 min at 70°C and 5 µg of protein was loaded onto Bio-Rad TGX stain-free fast-cast 12% polyacrylamide gels (Bio-Rad, USA) for SDS-PAGE. Samples from each group were counterbalanced across gels. Once resolved, gels were illuminated for 5 min under UV light for activation of Bio-Rad stain-free total protein determination. After gel activation, proteins were transferred onto PVDF membranes (EMD Millipore, USA), blocked for 1-hour in 5% BSA in TBS-T at room temperature, and incubated overnight in primary antibody. Primary antibodies used were anti-CB1 receptor (1:1000, ACR-001 Alamone
Labs), anti-PKA (catalytic subunit) (1:2000, 5661 Cell Signalling Technologies), anti-PSD95 (1:5000, NB300-556 Novus Biologicals), and anti-GAD67 (1:5000, MAB5406 Millipore). For experiment 2 (females), we also included primary antibodies directed at spinophilin (1:5000, 06-852 Millipore), glucocorticoid receptor (GR, 1:200, sc-1004 Santa Cruz Biotechnologies) and c-FOS (1:1000, sc-52 Santa Cruz Biotechnologies). After incubation in primary antibody, membranes were washed (3 X 5 min washes) in TBS-T and incubated in secondary antibody (AlexaFluor 488 and AlexaFluor 647; Life Technologies, USA) for 45 min at room temperature with gentle rocking. After incubation in secondary antibody, membranes were washed in TBS-T, and imaged wet using the Bio-Rad VersaDoc MP4000 (Bio-Rad, USA). Detected bands from each lane were normalized to total lane protein using Bio-Rad stain-free total protein determination (Bio-Rad, USA). Many commercially available CB1 receptor antibodies have been found to have non-specific binding properties (Ashton, 2012; Grimesy et al., 2008). Although the specificity of the CB1 receptor antibody used in the present study was verified by pre-treating the antibody with the supplied control peptide corresponding to amino acid residues 84-99 of the rat CB1 receptor (Accession P20272), results should be interpreted with caution.

**Social interaction test**

The 30 min social interaction was adapted from (Green et al., 2013) and involved three phases, allowing for measures of open field activity and interaction with a novel object, in addition to social interaction measures.

**Open field**
On PND 46 rats were tested for responses to a novel environment in an open field. The testing room contained four arenas, allowing for four animals (two pairs of cage partners) to be tested simultaneously. The testing arena consisted of white open-top melamine arenas (58 cm × 58 cm × 58 cm) illuminated indirectly by red light to attenuate anxiety related to bright illumination. The test session was 10 min in duration and occurred between 10:00h and 12:00h. Locomotor activity was recorded with a Sony digital video camera mounted from the ceiling and connected to a computer tracking system (Smart; Panlab, Spain) that measured distance travelled in centimeters, as well as the percentage of time spent in the centre of the test arena (12 cm away from any wall), and the latency to enter the centre of the test arena. At the end of the 10 min open field test, rats remained in the testing arena for a subsequent 10 min novel object test.

**Novel object**

A novel object (i.e. a small black, plastic fan, 8 cm × 12 cm) was placed into a corner of the test arena, and the latency to approach, and the time spent interacting with the novel objected were recorded. After the 10 min test, the novel object was removed from the arena, and the rats remained in the arenas for subsequent social interaction testing.

**Social interaction**

Social interaction testing lasted 10 min and involved the placement of a novel rat (Stimulus rat) into each testing arena occupied by a Test rat. For experiment 1 (males), Test rats were paired with same-sex Stimulus rats of the same experimental condition (i.e. same Confinement Stress and Drug condition). For experiment 2 (females), Test rats were paired with same-sex Stimulus rats naive to experimental testing (i.e. noACS-NoINJ
rats) to allow for increased sample size. For both experiments, rats were scored on the
time spent in an interaction initiated by the Test rat, and time spent in an interaction
initiated by the Stimulus rat. A social interaction was defined as any physical interaction
between the Test rat and Stimulus rat, with the initiation of each interaction being
assigned to whichever rat (Test or Stimulus) was the actor at the onset of the interaction.
After the 10 min test, all rats were removed from the arenas and placed back into their
homecage in the housing room. Arenas were cleaned with Virox disinfectant after each
test.

Statistical analyses

Statistical analysis consisted of between group and repeated measure analysis of
variance (ANOVA), and multivariate analysis of variance (MANOVA) when appropriate.
Post hoc analyses consisted of F tests for simple effects, and LSD tests where
appropriate. Analyses were performed using SPSS version 24 (IBM Corp, USA). An
alpha level of $p < 0.05$ (two-tailed) was used to determine statistical significance. Partial
eta squared ($\eta^2_p$) are reported as a measure of effect size (for which a large effect size is
$\sim .26$, medium effect is size $\sim .13$, and small effect size is $\sim .02$).

Results

Although experiment 1 (males) was conducted separately from that of experiment
2 (females), we show the results for both experiments measure by measure to allow for
some comparison of the sexes.

Weight

Males
There were no differences in weight among the groups on the first day of experimental procedures on PND 30 (n = 24 / group; all ps > 0.42) (data not shown). The percent weight gained from PND 30 to PND 44 by ACS rats was less than noACS rats (F_{1,138} = 12.98, p < 0.001, \eta^2_p = .086) and there was an effect of Drug (F_{2,138} = 7.51, p = 0.001, \eta^2_p = .098) that did not interact with Confinement Stress (p = 0.66); AM251 rats weighed less than did VEH (p = 0.024) and NoINJ (p < 0.001) rats, which did not differ (p = 0.118) (see Fig. 2A).

Females

There were no differences in weight among the groups on the first day of experimental procedures on PND 30 (n = 26-28 / group; all ps > 0.63) (data not shown). The percent weight gained from PND 30 to PND 44 by ACS rats was less than noACS rats (F_{1,134} = 13.00, p < 0.001, \eta^2_p = .088) and there was an effect of Drug (F_{2,134} = 14.36, p < 0.001, \eta^2_p = .176) that did not interact with Confinement Stress (p = 0.25): AM251 rats weighed less than did VEH (p < 0.001) and NoINJ (p < 0.001) rats, which did not differ (p = 0.50) (see Fig. 2B).

Behaviour in the homecage

Note that for both experiments, the noACS and ACS groups differ in terms of timing since injection, and thus are not directly comparable, with the effects of AM251 more likely to be evident in the ACS group (for which the drug had more time to take effects) than in the noACS group. Thus, data are analyzed for the two groups separately.

Males

For the noACS groups (n = 12 cages / group), the MANOVA on behaviour during the first 3 days of injection was significant (F_{12,56} = 3.60, p = 0.001, \eta^2_p = .436), and the
effect of Drug was significant for Social Active (NoINJ < VEH and AM251; VEH > AM251), Social Inactive (NoINJ > VEH and AM251), and Self-directed (NoINJ < VEH and AM251) (all ps < 0.05). There was no effect of Drug for Alone Active (p = 0.058), Digging (p = 0.44) and Atypical (p = 0.08). The MANOVA on behaviour during the last 3 days of injection was significant (F_{12,56} = 10.15, p < 0.001, \eta^2_p = .685), and the effect of Drug was significant for each behavioural category: Social Active (NoINJ < VEH and AM251; VEH > AM251), Alone Active (NoINJ < VEH and AM251), Social Inactive (NoINJ > VEH and AM251), Digging (AM251 > NoINJ and VEH), Self-directed (NoINJ < VEH and AM251), Atypical (AM251 > NoINJ and VEH) (all ps < 0.014). (see Fig. 3A).

For the ACS groups (n = 12 cages / group), the MANOVA on behaviour during the first 3 days of injection was significant (F_{12,54} = 4.92, p < 0.001, \eta^2_p = .52), and the effect of Drug was significant for Social Active (AM251 < NoINJ and VEH), Alone Active (AM251 < NoINJ and VEH), Digging (NoINJ < VEH and AM251), and Self-directed (AM251 > NoINJ and VEH) (all ps < 0.024). There was no effect of Drug for Social Inactive (p = 0.32) and Atypical (p = .06) (see Fig. 3A). The MANOVA on behaviour during the last 3 days of injection was significant (F_{12,54} = 10.49, p < 0.001, \eta^2_p = .45), and the effect of Drug was significant for Social Active (AM251 < NoINJ and VEH), Alone Active (NoINJ < VEH and AM251), Self-directed (AM251 > NoINJ and VEH), and Atypical (AM251 > NoINJ and VEH). There was no effect of Drug for Social Inactive (p = 0.84) and Digging (p = 0.77) (see Fig. 3A).

_Females_
For the noACS groups (13-14 cages / group), the MANOVA on behaviour during the first days of injection was significant ($F_{12,66} = 12.63$, $p < 0.001$, $\eta^2_p = .697$), and the effect of Drug was significant for each behavioural category: Social Active (NoINJ < VEH and AM251; AM251 < VEH), Alone Active (NoINJ < VEH and AM251), Social Inactive (NoINJ > VEH and AM251), Digging (NoINJ < VEH and AM251), Self-directed (NoINJ < VEH and AM251; VEH < AM251), Atypical (AM251 > NoINJ and VEH) (all $p$s < 0.012) (see Fig. 3B). The MANOVA on behaviour during the last days of injection was significant ($F_{12,66} = 17.66$, $p < 0.001$, $\eta^2_p = .762$), and the effect of Drug was significant for each behavioural category: Social Active (NoINJ < VEH and AM251; AM251 < VEH), Alone Active (NoINJ < VEH and AM251), Social Inactive (NoINJ > VEH and AM251), Digging (NoINJ < VEH and AM251), Self-directed (AM251 > NoINJ and VEH; VEH > NoINJ), Atypical (AM251 > NoINJ and VEH) (all $p$s < 0.026) (see Fig. 3B).

For the ACS groups (13 cages / group), the MANOVA on behaviour during the first days of injection was significant ($F_{12,64} = 7.26$, $p < 0.001$, $\eta^2_p = .577$), and the effect of Drug was significant for Social Active (AM251 < NoINJ and VEH), Alone Active (AM251 < NoINJ and VEH), Digging (AM251 < VEH), Self-directed (AM251 > NoINJ and VEH), and Atypical (AM251 > NoINJ and VEH) ($p$s < 0.004); there was no effect of Drug for Social Inactive ($p = 0.63$) (see Fig. 3B). The MANOVA on behaviour during the last days of injection was significant ($F_{12,64} = 3.73$, $p < 0.001$, $\eta^2_p = .411$), and the effect of Drug was significant for Social Active (AM251 < NoINJ and VEH; VEH < NoINJ), Alone Active (AM251 < NoINJ and VEH), Self-directed (AM251 > NoINJ and VEH),
and Atypical (AM251 > NoINJ and VEH) (ps < 0.021). There was no effect of Drug for Social Inactive (p = 0.12) or Digging (p = 0.79) (see Fig. 3B).

**Elevated plus maze PND 45**

**Males**

There was no effect of Confinement Stress, or Drug, or interaction between the two, for any of the measures of anxiety (all ps > 0.23; n = 8/group). ACS rats made more entries into the closed arms of the maze than noACS rats (F$_{1,47}$ = 11.154, p = 0.002, $\eta^2_p = .21$). No effect of Drug, or interaction between Confinement Stress and Drug, was found for entries to the closed arms (ps > 0.51; see Fig. 4A).

**Females**

There was no effect of Drug for time on the open arm or entries into the open arm (all ps > 0.07; n = 8/group). ACS rats made more entries into the closed arms than did noACS rats (F$_{1,42}$ = 4.54, p = 0.04, $\eta^2_p = .098$), and there was no effect of Confinement Stress (p = 0.19) nor interaction of Confinement Stress and Drug (p = 0.83). There were no group differences for time in the closed arm (all ps > 0.10) (see Fig. 4B).

**Plasma corticosterone concentrations PND 45**

**Males**

A Confinement Stress X Drug X Time (baseline, immediately after confinement, one hour after confinement; n = 7 - 8 / group) ANOVA found a main effect of Time (F$_{2,124}$ = 169.49, p < 0.001, $\eta^2_p = .732$) (see Fig. 5A). Analyses at the separate time points indicated that immediately after confinement, ACS rats had lower plasma CORT than did noACS rats (p = 0.02) (see Fig. 5A) with no effect of Confinement Stress or Drug, or
interaction between the two, on plasma CORT at baseline or 60 min after confinement (ps > 0.05) (see Fig. 5A).

Females

A Confinement Stress X Drug X Time (baseline, immediately after confinement, one hour after confinement; n = 5 – 10 / group) ANOVA found a main effect of Time (F_{2,117} = 89.31, p < 0.001, \eta^2_p = .604), and an interaction of Drug X Time (F_{4,117} = 2.638, p = 0.037) (other ps > 0.17) (see Fig. 5B). Analyses at the separate time points indicated that at baseline, VEH rats had higher CORT than did NoINJ (p = 0.03) rats; VEH and AM251 rats did not differ (p = 0.60). There were no differences among Drug groups immediately after confinement, (ps > 0.06). After 60 min back in the homecage, ACS rats had higher CORT than noACS rats (p < 0.001), and the Drug groups did not differ (ps > 0.78) (see Fig. 5B).

Social interaction test PND 46

Males

There were no group differences for latency to enter the centre of the open field, time spent in the centre of the open field, or for total distance travelled in the open field (all ps > 0.37) (see Table 1).

There were no group differences in latency to approach an object placed in the open field, or in time spent with the object (all ps > 0.31) (see Table 1).

A mixed model ANOVA with rat (Test or Stimulus) as the within group factor, and Confinement Stress and Drug as the between group factors (n = 4 pairs / group) found that Test Rats initiated more social interaction than did Stimulus Rats (F_{1,18} = 36.77, p < 0.001, \eta^2_p = .671). The effect of Drug was significant (F_{2,18} = 4.18, p = 0.032,
VEH rats spent more time engaging in social interactions than did NoINJ rats (see Fig. 6A).

**Females**

There were no group differences for latency to enter the centre of the open field, time spent in the centre of the open field, or for total distance travelled in the open field (all ps > 0.16) (see Table 1). There were no group differences in latency to approach an object placed in the open field, or in time spent with the object (all ps > 0.22) (see Table 1).

A Confinement Stress X Drug X Individual (Test Rat vs Stimulus Rat) ANOVA indicated that the Test Rats initiated more time in social interaction than did Stimulus rats ($F_{1,42} = 211.49, p < 0.001, \eta^2_p = .834$), and that the interaction of Drug and Individual was significant ($F_{2,42} = 3.41, p = 0.042, \eta^2_p = .14$; other ps > .20). Post hoc analysis indicated that among Test Rats, AM251 rats initiated more time in social interaction than did NoINJ rats ($p = 0.035$, other ps > 0.14). Among Stimulus rats, those interacting with AM251 rats spent less time initiating social interactions than did Stimulus rats interacting with VEH ($p = 0.045$) rats (see Fig. 6B).

**Corticolimbic protein expression**

**Males**

In the ventral hippocampus, the only effect of Confinement Stress for any of the 4 proteins investigated was that of greater GAD67 expression in ACS rats compared to noACS rats ($F_{1,46} = 19.451, p < 0.001, \eta^2_p = .33$) (see Fig. 7B). There was no effect of Drug for any of the 4 proteins in the ventral hippocampus (all ps > 0.085). There were no effects of Confinement Stress or of Drug, or interaction between the two, for any of the 4
proteins investigated in the dorsal hippocampus (all ps > 0.08) or PFC (all ps > 0.16) (see Table 2).

**Females**

In the PFC, the only effect of Confinement Stress for any of the 7 proteins was that of higher FOS expression in ACS than in noACS females ($F_{1,43} = 10.46, p = 0.002, \eta^2_p = .196$; all other ps > 0.09) (see Fig. 7B). The only effect of Drug was for GAD67 ($F_{2,43} = 7.13, p = 0.002, \eta^2_p = .249$; other ps > 0.40), whereby AM251 rats had higher GAD67 expression than did VEH ($p = 0.006$) and NoINJ ($p < 0.001$) females (see Fig. 7A). No interaction of the two factors was significant (ps > 0.14) (see Table 2).

In the dorsal hippocampus, there was lower spinophilin and higher GR expression in ACS than in noACS females ($F_{1,44} = 5.01, p = 0.029, \eta^2_p = .104$, and $F_{1,44} = 5.04, p = 0.03, \eta^2_p = .103$, respectively; all other ps > 0.14) (see Table 2 and Fig. 7B, respectively). The only effect of Drug was for CB1 ($F_{2,44} = 5.18, p = 0.01, \eta^2_p = .19$; other ps > 0.06), whereby AM251 rats had lower CB1 expression than did VEH ($p = 0.049$) and NoINJ ($p = 0.002$) females (see Fig. 7A). No interaction of the two factors was significant (ps > 0.10). The effect of Drug did not reach statistical significance for spinophilin ($p = 0.066$) or GAD67 (ps > 0.063) (see Table 2).

In the ventral hippocampus, there was lower GAD67 expression in ACS than in noACS females ($F_{1,42} = 4.48, p = 0.04, \eta^2_p = .096$; other ps > 0.26) (see Fig. 7B). There was no effect of Drug on any of the protein measures (ps > 0.068) (see Table 2).

**Discussion**

In contrast to our predictions that confinement stress exposures would augment the effects of CB1 receptor antagonism on subsequent function (i.e., > 24 h after
treatments), the two manipulations had independent effects. Further, the effects were sex-specific, with the effects of CB1 receptor antagonism more evident in females than in males (Table 3 summarizes the results).

**Effects of CB1 receptor antagonism**

*Elevated plus maze*

In the present experiments, adolescent CB1 receptor antagonism/inverse agonism had no effect on anxiety in the EPM in male or female rats tested 24 hours after repeated drug exposure. Although this result contrasts reports of increased anxiety after CB1 receptor antagonism in adult males (Haller et al., 2002; Navarro et al., 1997; Simone et al., 2015a) and females (Hill et al., 2007; Simone et al., 2015b), those studies measured behaviour 30-60 min after administration, whereas we measured behaviour 24 hours after the final exposure to the drug. Our findings are consistent with the report of no effect of an acute administration of 0.25 mg/kg AM251 on anxiety in an open field test in adolescent male rats (Pandolfo et al., 2007) and with the report that adult male rats treated repeatedly with 5 mg/kg AM251 during adolescence did not differ from controls in the EPM when tested in a drug-free state in adulthood (Lee et al., 2015). Nevertheless, it is possible that desensitization of CB1 receptors prevented a difference in anxiety (O’Brien et al., 2013) or that differences would have been found for other measures of affective state (Beyer et al., 2010).

*Social interactions*

AM251 females in the present study spent more time in social interactions than did NoINJ females, irrespective of Confinement Stress condition; thus, repeated CB1 receptor antagonism during adolescence may lead to reduced social anxiety (more time in
social interaction corresponds to reduced social anxiety; (File and Seth, 2003). There was no effect of either AM251 or Confinement Stress in males even though males were tested in pairings of rats from the same treatment condition (which should enhance any treatment effect, see for example (Green et al., 2013)) whereas females were paired with non-treated Stimulus females; thus, female adolescents’ social behaviour may be more susceptible to repeated AM251 treatment.

Pharmacological enhancement of endocannabinoid signalling via inhibition of endocannabinoid hydrolysis and re-uptake is associated with increased social play in adolescent rats, whereas direct cannabinoid receptor agonists typically reduce social play (Trezza et al., 2010). The discrepancy in the effects of direct versus indirect CB1 receptor agonists is likely the result of the on-demand nature of endocannabinoid signalling; whereas direct agonists activate CB1 receptors indiscriminately throughout the organism, endocannabinoid hydrolysis and re-uptake inhibitors only have effects where endocannabinoids are being released (Trezza et al., 2010). Our observations of increased social interactions with a CB1 receptor antagonist are thus consistent with the findings of reduced social interactions in both male (O'Shea et al., 2006; Schneider et al., 2008) and female (O'Shea et al., 2004) rats after treatment with direct CB1 receptor agonists and tested after a drug washout period of at least 3 weeks. Conversely, that the effects of previous exposure to AM251 in the present study are like those reported with acute administrations of endocannabinoid hydrolysis and re-uptake inhibitors (Trezza et al., 2010) suggests that repeated AM251 treatment caused CB1 receptor sensitization, thereby enhancing endogenous CB1 transmission.

*Neuroendocrine stress responses*
In contrast to our predictions of increased neuroendocrine stress responses, we found no effect 24 h after AM251 treatment (i.e., in the absence of drug) on CORT release to confinement stress in either sex. A previous study found that adolescent male rats exposed to daily injections of AM251 had increased habituation of CORT responses to repeated restraint stress relative to vehicle treated males when tested as adults (i.e., in a drug-free state) (Lee et al., 2015). Thus, the developmental effects of adolescent CB1 receptor antagonism on neuroendocrine stress responses in males may emerge with time. Alternatively, given the 1 h duration of the confinement stress in the present study and given that peak CORT concentrations have been reported after 30 min of restraint stress, it is possible that we are capturing the recovery phase, and not the peak, of the stress response and that group differences may have been observed at an earlier timepoint after the onset of the stressor. Thus, given that we collected measures pertaining to stress responses 24 hours after the final drug exposure, our results suggest that there are no immediate residual effects of CB1 receptor antagonism on neuroendocrine stress responses in either sex, though we cannot rule out effects of AM251 while it is in the system.

*Corticolimbic protein expression*

We found no effects of AM251 on markers of synaptic plasticity in male (PSD95) or female (PSD95, spinophilin) rats despite a previous report of increased PSD95 expression in the PFC of adolescent female rats repeatedly exposed to AM251 (Rubino et al., 2015). AM251 treatment in females in the present study reduced CB1 receptor expression in the dorsal hippocampus and increased GAD67 (marker of GABAergic neurons) expression in the prefrontal cortex relative to VEH and NoINJ females. In adult
male mice, repeated exposure to AM251 reduced hippocampal CB1 receptor expression and increased prefrontal cortical CB1 receptor expression, and these changes were associated with altered behavioural responding to a CB1 receptor agonist (Tambaro et al., 2013). Thus, the behavioural effects of AM251 in females in the present study may involve enduring alterations to the expression and/or sensitivity of CB1 receptors. Nevertheless, why our effects were sex-specific remains to be determined. The changes in protein expression observed in AM251 females may be long-lasting; the previous report of increases in glutamatergic markers within the PFC after repeated adolescent CB1 receptor antagonism were maintained into adulthood (Rubino et al., 2015).

During adolescence, corticolimbic GABAergic systems continue to mature, and the expression of GABAergic cells increases (Caballero et al., 2013; Caballero et al., 2014). Disruption of endocannabinoid signalling via repeated exposure to the CB1 receptor agonist WIN 55,212-2 reduced GABAergic transmission within the PFC of adult male rats (Cass et al., 2014). Similarly, repeated exposure to THC during adolescence reduced the expression of GAD67 in the PFC of adult male rats (Zamberletti et al., 2014). Thus, our finding of a CB1 receptor antagonist-induced increase in PFC GAD67 expression is consistent with an inhibitory role of CB1 receptor signalling in corticolimbic GABAergic transmission during adolescence.

*Effects of repeated confinement stress*

*Elevated plus maze*

Although there were no observable effects of confinement stress on anxiety-like behaviours in either male or female rats in the present study, ACS rats of both sexes made more entries onto the closed arms of the EPM relative to noACS rats, indicative of
increased locomotor behaviour. Our findings of increased locomotor activity on the EPM are consistent with reports of stress-induced increases in locomotor activity in adult rats (Grønli et al., 2005) and mice (Keeney et al., 2001). The increase in locomotor activity in the present study, however, was specific to the EPM, with no differences observed in the open field, which makes the interpretation of the increased locomotion in the EPM difficult to interpret.

*Neuroendocrine stress responses*

When repeatedly exposed to a homotypic stressor, stress-induced CORT release typically diminishes, a process referred to as habituation (Grissom and Bhatnagar, 2009; Herman, 2013). In the present study, there was habituation of CORT responses in males undergoing a 16th bout of confinement stress compared with males undergoing their first bout, consistent with a previous report in post-pubertal (> post-natal day 42) adolescent male rats (McCormick et al., 2007). Shorter bouts (30 min) of restraint stress for 7 days did not result in habituation in pre-pubertal rats (28 days-of-age at time of testing) (Romeo et al., 2006). In contrast to our previous report of habituation to repeated confinement in adolescent females (McCormick et al., 2007), ACS females in the present study did not differ from noACS females in their CORT response immediately after confinement, and they had an enhanced recovery to baseline CORT concentrations when measured 60 min after the cessation of the stressor. There is a paucity of studies of neuroendocrine responses to repeated stressors in adolescents, and any differences across studies likely depend on the strain, sex, and age of the rats, and on the duration and severity of the stressor (McCormick et al., 2017). In studies with adult rats, there are reports of sex differences in habituation, with females less likely to show habituation than
are males (Bhatnagar et al., 2005). Although organizational and activational effects of sex hormones are implicated in the sex differences in stress responses (Handa and Weiser, 2014), the mechanisms driving differences in the adaptation to a repeated homotypic stressor are not known.

*Corticolimbic protein expression*

There was no effect of repeated confinement stress on hippocampal GR expression in adolescent males, consistent with no effect of repeated restraint on GR mRNA expression in pre-pubertal males (Romeo et al., 2008). In adult males, repeated stress typically is associated with reductions in corticolimbic GR expression (Chiba et al., 2012; Makino et al., 1995; Uchida et al., 2008). Less is known, however, regarding repeated stress effects on GR expression in females. In the present study, ACS females had greater expression of GR in the dorsal hippocampus, which may be the basis of their enhanced recovery of CORT concentrations after stress compared to noACS females; negative feedback of CORT release involves actions at hippocampal GR (Herman et al., 2016). ACS females had increased FOS expression in the PFC relative to noACS females. As the PFC is also associated with inhibition of neuroendocrine stress responses, the increased basal FOS expression observed in ACS females may reflect increased prefrontal cortical output to stress-regulatory brain regions, and thus may explain, in part, the enhanced recovery from stress observed in ACS females. Nevertheless, further studies are required to determine if the observed changes in corticolimbic protein expression are causally linked to the changes in neuroendocrine stress responses.

In the ventral hippocampus, stress effects on GABAergic systems were evident in both male and female rats, albeit in opposite directions. Whereas repeated confinement
stress increased GAD67 expression in the ventral hippocampus of males, it decreased expression in females. Like the PFC (as outlined above), hippocampal GABAergic systems undergo protracted development during adolescence (Caballero et al., 2013), and thus, are sensitive to environmental influences such as stress exposure. The mechanisms driving the different pattern of effects in males and females, as well as the functional consequences of these differences, however, are not known.

**Effects of repeated injection stress**

An important caveat of pharmacological investigations in adolescent rats is the susceptibility to the effects of injection stress (Keeley et al., 2015a; Keeley et al., 2015b; Waters and McCormick, 2011). In the present study, injection stress effects were observed in both males and females. In males, injection stress effects were most evident behaviourally, with VEH rats spending more time initiating social interactions than NoINJ rats, whereas injection stress effects were absent in females. Together, our findings provide evidence of altered social behaviour in adolescent male rats exposed to daily injection stress and highlight potential sex-differences in the effects of adolescent injection stress.

**Potential mechanisms underlying sex-specific effects of repeated CB1 receptor antagonism**

Across measures, we observed sex-specific effects of repeated CB1 receptor antagonism in adolescent rats, with effects more evident in females than males. Previous reports have described sex differences in behavioural responses to cannabinoid drugs with females typically having a greater sensitivity, and thus, lower effective doses than males (Craft et al., 2012; Tseng and Craft, 2001; Tseng et al., 2004). As such, it is
possible that effects may have been observed in males with a larger dose of AM251.

Nevertheless, the dose of 1 mg / kg used in the present study caused reductions in weight gain and reduced social behaviours and increased Self-directed and Atypical behaviours when in the homecage in both males and females. The increase in Self-directed behaviours was driven mainly by increases in AM251-induced pruritic responses, consistent with a previous report of increased scratching after administration of AM251 (Tallett et al., 2009). The sex-specific effects of repeated CB1 receptor antagonism may reflect sex-specific regulation of downstream signal transduction pathways by the endocannabinoid system. Sex-differences in the pharmacokinetics and pharmacodynamics of cannabinoid drugs and in CB1 receptor sensitization and expression also are likely contributing factors.

Conclusions

The results of the present study suggest that females are more susceptible to the effects of altered endocannabinoid signalling that endure post-treatment. Specifically, AM251 females had reduced CB1 receptor expression and increased GAD67 expression in the hippocampus 24 hours after, and increased social interactions 48 hours after, the final drug exposure; there were no effects of AM251 on any measure in males. Given that the half-life of AM251 is ~22 h in adult rats (McLaughlin et al., 2003), and that we tested rats 24 or 48 h after their final exposure to AM251, it is possible that the observed drug effects are because of effects on downstream neurodevelopmental processes. Indeed, adolescence is a critical period for neurodevelopment that involves the maturation of excitatory and inhibitory circuits within corticolimbic regions involved in regulation of anxiety behaviours and physiological stress responses (Crews et al., 2007). Likewise,
endogenous cannabinoid signalling also undergoes extensive maturation and refinement across the adolescent period (Lee and Gorzalka, 2015), and given the role of the ECS in regulation of stress and anxiety responses, developmental changes in endocannabinoid signalling may underlie changes in emotional reactivity. Alternatively, we cannot rule out the possibility that the sex-specific effects of AM251 involve differences in absorption, distribution, and/or metabolism of the drug. Nevertheless, our results of immediate (e.g., within 48 h of the final drug exposure), sex-specific effects of adolescent CB1 receptor antagonism, along with results of long-lasting effects on brain and behaviour in studies by other researchers (Lee et al., 2015; Rubino et al., 2015) highlight the importance of understanding the role of endogenous cannabinoid signalling during the adolescent period.
Tables

Table 2-1. Means (including S.E.M.) for time spent in the centre, latency to approach the centre, and total distance travelled in the open field, and for latency to approach, and time spent interacting with a novel object for both male (left) and female (right) rats.
<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Open field</td>
<td>Novel object</td>
</tr>
<tr>
<td></td>
<td>Time in centre (s)</td>
<td>Latency to approach (s)</td>
</tr>
<tr>
<td></td>
<td>Latency to centre (s)</td>
<td>Time investigating (s)</td>
</tr>
<tr>
<td></td>
<td>Total distance travelled (m)</td>
<td></td>
</tr>
<tr>
<td>noACS</td>
<td>65.58 (18.73)</td>
<td>192.33 (137.22)</td>
</tr>
<tr>
<td>NoINJ</td>
<td>24.65 (10.58)</td>
<td>81.39 (36.88)</td>
</tr>
<tr>
<td></td>
<td>5.14 (0.39)</td>
<td>(18.73)</td>
</tr>
<tr>
<td></td>
<td>192.33 (137.22)</td>
<td>81.39 (36.88)</td>
</tr>
<tr>
<td>VEH</td>
<td>99.13 (40.52)</td>
<td>30.03 (16.38)</td>
</tr>
<tr>
<td></td>
<td>39.50 (6.34)</td>
<td>176.34 (22.44)</td>
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<td></td>
<td>5.12 (0.21)</td>
<td>(9.83 (10.19)</td>
</tr>
<tr>
<td></td>
<td>30.03 (16.38)</td>
<td>176.34 (22.44)</td>
</tr>
<tr>
<td>AM251</td>
<td>78.00 (25.95)</td>
<td>195.13 (137.78)</td>
</tr>
<tr>
<td></td>
<td>40.30 (16.90)</td>
<td>100.57 (39.61)</td>
</tr>
<tr>
<td></td>
<td>4.58 (0.12)</td>
<td>(7.75 (4.64)</td>
</tr>
<tr>
<td></td>
<td>195.13 (137.78)</td>
<td>100.57 (39.61)</td>
</tr>
<tr>
<td>ACS</td>
<td>49.00 (6.75)</td>
<td>29.35 (13.75)</td>
</tr>
<tr>
<td>NoINJ</td>
<td>4.45 (20.60)</td>
<td>109.73 (32.75)</td>
</tr>
<tr>
<td></td>
<td>5.30 (0.30)</td>
<td>(11.59 (6.47)</td>
</tr>
<tr>
<td></td>
<td>29.35 (13.75)</td>
<td>109.73 (32.75)</td>
</tr>
<tr>
<td>VEH</td>
<td>77.70 (31.28)</td>
<td>161.20 (146.58)</td>
</tr>
<tr>
<td></td>
<td>28.90 (10.44)</td>
<td>116.77 (57.52)</td>
</tr>
<tr>
<td></td>
<td>5.01 (0.13)</td>
<td>(6.59 (9.38)</td>
</tr>
<tr>
<td></td>
<td>161.20 (146.58)</td>
<td>116.77 (57.52)</td>
</tr>
<tr>
<td>AM251</td>
<td>70.13 (3.67)</td>
<td>68.76 (51.46)</td>
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<td></td>
<td>30.40 (6.20)</td>
<td>181.65 (100.41)</td>
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<td></td>
<td>5.57 (0.36)</td>
<td>(7.94 (6.70)</td>
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<td>68.76 (51.46)</td>
<td>181.65 (100.41)</td>
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<td></td>
<td>63.74 (3.67)</td>
<td>(1.92 (16.35)</td>
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<tr>
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<td>63.74 (3.67)</td>
<td>(1.92 (16.35)</td>
</tr>
</tbody>
</table>
Table 2-2. Means (including S.E.M.) for the different protein measures in the dorsal hippocampus, ventral hippocampus, and prefrontal cortex of male (left) and female (right) rats.

<table>
<thead>
<tr>
<th>Dorsal hippocampus</th>
<th>Dorsal hippocampus</th>
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<tbody>
<tr>
<td>CB1</td>
<td>PKA</td>
</tr>
<tr>
<td>noACS</td>
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</tr>
<tr>
<td>N=9</td>
<td>58.14 (25.57)</td>
</tr>
<tr>
<td>VEH</td>
<td>56.06 (18.62)</td>
</tr>
<tr>
<td>AM251</td>
<td>53.33 (9.32)</td>
</tr>
<tr>
<td>ACS</td>
<td>N=9</td>
</tr>
<tr>
<td>N=9</td>
<td>64.57 (26.01)</td>
</tr>
<tr>
<td>VEH</td>
<td>56.75 (22.42)</td>
</tr>
<tr>
<td>AM251</td>
<td>55.36 (22.17)</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Ventral hippocampus</th>
<th>Ventral hippocampus</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB1</td>
<td>PKA</td>
</tr>
<tr>
<td>noACS</td>
<td>N=9</td>
</tr>
<tr>
<td>N=9</td>
<td>15.34 (35.61)</td>
</tr>
<tr>
<td>VEH</td>
<td>15.61 (52.13)</td>
</tr>
<tr>
<td>AM251</td>
<td>15.24 (22.75)</td>
</tr>
<tr>
<td>ACS</td>
<td>N=9</td>
</tr>
<tr>
<td>N=9</td>
<td>16.64 (34.70)</td>
</tr>
<tr>
<td>VEH</td>
<td>18.31 (34.52)</td>
</tr>
<tr>
<td>AM251</td>
<td>18.21 (28.13)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Prefrontal cortex</th>
<th>Prefrontal cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB1</td>
<td>PKA</td>
</tr>
<tr>
<td>noACS</td>
<td>N=9</td>
</tr>
<tr>
<td>N=9</td>
<td>49.67 (30.08)</td>
</tr>
<tr>
<td>VEH</td>
<td>44.23 (32.80)</td>
</tr>
<tr>
<td>AM251</td>
<td>48.78 (32.71)</td>
</tr>
<tr>
<td>ACS</td>
<td>N=9</td>
</tr>
<tr>
<td>N=9</td>
<td>44.57 (32.48)</td>
</tr>
<tr>
<td>VEH</td>
<td>42.13 (31.57)</td>
</tr>
<tr>
<td>AM251</td>
<td>52.21 (35.98)</td>
</tr>
</tbody>
</table>

*a Denotes a significant main effect of Drug (p < 0.05).
*b Denotes a significant main effect of Stress (p < 0.05).
Figures

Fig. 2-1. Experimental design and timeline of experimental procedures.
Fig. 2-2. Mean (+/- S.E.M.) percent weight gain from the first day of treatment (PND 30) to the last day of treatment (PND 44) in (A.) male and (B.) female rats. † main effect of stress, p < 0.05. * compared with NoINJ, p < 0.05. # compared with VEH, p < 0.05.
Fig. 2-3. Mean (+/- S.E.M) number of observations for each behavioural category for the first three days of treatment (PND 30-32) and for the last three days of treatment (PND 42-44) in (A.) male and (B.) female rats. * compared with NoINJ, # compared with VEH, p < 0.05.
Fig. 2-4. Mean (+/- S.E.M.) for behavioural measures in the elevated plus maze on PND 45 (24 h after final treatment) in (A) male and (B) female rats. † main effect of Stress, p < 0.05.
Fig. 2-5. Mean (+/- S.E.M.) plasma corticosterone concentrations (µg / dL) on PND 45 immediately before, immediately after, and 60 min after the first (noACS) or 16th (ACS) exposure to 1 h confinement stress in (A.) male and (B.) female rats. Lower figures depict the post hoc comparisons for significant interactions for each sex.
Fig. 2-6 Mean (+/- S.E.M.) time spent engaging in social interactions initiated by either the Test or Stimulus rat in (A.) male and (B.) female rats. Right-sided figures depict the significant main effect of Drug in males and the post hoc comparisons for the significant interaction in females.
Fig. 2-7. Significant main effects of (A.) AM251 and (B.) confinement stress on neural protein measures in the dorsal hippocampus (DH), ventral hippocampus (VH), and prefrontal cortex (PFC) of male and female rats. * compared with NoINJ, # compared with VEH, † compared with noACS, ps < 0.05.
Chapter 3: Long-term effects of adolescent CB1 receptor antagonism on emotion and cognition in male and female rats.
This chapter is submitted for publication in the journal *International Journal of Developmental Neuroscience* as:

Simone, J. J., Baumbach, J. L., & McCormick, C. M. Sex-specific effects of CB1 receptor antagonism and stress in adolescence on anxiety, corticosterone concentrations, and contextual fear in adulthood in rats.

Author contribution: I was the primary investigator of this work, responsible for the majority of study design, data collection, data analysis, and writing of the manuscript. I performed the injections and stress procedures, as well as the behavioural testing and scoring, hormone extractions and CORT assays, and protein extractions and Western blot experiments.

Jennet Baumbach assisted with the behavioural testing and scoring of elevated plus maze videos. Dr. Cheryl McCormick assisted in designing the experiments (30%), analyzing the data, and editing the manuscript.
Introduction

The contributions of endocannabinoid signalling in adolescence to the normative development of emotional behaviours and stress reactivity are not well understood. Long-term developmental effects of adolescent exposure to cannabinoid drugs, however, are well documented in both humans (Crean et al., 2011) and rodents (Rubino and Parolaro, 2008). Developmental effects of cannabinoid drugs on brain and behaviour are attributed to actions at the cannabinoid type-1 (CB1) receptor (Renard et al., 2016a). Consistent with effects of cannabinoid drugs on emotional behaviours and neuroendocrine stress responses (McLaughlin and Gobbi, 2012), CB1 receptors are densely expressed in corticolimbic brain regions such as the prefrontal cortex (PFC) and hippocampus (de Fonseca et al., 1993; Tsou et al., 1998; Tsou et al., 1999), and there are changes in expression in adolescence. For example, in the PFC, CB1 receptor expression decreased in male rats from PND 29-50 (Ellgren et al., 2008), whereas expression increased in females from PND 46-60, then decreased to adult levels by PND 75 (Rubino et al., 2015).

Behaviourally, repeated exposure to CB1 receptor agonists during adolescence is associated with long-term reductions in social behaviours and object recognition memory in rats (O'Shea et al., 2006; O'Shea et al., 2004; Renard et al., 2013; Renard et al., 2017; Schneider et al., 2008). In accordance with effects on behaviour, adolescent exposure to cannabinoid drugs is associated with long-term alterations to brain structure and function that are evident into adulthood. In male rats, repeated exposure to the CB1 receptor agonist Δ⁹-tetrahydrocannabinol (THC) during adolescence induced a state of dopaminergic hyperactivity in the mesocorticolimbic system, as well as reduced the activity of the Akt/mTOR signalling pathway within the PFC when measured in
adulthood (Renard et al., 2017). Similarly, male rats exposed to the synthetic CB1 receptor agonist CP55,940 in adolescence were found to have reduced PFC dendritic complexity, reduced hippocampal-induced PFC plasticity, and reductions in the glutamatergic synaptic marker PSD95 in the PFC in adulthood (Renard et al., 2016b). In female rats repeatedly exposed to THC during adolescence there was a similar reduction in dendritic complexity within the PFC, however, the effects on markers of glutamatergic synapses were opposite to those previously reported in males; THC treated females had increased expression relative to vehicle-treated females (Rubino et al., 2015). That disruptions to normative CB1 receptor signalling via administration of agonists leads to altered neuronal and behavioural phenotypes is not surprising given the role of CB1 receptors in regulating trans-synaptic communication (Viveros et al., 2007). Nevertheless, the role of endogenous cannabinoid signalling during adolescence on brain and behaviour development is poorly understood.

Under normative physiological conditions, CB1 receptors are activated by a family of lipid neuromodulators known as endocannabinoids (Di Marzo, 2011). Although endocannabinoid signalling in adolescence has not been well characterized, studies investigating the effects of repeated administration of the CB1 receptor selective antagonist / inverse agonist AM251 during adolescence have confirmed a role for endocannabinoid signalling during this period of development. In male rats, adolescent AM251 exposure (5 mg / kg, PND 35-45) resulted in increases in risk-assessment and stress-coping behaviours, as well as increases in PFC CB1 receptor expression, when tested several weeks after the final drug exposure (Lee et al., 2015). In female rats, repeated AM251 exposure during adolescence (0.5 mg / kg, PND 35-45) prevented
developmental decreases in glutamatergic markers within the PFC into adulthood (Rubino et al., 2015). Previously, we reported sex-specific effects of repeated adolescent CB1 receptor antagonism (1 mg / kg, PND 30–44) on brain and behaviour when tested 24–48 h after the final exposure to the antagonist, and drug effects were present whether exposure occurred alone or in tandem with a psychological stressor (Simone et al., 2018). Specifically, AM251 increased time spent in social interactions, increased the expression of the PFC GABAergic marker GAD67, and reduced dorsal hippocampal CB1 receptor expression in female, but not in male, rats (Simone et al., 2018). Nevertheless, many manipulations in adolescence require an incubation period before their effects are manifested (Isgor et al., 2004; Lee et al., 2015; Trauth et al., 2000). Thus, in experiment 1, we tested the hypotheses that (1) repeated adolescent CB1 receptor antagonism will alter neurodevelopment leading to changes in anxiety-like behaviour and neuroendocrine stress responses in adulthood, and that (2) effects would be more pronounced when antagonism occurred in tandem with a stressor than when alone because of the on-demand nature of endocannabinoid signalling (i.e., an increase in endocannabinoid release is expected in response to stress (Hillard, 2014)). To address these questions, male (experiment 1A) and female (experiment 1B) rats were treated with either the CB1 receptor selective antagonist / inverse agonist AM251 or vehicle, or were untreated, on PND 30-44. Males and females were investigated in separate experiments because of the high number of comparisons within each sex, and because it is expected that any effects observed would be sex-specific. To investigate endocannabinoid-stress interactions, half of all rats from each experiment received treatment and were immediately placed back into their homecages, whereas the other half received treatment immediately before 1 h of
confinement stress. Measures of anxiety (elevated plus maze), neuroendocrine stress responses (plasma corticosterone), and neural protein markers of the endocannabinoid system (CB1 receptors, PKA), GABAergic system (GAD67), glutamatergic system (PSD95), as well as a marker for synaptic plasticity (spinophilin), were obtained in adulthood (PND 70-74) several weeks after the final exposure to AM251. In a second experiment, we investigated the long-term effects of adolescent AM251 on cognitive behaviours in male (experiment 2A) and female (experiment 2B) rats. We previously reported AM251-mediated increases in PFC GAD67 expression and decreases in dorsal hippocampal CB1 receptor expression (Simone et al., 2018), thus we tested rats in a contextual fear conditioning and extinction paradigm; contextual fear conditioning and extinction have been shown to critically depend on dorsal hippocampal and PFC signalling (Maren, 2001; Quirk and Mueller, 2008; Sierra-Mercado et al., 2011). Because the behavioural effects of AM251 observed in experiment 1 were independent of stress exposures, we limited our comparisons in experiment 2 to vehicle and AM251 rats only, allowing for an increase in statistical power and a reduced number of rats.

Methods

Experiment 1

See Fig. 1 for the experimental design and timeline. Testing procedures were identical for experiments 1A (males) and 1B (females).

Animals

Forty-eight male (experiment 1A) and 48 female (experiment 1B) Long-Evans rats (Charles River, Kingston, New York, USA) arrived at the Brock University Comparative Bioscience Facility on PND 25 and were housed in same-sex pairs. Rats
were kept on a 12-hour light-dark cycle (lights on at 8:00 h) and were provided access to food and water *ad libitum*. All procedures were approved by the Brock University Institutional Animal Care Committee and were in accordance with the Canadian Council on Animal Care and with the National Institutes of Health guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1996). All efforts were made to minimize the number of animals used and their suffering.

**Weights**

Rats were weighed daily during the light phase immediately before drug or stress exposures each of the 15 days of treatment (PND 30-44). Rats were weighed again on PND 70 approximately two hours before behavioural testing.

**Adolescent confinement stress**

Adolescence in rats has been defined liberally as spanning from PND 21-59 (Tirelli et al., 2003). The average age of pubertal onset in males (assessed by balanopreputial separation) is PND 40, and in females (assessed by vaginal opening) is PND 35. For the present experiment, we chose a peri-pubertal range of PND 30-45 that corresponds with the period of mid-late adolescence and is known to be a sensitive period for the effects of stressors (Burke et al., 2017). Rats were assigned to either the Adolescent Confinement Stress (ACS) protocol or to the No Confinement Stress (noACS) protocol daily for 15 days (PND 30-44). ACS rats were removed from their homecage and placed individually into small, ventilated plastic containers (14 cm in height, 10 cm in diameter) in a separate room from the housing colony for 1 h, a procedure that results in high concentrations of plasma corticosterone (Hodges and McCormick, 2015). After 1 h of confinement stress, rats were returned to their homecage.
The stress protocol was performed at a different time each day (09:00-20:00 h) during the light cycle to minimize habituation. Rats in the noACS group remained undisturbed in their homecages during the confinement stress procedure.

**Drugs and injections**

The CB1 receptor antagonist / inverse agonist AM251 (Cayman Chemical, USA) was dissolved in a 1:1:18 dilution of DMSO, Tween-80, and 0.9% saline and administered i.p. at a concentration of 1.0 mg / kg. The vehicle was a 1:1:18 mixture of DMSO, Tween-80, and 0.9% saline in the absence of AM251. The dose of AM251 was chosen based on previous work from our lab (Simone et al., 2018). Injections were administered at a volume of 1.0 mL / kg. Vehicle (VEH) and AM251 injected rats in the noACS groups were returned directly to their homecages after injection. VEH and AM251 injected rats in the ACS groups were injected immediately before the confinement stress procedure each day. To account for any stress of injection effects we also included a no injection (NoINJ) comparison group for both the noACS and ACS conditions.

**Elevated plus maze**

The elevated plus maze is a test of unconditioned avoidance behaviour that has been well-validated as a measure of anxiety in rodents (Wall and Messier, 2001). The apparatus consisted of two open arms and two closed arms extended from a common central platform 80 cm in height. The maze was constructed of grey plastic and was situated in the centre of the testing room. Rats were tested in the elevated plus maze on PND 70 (see Fig. 1 for experimental timeline). Rats were individually transported from the housing room to the testing room in an empty cage lined with paper towel, placed
onto a closed arm of the maze, and left to explore the maze for 5 min. Testing took place under low illumination and occurred between 10:00 h and 12:00 h. Behaviours were recorded by an overhead camera, and were scored by an observer blind to experimental condition. The behavioural measures analyzed were the time spent on the open arms, time spent on the closed arms, number of entries onto an open arm, and number of entries onto a closed arm. An arm entry was recorded when the two front paws of the animal were in the arm. The time spent on the open arms of the maze and the number of entries onto an open arm are the standard measures of anxiety-like behaviour in this test (Wall and Messier, 2001). The number of entries onto the closed arms of the maze is used as a measure of locomotor activity (Cruz et al., 1994; Rodgers and Dalvi, 1997). The maze was cleaned with Virox disinfectant between each test session. To minimize effects of separation of cage partners on performance, cage partners were tested 2 h apart to allow recovery from any stress attributable to the cage partner’s absence of approximately 7 min.

Restraint stress, blood collection, and plasma hormone analysis on PND 72

On PND 72 (48 h after EPM testing), rats were placed into small plastic restraint cylinders for 30 min. Blood was collected from each rat immediately before placement into the restrainers (baseline; BL), immediately after (T0), 45 min after (T45), and 90 min after (T90) 30 min of restraint stress. Blood samples were collected from a tail nick into chilled microcentrifuge tubes containing EDTA powder (Sarstedt). Samples were centrifuged at 1730 x g for 10 min and plasma (supernatant) was collected and stored at -20°C until the time of assay.

Enzyme-linked immunosorbent assays
Steroid hormones were extracted from plasma samples using diethyl ether, and the samples were reconstituted in buffer provided in the enzyme-linked immunosorbent assay (ELISA) kits (Neogen, Lansing, MI, USA) for the measurement of corticosterone. The assays were conducted according to kit instructions and using a BioTek Synergy plate reader. Assay sensitivity was 0.05 ng / mL for corticosterone.

**Brain collection, tissue processing and protein extraction**

The brains were taken from rats on PND 73 (24 h after restraint stress) directly from the homecage for a baseline measure of neural protein expression. Brains were cut down the midline on ice with a razor blade, rapidly frozen in isopentane, and each hemisphere was stored at -80°C until processing. The left hemisphere of each animal was used for Western blot experiments. The dorsal hippocampus (-3.12 mm from bregma) and medial prefrontal cortex (+3.24 mm from bregma) were dissected from the left hemisphere of each sample, and each dissected brain region was placed into a separate microcentrifuge tube on dry ice. Glass beads (0.5 mm diameter) and ice-cold whole-cell lysis buffer (1 M Tris, 150 mM NaCl, 0.1% Triton-X) with protease and phosphatase inhibitors (Roche, USA) were added into each tube containing tissue, and samples were homogenized using a BulletBlender (NextAdvance, USA) at 4°C for 3 min at a power setting of 7. After homogenization, samples were incubated on ice for 30 min with gentle rocking. After incubation, samples were centrifuged at 4°C at 16,000 X g for 10 min. After centrifugation, supernatant was collected as whole-cell soluble protein. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, USA) and a BioTek Synergy plate reader. Samples were then diluted to equal concentrations using whole-cell lysis buffer, and heated 2X Laemmli buffer was added to each sample at a 1:1
ratio. Samples were then heated for 5 min at 70°C, centrifuged at 4°C at 16,000 X g for 10 min, and stored at -20°C until use in Western blot experiments.

**Western blots**

For Western blot experiments, processed samples were heated for 5 min at 70°C and 10 µg of protein was loaded onto Bio-Rad TGX stain-free fast-cast 12% polyacrylamide gels (Bio-Rad, USA) for SDS-PAGE. Samples from each group were counterbalanced across gels. Once resolved, gels were illuminated for 5 min under UV light for activation of Bio-Rad stain-free total protein determination. After gel activation, proteins were transferred onto PVDF membranes (EMD Millipore, USA), blocked for 1-hour in 5% BSA in TBS-T at room temperature, and incubated overnight in primary antibody. Primary antibodies were directed at CB1 receptor (1:1000, ACR-001 Alamone Labs), phosphoPKA (catalytic subunit) (1:2000, 5661 Cell Signalling Technologies), PSD95 (1:5000, NB300-556 Novus Biologicals), GAD67 (1:5000, MAB5406 Millipore), spinophilin (1:5000, 06-852 Millipore). After incubation in primary antibody, membranes were washed (3 X 5 min washes) in TBS-T and incubated in secondary antibody (AlexaFluor 488 and AlexaFluor 647; Life Technologies, USA) for 45 min at room temperature with gentle rocking. After incubation in secondary antibody, membranes were washed in TBS-T, and imaged wet using the Bio-Rad VersaDoc MP4000 (Bio-Rad, USA). Detected bands from each lane were normalized to total lane protein using Bio-Rad stain-free total protein determination (Bio-Rad, USA).

**Statistical analyses**

Statistical analyses consisted of between group (Stress: noACS, ACS; Drug: NoINJ, VEH, AM251) and repeated measures (blood samples) analysis of variance
(ANOVA). Post hoc analyses consisted of F tests for simple effects, Tukey’s tests, and t-tests, where appropriate. Analyses were performed using SPSS version 24 (IBM Corp, USA). An alpha level of p < 0.05 (two-tailed) was used to determine statistical significance. Partial eta squared ($\eta^2_p$) are reported as a measure of effect size (for which a large effect size is 0.14, medium effect is size 0.06, and small effect size is 0.01) (Cohen, 1988).

**Experiment 2**

**Animals**

Twenty-four male (experiment 2A) and 24 female (experiment 2B) Long-Evans rats (Charles River, Kingston, New York, USA) arrived at the Brock University Comparative Bioscience Facility on PND 25 and were housed in same-sex pairs. Rats were kept on a 12-hour light-dark cycle (lights on at 8:00 h) and were provided access to food and water *ad libitum*. All procedures were approved by the Brock University Institutional Animal Care Committee and were in accordance with the Canadian Council on Animal Care and National Institutes of Health guide for Care and Use of Laboratory Animals (Publications No. 8023, revised 1978). All efforts were made to minimize the number of animals used and their suffering. As such, one VEH and two AM251 male rats, and one animal each from VEH and AM251 females were removed from the study for health reasons, reducing the sample size in the analyses.

**Drugs and injections**

AM251 and vehicle were prepared and administered as described for noACS rats in Experiment 1.

**Contextual fear conditioning**
On PND 75, rats underwent contextual fear conditioning, consisting of an initial 3 min exploration period followed by the presentation of three 1 s foot shocks with an inter-shock interval of 1 min. Rats remained in the conditioning chamber for 1 min after the last foot shock. Fear conditioning took place in a testing room illuminated by low fluorescent light. Animals were transported to the testing room in their home cages on a metal cart and trained in one of four identical chambers (30×37×25cm), each situated in a larger, ventilated sound-attenuating chamber. The outer chambers consisted of a small window (8 cm diameter) on the door that was covered by a clear Plexiglas® pane (all equipment from PanLab, Spain). The inner chamber walls were made of black stainless steel, and the door of clear Plexiglas®. The floor of the inner chamber consisted of 20 stainless steel rods wired to a shock source and a solid-state scrambler that allowed for the delivery of the unconditioned stimulus, a 0.5 mA, 1 s foot shock. The grid floor rested approximately 1 cm on top of a black stainless-steel base that was placed on weight transducers that connected to a load cell coupler designed to detect fine movement. The computer software (Freezing, PanLab) displayed the percentage of time spent freezing for each individual component of the protocols of each session. To record a freezing event (the measure of fear), the load cell coupler weight transducer required activity to be below the activity threshold for a minimum of 3000 ms.

Recall and extinction

Twenty-four hours after contextual fear conditioning (PND 76) rats were returned to the conditioning chambers for a 3 min test of contextual fear recall. Beginning 24 h after fear recall, rats were returned to the conditioning chambers once per day for 4 days (PND 77-80) for a 10 min test of fear extinction. After the last day of extinction training
(PND 80) rats were left undisturbed for 10 days. After the 10-day rest period (PND 91), rats were returned to the conditioning chambers for a 3 min test of long-term extinction memory.

**Reconditioning and recall of reconditioning**

Twenty-four hours after the long-term extinction memory test (PND 92) rats were placed back into the conditioning chambers for 3 min before receiving a single 0.5 mA 1 s foot shock. Rats remained in the condition chambers for 1 min after the foot shock. Twenty-four hours after reconditioning (PND 93) rats were returned to the conditioning chambers for a 3 min test of recall of reconditioning.

**Statistical analyses**

Statistical analyses consisted independent samples t-tests and repeated measures analysis of variance (ANOVA). Analyses were performed using SPSS version 24 (IBM Corp, USA). An alpha level of p < 0.05 (two-tailed) was used to determine statistical significance. Partial eta squared ($\eta_p^2$) are reported as a measure of effect size.

**Results**

**Experiment 1**

**Weight: Males**

Confinement stress and injection stress resulted in reduced weight gain. On PND 30 (first day of adolescent treatment) no weight differences were observed among groups (all $p > 0.321; n = 8$/group) (data not shown). On the last day of treatment (PND 44), noACS rats weighed more than ACS rats ($F_{1,42} = 9.639, p = 0.003, \eta_p^2 = .187$). There was a main effect of Drug ($AM251 < VEH = NoINJ, F_{2,42} = 3.417, p = 0.042, \eta_p^2 = .140$) whereby AM251 males weighed less than NoINJ males ($p = 0.044$) and did not differ
from VEH males (p = 0.850). VEH and NoINJ males did not differ significantly in weight on PND 44 (p = .140). The interaction of Confinement Stress and Drug was not significant (p = 0.885). On PND 70, no main effect nor interaction was significant (all p > 0.10) (see Fig. 2A).

Weight: Females

AM251 resulted in reduced weight gain in adulthood only in non-stressed females. On PND 30 (first day of adolescent treatment) no weight differences were observed among groups (all p > 0.569; n = 8/group) (data not shown). On the last day of treatment (PND 44) AM251 females tended to weigh less than VEH (p = 0.048) females, however, the effect did not meet statistical significance (F2,42 = 3.172, p = 0.052, ηp² = .131). There was no effect of Confinement Stress, nor was there an interaction of Confinement Stress and Drug for PND 44 weights (all p > 0.58). On PND 70, there was a significant interaction of Confinement Stress and Drug (F2,42 = 3.515, p = 0.039, ηp² = .143). Within the noACS condition, AM251 females weighed less than NoINJ females, but not significantly (p = 0.054), and, neither AM251 or NoINJ differed in weight from VEH (p = .281 and p = 0.634, respectively). There were no differences in PND 70 weight between any of the Drug groups in the ACS condition (all p > 0.180) (see Fig. 2B).

Elevated plus maze: Males

AM251 decreased anxiety in the elevated plus maze. There was an effect of drug on the time spent on the open arms of the maze (F2,42 = 3.986, p = 0.026, ηp² = .160; n = 8/group) whereby AM251 males spent more time on the open arms than did NoINJ males (p = 0.021), with no difference between AM251 and VEH males (p = 0.206) nor between NoINJ and VEH males (p = 0.538). There was no effect of Confinement Stress, nor
interaction between Confinement Stress and Drug, for any of the measures collected (all ps > 0.64) (see Fig. 3A).

**Elevated plus maze: Females**

There was no effect of Confinement Stress, Drug treatment, or interaction between the two, for any of the measures obtained (all p > 0.298; n = 8/group) (see Fig. 3B).

**Corticosterone response to acute restraint stress: Males**

Injection stress increased baseline corticosterone concentrations, with no effect of AM251 treatment or confinement stress at any time point. For the within-subjects measure of Time (BL, T0, T45, T90), there was a main effect of time, such that plasma CORT concentrations increased after restraint (BL to T0) and decreased during the recovery phase (T0 to T45) ($F_{3,123} = 219.885$, $p < 0.001$, $\eta_p^2 = .843$; n = 7-8/group). There was also a significant three-way interaction between Time, Confinement Stress, and Drug ($F_{6,123} = 2.876$, $p = 0.012$, $\eta_p^2 = .123$). Mixed factor ANOVAs performed on the separate time points (BL, T0, T45, T90) revealed a main effect of Drug on baseline CORT concentrations ($F_{2,41} = 3.976$, $p = 0.026$, $\eta_p^2 = .162$). Post-hoc Tukey’s tests indicated that the lower baseline CORT in NoINJ rats compared with VEH ($p = 0.051$) and AM251 ($p = 0.051$) rats did not meet statistical significance, and VEH and AM251 rats did not differ ($p = 0.999$). There was a significant Confinement Stress by Drug interaction immediately after restraint stress (T0) ($F_{2,41} = 4.635$, $p = 0.015$, $\eta_p^2 = .184$) that was attributable to higher plasma CORT concentrations in VEH rats in the ACS group compared with VEH rats in the noACS group ($p = 0.031$) (all other comparisons p > 0.112) (see Fig. 4A).

**Corticosterone response to acute restraint stress: Females**
For the within-subject measure of Time (BL, T0, T45, T90), there was a main effect such that plasma CORT concentrations increased after restraint (BL to T0) and decreased during the recovery phase (T0 to T45) ($F_{3,117} = 112.012$, $p < 0.001$, $\eta^2_p = .742$; $n = 6-8$/group). No other main effect or interaction was significant ($p > 0.11$) (see Fig. 4B).

**Neural protein expression: Males**

In the dorsal hippocampus there was a main effect of Drug ($F_{2,42} = 3.462$, $p = 0.042$, $\eta^2_p = .140$; $n = 8$/group) and a significant Stress by Drug interaction ($F_{2,42} = 5.038$, $p = 0.011$, $\eta^2_p = .193$) on PKA activation. ANOVAs performed separately on the Confinement Stress groups found that AM251 males had higher PKA phosphorylation than did VEH ($p = 0.009$) males only in the ACS group (there were no differences between AM251 and NoINJ or between NoINJ and VEH males, all $p > 0.134$), with no effect in the noACS group ($p = 0.232$). There was no effect of Stress, or Drug, or interaction between the two for any of the other protein measures obtained in the dorsal hippocampus (all $p > 0.080$) (see Table 1). In the prefrontal cortex there was no effect of Stress, Drug, or interaction between the two for any of the neural protein measures obtained (all $p > 0.248$; $n = 8$/group) (see Table 1).

**Neural protein expression: Females**

In the dorsal hippocampus, there was a main effect of Stress ($F_{1,42} = 4.25$, $p = 0.045$, $\eta^2_p = .092$; $n = 8$/group) and a Stress by Drug interaction ($F_{2,42} = 5.705$, $p = 0.006$, $\eta^2_p = .214$) for spinophilin expression. ANOVAs performed on the Confinement Stress groups separately revealed a stress of injection effect whereby VEH rats in the noACS group had lower spinophilin expression relative to NoINJ ($p = 0.01$) and AM251 ($p =$
0.032) females, which did not differ (p = 0.198). There was no effect of Confinement Stress, Drug, or interaction between the two, for any of the other neural protein measures obtained in the dorsal hippocampus for female rats (all p > 0.115) (see table 1). In the prefrontal cortex, there were no effects of Confinement Stress, Drug, or interaction between the two terms on any protein measures obtained (all p > 0.11; n = 7-8/group) (see table 1).

**Experiment 2A: Males**

**Fear conditioning**

VEH and AM251 rats did not differ in percent time freezing during the exploration (p = 0.420) or conditioning phase (p = 0.205; 10-11/group) (see Fig. 5A).

**Recall**

Twenty-four hours after fear conditioning, VEH and AM251 rats did not differ in freezing during the 3 min test of contextual fear recall (p = 0.735) (see Fig. 5A).

**Extinction**

The percentage of time spent freezing decreased in both VEH and AM251 males across extinction days ($F_{3,57} = 67.605$, $p < 0.001$, $\eta^2_p = .781$). There was no interaction between Day and Drug on freezing behaviour across the 4 days of extinction training (p = 0.818), nor was there any main effect of Drug (p = 0.304). VEH and AM251 rats did not differ in long-term extinction memory recall when tested 10 days after the final extinction training day (p = 0.128) (see Fig. 5A).

**Reconditioning**

VEH and AM251 rats did not differ in percent time freezing during the exploration (p = 0.396) or the reconditioning phase (p = 0.364) (see Fig. 5A).
Recall of reconditioning

Twenty-four hours after reconditioning, VEH and AM251 rats did not differ freezing during the 3 min recall of reconditioning test (p = 0.718) (see Fig. 5A).

Experiment 2B: Females

Fear conditioning

VEH and AM251 rats did not differ in percent time freezing during the exploration (p = 0.316) or conditioning phase (p = 0.952; 11/group) (see Fig. 5B).

Recall

Twenty-four hours after conditioning, AM251 females froze more during the 3 min contextual recall test than did VEH females (t = -2.392, df = 20, p = 0.027) (see Fig. 5B).

Extinction

Because of equipment failure, data from four AM251 females were lost on Extinction Day 2. Thus, to maintain the larger sample size across extinction days in analyses, we omitted Extinction Day 2 from analysis but have included the data for the remaining rats for that day in the graph (see Fig. 5B).

The percentage of time spent freezing decreased in both VEH and AM251 males across extinction days (F_{2,40} = 192.547, p < 0.001, \eta^2_p = .906). There was no interaction of Day and Drug on freezing behaviour across the 4 days of extinction training (p = 0.844), nor was there a main effect of Drug (p = 0.852). There were no group differences in long-term extinction memory recall when tested 10 days after the final extinction training day (p = 0.431) (see Fig. 5B).
There were no group differences in freezing during the initial 3 min exploration period (p = 0.152). AM251 females froze more during reconditioning (from the onset of the shock to the end of the session) compared with VEH females (t = -4.233, df = 20, p < 0.001) (see Fig. 5B).

**Recall of reconditioning**

AM251 females tended to freeze more than VEH females 24 h after reconditioning, though the effect failed to reach significance (t = -1.975, df = 20, p = 0.062) (see Fig. 5B).

**Discussion**

We have previously reported that repeated CB1 receptor antagonism (1 mg / kg AM251) in adolescence (PND 30-44) increased social interactions, increased PFC GAD67 expression, and reduced DH CB1 receptor expression in female rats soon after cessation of treatment (i.e., 24-48 h after final drug exposure), with no effects observed in males (Simone et al., 2018). Our present findings of reduced anxiety-like behaviour in adult males and increased contextual fear recall and reconditioning in adult females expands upon our previous findings by demonstrating that whereas effects of adolescent CB1 receptor antagonism in females are evident both soon (24-48 h) and long (~ 4 weeks) after exposure, effects in males emerge with time. Further, that effects in males were specific to generalized anxiety-like behaviours, whereas effects in females were specific to contextual fear memory, suggests that adolescent CB1 receptor signalling may have a greater involvement in the development of affective behaviours in males and of cognitive behaviours in females.

**Experiment 1: Long-term effects of AM251 and stress exposures in adolescence**
Anxiety-like behaviours

Endocannabinoid regulation of anxiety-like behaviours is well-documented, although most investigations have involved the effects of agonism or antagonism while the drugs were in the system (Bellocchio et al., 2013; Bitencourt et al., 2008; Griebel et al., 2005; Haller et al., 2002; Haller et al., 2004; Litvin et al., 2013; Moreira et al., 2008; Navarro et al., 1997; Rey et al., 2012; Rubino et al., 2008a; Simone et al., 2015a; Simone et al., 2015b; Tambaro et al., 2013). In general, acute administration of CB1 receptor agonists decrease, whereas antagonists increase, anxiety-like behaviour in adult rats (Lafenetre et al., 2007). Less is known about endocannabinoid involvement in the development of anxiety-like behaviours, however, studies involving repeated agonism during adolescence have provided support for CB1 receptor contributions (O’Shea et al., 2006; O’Shea et al., 2004; Renard et al., 2013; Renard et al., 2017; Renard et al., 2016b; Rubino and Parolaro, 2008; Rubino et al., 2015; Rubino et al., 2009a; Rubino et al., 2009b; Rubino et al., 2008b; Schneider and Koch, 2003; Schneider et al., 2008).

Nevertheless, studies with agonists are limited in their ability to discern the role of endogenous CB1 receptor signalling, particularly given the contrast in spatio-temporal signalling between exogenous and endogenous cannabinoid agonists. Studies with antagonists better allow for investigation of the role of endogenous CB1 receptor signalling. Here, we demonstrate modest reductions in anxiety-like behaviour (as measured by time spent in the open arms of the EPM) in adult male rats previously exposed to AM251 in adolescence, with no effect in females. Although acute exposure to AM251 tends to be anxiogenic in adult male rats (Haller et al., 2002; Navarro et al., 1997; Simone et al., 2015a; Sink et al., 2010b), the anxiolytic effects in the present study,
though only mild, were observed several weeks after the last AM251 exposure. We previously reported no effect of adolescent AM251 on anxiety-like behaviours in the EPM of male rats tested 24 hours after the final drug exposure (Simone et al., 2018). Thus, our present findings suggest that, in males, adolescent AM251 effects emerge with time and likely involve altered neurodevelopment and hardwiring of the neural circuits subserving anxiety. Another explanation for the anxiolytic phenotype observed in males of the present study is that repeated antagonism during adolescence caused a sensitization of CB1 receptors in brain regions associated with anxiety regulation, and thus potentiated the effects of endogenous cannabinoids; pharmacological enhancement of endocannabinoid signalling via reuptake and hydrolysis inhibitors is associated with reduced anxiety-like behaviours in rodents (Kinsey et al., 2011; Marco et al., 2015; Moreira et al., 2008; Sciolino et al., 2011). In contrast to our findings, a study in male Sprague-Dawley rats reported no effect of adolescent AM251 exposure (5 mg / kg) on anxiety-like behaviours in adulthood (Lee et al., 2015). The lack of effect of adolescent AM251 reported by Lee and colleagues (2015) may reflect strain differences in the effects of cannabinoid drugs (Coria et al., 2014; Keeley et al., 2015b; Manduca et al., 2014). Alternatively, the dose of AM251 used in the study by Lee et al., was greater (5 mg / kg vs 1 mg / kg), with fewer daily injections (10 vs 15 days) and a shorter age range of exposures (PND 35-44 vs PND 30-44). Thus, the long-term anxiolytic effects of CB1 receptor antagonism in male rats may require a longer duration of antagonism and/or developmentally earlier exposures to induce long-term behavioural changes.

Although we did not observe any long-term effects of AM251 on anxiety-like behaviour in females, AM251 reduced weight-gain relative to non-injected females in the
noACS group, demonstrating long-term physiological effects of the drug, and confirming that females are indeed sensitive to this manipulation. The absence of AM251 effects on anxiety-like behaviours in female rats of the present study may be explained by sex-differences in the behavioural and neuropharmacological effects of cannabinoid drugs (Craft, 2005; Craft et al., 2012; Tseng and Craft, 2001; Tseng et al., 2004; Wakley et al., 2014). Conversely, the lack of effects of AM251 on anxiety-like behaviours in females may be independent of sex-differences in pharmacokinetics and pharmacodynamics, and instead may involve sex-specific neurodevelopmental disruptions. Although adolescence is considered a critical period of neurodevelopment in both male and female animals, the specific developmental alterations that occur, and the mechanisms driving these alterations, can differ by sex and often involve gonadal hormone signalling (Sisk and Zehr, 2005). Further, onset of puberty is about a week earlier in females, and thus although of the same age, the sexes differ in developmental status. Activation of CB1 receptors via exogenous or endogenous cannabinoid ligands has been found to reduce circulating concentrations of androgens and estrogens via reductions in luteinizing hormone and gonadotropin releasing hormone (Gorzalka and Dang, 2012). Thus, inhibition of CB1 receptor signalling during adolescence may elicit sex-specific organizational effects on the brain via regulation of circulating gonadal hormones.

**Stress exposures**

Contrary to our prediction that AM251 administered in tandem with a stressor would potentiate the effects of AM251 on its own, we observed no interaction of the two manipulations on behaviour in the EPM, nor on plasma CORT concentrations either at baseline or after 30 min restraint stress. The lack of interaction between repeated stress
and repeated AM251 in adolescence on adult measures of emotional behaviour and stress reactivity is consistent with our previous findings in adolescent rats (Simone et al., 2018), and suggests that the behavioural effects of AM251 are independent of hypothalamic-pituitary-adrenal axis stress responses. Similarly, we observed no effect of repeated confinement stress on any measures in either sex. Although modest long-term effects of adolescent stress have been reported across a variety of measures in both male and female rats, effects are often transient and greatest when observed soon after cessation of the stressor (McCormick and Mathews, 2007). It should be noted, however, that although we did not observe any long-term effects of repeated confinement stress in adolescence, we did observe long-term stress of injection effects in both sexes that may reflect a greater vulnerability of adolescent animals to repeated physical versus psychological stress exposures.

**Neural protein expression**

Adolescence is a sensitive period for neurodevelopment, and studies from our lab (Simone et al., 2018) and others (Lee et al., 2015; Rubino et al., 2015) have demonstrated altered neural protein expression in male and female rats repeatedly exposed to a CB1 receptor antagonist in adolescence; effects of adolescent CB1 receptor antagonism have been observed across markers of endocannabinoidergic, glutamatergic, and GABAergic signalling. We previously demonstrated that repeated CB1 receptor antagonism in adolescence reduced dorsal hippocampal CB1 receptor expression and increased prefrontal cortical GAD67 expression in female rats 24 h after their last treatment, with no effects in males (Simone et al., 2018). In the present study, we observed no differences in CB1 receptor or GAD67 expression in the dorsal hippocampus (DH) or PFC in either
sex, suggesting that although adolescent CB1 receptor antagonism leads to more immediate neural effects in females, these effects are transient. Further, we failed to observe any differences in the expression of the glutamatergic marker PSD95 in either brain region for males or females, despite a previous report of increased PSD95 expression in the PFC of adult females repeatedly exposed to AM251 in adolescence (Rubino et al., 2015).

Although we found no effect of AM251 on CB1 receptor expression, long-term effects of adolescent AM251 have been reported for CB1 receptor binding in the PFC of adult male rats (Lee et al., 2015). One limitation of the present results is the use of Western blot for the detection of CB1 receptor expression; many commercially available antibodies targeting CB1 receptors demonstrate non-specific binding properties (Ashton, 2012; Grimsey et al., 2008). Although the specificity of the CB1 receptor antibody used in the present study was verified by pre-treating the antibody with the supplied control peptide (corresponding to amino acid residues 84-99 of the rat CB1 receptor; Accession P20272), results should be interpreted with caution. As an indirect measure of CB1 receptor activation, we measured the phosphorylation of the catalytic subunit of cyclic adenosine monophosphate (cAMP)-dependent Protein Kinase A (PKA). The canonical pathway of CB1 receptor activation involves direct inhibition of adenylyl cyclase and subsequent reductions in intracellular cAMP (Andersson et al., 2005; Kaminski, 1998; Robbe et al., 2001). Thus, CB1 receptor activation leads to reductions in the activation of PKA via reductions in cAMP. Because we observed increased PKA activation in the DH of AM251-treated ACS males, our findings may reflect a sustained decrease in endocannabinoid signalling at DH CB1 receptors; adolescent AM251 exposure was
found to alter brain endocannabinoid content in adult male rats in a region-dependent manner (Lee et al., 2015). Further, that we reported no differences in PKA phosphorylation in ACS males 24 h after the cessation of treatment (Simone et al., 2018) yet we found a difference approximately 4 weeks later suggests emergent effects like those observed for anxiety-like behaviours in males.

**Experiment 2: Long-term effects of AM251 in adolescence on contextual fear conditioning and extinction**

Because only modest behavioural effects of AM251 exposure were evident in experiment 1, we sought to further characterize the adult behavioural phenotype associated with downregulated adolescent CB1 receptor signalling in experiment 2. The behavioural effects of AM251 were sex-specific; AM251 increased fear recall in females 24 h after fear conditioning compared with vehicle-treated females, with no effects observed in males. Although no effects on within- or between-session extinction were observed in either sex, AM251 females displayed significantly greater fear during a single reconditioning trial (1 x 1 s foot shock) and showed evidence of greater fear recall 24 h after reconditioning (though not statistically significant), compared with vehicle females. Thus, the present findings suggest that repeated antagonism of CB1 receptor signalling in adolescence disrupts the development of neural systems involved in contextual fear learning and memory processes in a sex-specific manner, leading to increased fear memory recall and reconditioning in females.

Sex-differences in conditioned fear are well-documented (Baran et al., 2009; Dalla et al., 2011; Gupta et al., 2001; Maren et al., 1994; Markus and Zecevic, 1997; Pryce et al., 1999), and are attributed, in part, to differences in the activation of
intracellular signalling pathways (Mizuno and Giese, 2010). One candidate pathway that may be involved in the sex-specific effects of AM251 on fear recall and reconditioning is the extracellular-signal regulated protein kinase-1 and -2 (ERK1/2) pathway. ERK1/2 signalling is modulated by CB1 receptors (Dalton and Howlett, 2012; Dalton et al., 2009), and sex-differences in fear-induced activation of ERK1/2 were reported (Gresack et al., 2009; Howlett, 2004; Matsuda et al., 2015). Alternatively, as adolescent CB1 receptor antagonism was shown to increase markers of glutamatergic signalling in the PFC of adult female rats (Rubino et al., 2015), it is possible that the greater fear recall observed in AM251 females is because of greater excitatory drive within fear-promoting brain regions. One such candidate region is the prelimbic division of the PFC (PL-PFC). Excitatory signalling from the PL-PFC onto the amygdala is a critical process for the expression of conditioned fear (Burgos-Robles et al., 2009; Burgos-Robles et al., 2007; Choi et al., 2010; Gabbott et al., 2005; Sierra-Mercado et al., 2011; Vertes, 2004; Vidal-Gonzalez et al., 2006). Although the study by Rubino and colleagues (2015) did not differentiate between subdivisions of the PFC, it is possible that exposure to AM251 in adolescence selectively increases glutamatergic signalling from the PL-PFC to the amygdala leading to increased fear recall and reconditioning. Nevertheless, the increased fear observed in adolescent females may reflect, to a degree, stress of injection effects based on our finding that VEH females had greater DH spinophilin expression relative to AM251 females in the noACS condition (experiment 1). This is unlikely, however, as dendritic density within the hippocampus is positively associated with contextual fear learning and memory processes (Brigman et al., 2010), and thus, we would predict VEH females to demonstrate greater fear learning and memory than AM251 females.
Conclusions

The present findings suggest that repeated CB1 receptor blockade in adolescence acts to alter the organization of the developing brain, leading to long-term behavioural and physiological changes. These results are consistent with the hypothesis of adolescence as a sensitive period during which the developmental trajectory is malleable and contribute to an understanding of the role of the endocannabinoid system in adolescent development. Whereas long-term effects on anxiety-like behaviour and neural protein expression were specific to males, effects on fear learning and memory were specific to females, suggesting a greater involvement of adolescent CB1 receptor signalling in the development of affective behaviours in males, and cognitive behaviours in females.
Table 3-1. Means (including S.E.M.) for the different protein measures in the dorsal hippocampus and prefrontal cortex of male (left) and female (right) rats.

<table>
<thead>
<tr>
<th></th>
<th>Males Dorsal hippocampus</th>
<th>Females Dorsal hippocampus</th>
<th>Males Prefrontal Cortex</th>
<th>Females Prefrontal Cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CB1</td>
<td>PKA</td>
<td>GAD67</td>
<td>PSD95</td>
</tr>
<tr>
<td>NoACS NoNJ</td>
<td>10.85</td>
<td>35.54</td>
<td>38.03</td>
<td>9.91</td>
</tr>
<tr>
<td>VEH</td>
<td>(0.57)</td>
<td>(1.79)</td>
<td>(4.75)</td>
<td>(0.78)</td>
</tr>
<tr>
<td>AM251</td>
<td>(0.83)</td>
<td>(2.41)</td>
<td>(4.83)</td>
<td>(0.96)</td>
</tr>
<tr>
<td>VEH</td>
<td>(0.90)</td>
<td>(2.17)</td>
<td>(3.79)</td>
<td>(1.00)</td>
</tr>
<tr>
<td>AM251</td>
<td>11.60</td>
<td>42.12</td>
<td>38.34</td>
<td>8.77</td>
</tr>
</tbody>
</table>

* main effect of Drug (p < 0.05).
† main effect of Stress (p < 0.05).

Matching letters represent significantly different from each other (p < 0.05).
Fig. 3-1. Experimental design and timeline of experimental procedures.
Fig. 3-2. Mean (+/- S.E.M.) weights on the last day of treatment (PND 44; left panel) and in adulthood (PND 70; right panel) for (A.) male and (B.) female rats. † main effect of stress, p < 0.05. * compared with noACS-NoINJ, p < 0.05.
Fig. 3-3. Mean (+/- S.E.M.) for behavioural measures in the elevated plus maze on PND 70 in (A.) male and (B.) female rats.
Fig. 3-4. Mean (+/- S.E.M.) plasma CORT concentrations (µg / dL) on PND 73 immediately before (BL, immediately after (T0), 45 min after (T45), and 90 min after (T90) 30 min restraint stress in (A.) male and (B.) female rats. Lower figures in (A.) depict the significant main effect and interaction. † main effect of stress, p < 0.05.
Matching letters indicate significantly different from each other, $p < 0.05$, for the main effect of timepoint.
Fig. 3-5. Mean (+/− S.E.M.) percent freezing during the conditioning, recall, extinction, long-term extinction recall, reconditioning, and recall of reconditioning in (A.) male and (B.) female rats. * compared with VEH, p < 0.05.
Chapter 4: Adolescent endocannabinoid signalling contributes to the development of social behaviours in female rats.
This chapter is in preparation for journal submission as:
Simone, J. J., McPherson, J. L., Baumbach, J. L., Zeidan, M., & McCormick, C. M.

Repeated CB1 receptor antagonism in adolescence increases social interactions and EGR-1 expression in the nucleus accumbens shell of female rats.

Author contribution: I was the primary investigator of this work, responsible for the majority of study design, data collection, data analysis, and writing of the manuscript. I performed the injections, the behavioural testing and scoring, brain collections and processing, and the immunohistochemistry experiments.

Jennifer McPherson assisted with the behavioural experiments. Jennet Baumbach assisted with statistical analyses, EGR-1 cell counts, and editing of the manuscript. Mostafa Zeidan assisted with brain collections and immunohistochemistry experiments. Dr. Cheryl McCormick assisted in designing the experiments, analyzing the data, and editing the manuscript.
Introduction

Adolescence is a transitional period of development between childhood and adulthood that involves extensive maturation and refinement of corticolimbic networks within the brain (Sisk and Zehr, 2005; Spear, 2000). The plastic nature of the developing adolescent brain is thought to underlie the greater sensitivity of adolescent than adult animals to external factors and to the development of psychopathologies such as generalized and social anxiety disorders (Crews et al., 2007; McCormick et al., 2015). Consequently, there is considerable interest in understanding the mechanisms that drive the development and maturation of social behaviours and their underlying neural networks in adolescence.

Over the last decade and a half, the endogenous cannabinoid system (ECS) has emerged as a likely regulator of normative social development in adolescent rats (O'Shea et al., 2006; O'Shea et al., 2004; Renard et al., 2017; Schneider et al., 2008; Simone et al., 2018). The ECS consists of two main receptors, the cannabinoid type-1 (CB1) and type-2 (CB2) receptors, their endogenous ligands, namely \( \text{N}-\text{arachidonylethanolamine} \) (AEA) and \( 2-\text{arachidonyl glycerol} \) (2-AG), and the enzymatic machinery involved in endocannabinoid metabolism and transport (Mechoulam and Parker, 2013). The expression of CB1 receptors, the predominant neural cannabinoid receptor, in adolescence increases from the juvenile period to peak expression levels in early-adolescence, before declining from mid-adolescence into adulthood where expression remains relatively stable (Lee and Gorzalka, 2015). The dynamic expression of CB1 receptors in adolescence coincides with changes in major neurotransmitter systems such as the glutamatergic (Rubino et al., 2015) and GABAergic (Caballero et al., 2014; Cass et al., 2014) systems and with the development and maturation of social behaviours.
(Blakemore, 2008; Sisk and Zehr, 2005; Spear, 2000). Thus, it is hypothesized that developmental shifts in the expression of the various components of the ECS in adolescence contribute to the maturation and refinement of the adolescent brain, and thus to the normative development of adolescent behaviours.

In support of CB1 receptor contributions to social development, studies with agonists have demonstrated long-term effects of CB1 receptor modulation on social behaviours; repeated exposure to CB1 receptor agonists in adolescence resulted in reduced time spent in social interactions in both male (O'Shea et al., 2006; Renard et al., 2017; Schneider et al., 2008) and female (O'Shea et al., 2004) rats when tested several weeks after the cessation of treatment. Further, we previously reported that female rats exposed daily to the highly-selective CB1 receptor antagonist / inverse agonist AM251 in adolescence (1 mg / kg; postnatal days (PND) 30-44) spent more time in social interactions when paired with an unfamiliar experiment-naïve conspecific compared with vehicle-treated females (Simone et al., 2018). Conversely, we found no effect of adolescent AM251 pre-treatment on social interactions in males when paired with an unfamiliar age- and treatment-matched stimulus male (Simone et al., 2018). Although our previous findings suggest a sex-specific role of endogenous CB1 receptor signalling in social behaviour development, the differences in experimental design between males and females (i.e., social interactions when paired with a treatment-matched conspecific versus when paired with an experimental-naïve conspecific) may account for the observed sex-specific effects. Further, the increased social interactions in female rats previously exposed to AM251 were observed 48 h after the last exposure to the antagonist (Simone et al., 2018). Based on the half-life of AM251 (~22 hours; (McLaughlin et al., 2003)), it
is likely that our prior findings of AM251-mediated increases in sociality are because of neurodevelopmental alterations to the social brain. Nevertheless, we cannot completely rule out the possibility that the effects on social interactions were because of acute pharmacological effects resulting from residual drug in the system.

In the present study we sought to further investigate sex-differences in CB1 receptor-dependent social development and identify candidate brain regions involved in the developmental effects of AM251 on social behaviour. In experiment 1, male and female rats were treated daily (PND 30-44) with the CB1 receptor antagonist AM251 (1 mg / kg) or with vehicle and then tested in a social interaction paradigm after a 5-day drug washout period; both male and female rats were tested for social interactions with an age- and sex-matched conspecific naive to experimental manipulation. Consistent with our previous report (Simone et al., 2018), adolescent AM251 treatment led to an increase in social interactions only in female rats, with no observed effects in males. In a second experiment, we sought to determine candidate brain regions involved in the AM251-induced increases in social interactions in females. Based on our previous observation of reduced CB1 receptor expression in the dorsal hippocampus of female rats exposed to AM251 in adolescence (Simone et al., 2018), and because both CB1 receptor (Haller et al., 2004; Trezza et al., 2012; Trezza and Vanderschuren, 2009; Wei et al., 2017; Wei et al., 2015) and dorsal hippocampal [File, 2003 #1604;Hitti, 2014 #1870;Stevenson, 2014 #1869;Alexander, 2016 #1898] signalling are regulators of social behaviours, we investigated the extent to which dorsal hippocampal activity was associated with social interaction. Additionally, previous research found long-term impairments in social behaviour after adolescent THC treatment that were associated with a state of
dopaminergic hyperactivity along the mesocorticolimbic dopamine pathway (Renard et al., 2017), suggesting that CB1 receptor signalling is involved in social-reward development. Based on our finding that the increase in social interactions both in experiment 1 of the present study and in our previous report (Simone et al., 2018) were independent of generalized anxiety-like or novelty-seeking behaviours, we hypothesized that adolescent AM251 treatment would alter the normative development of the brain’s reward circuitry leading to increases in the reward value of social interactions. Thus, we also collected measures in the nucleus accumbens (NAc), a region highly implicated in social reward processing (Dölen et al., 2013; Kohls et al., 2013; Trezza et al., 2011; Trezza et al., 2012). To this end, female rats were treated daily with either vehicle or AM251 in adolescence (PND 30-44) and tested for social interactions after a 5-day drug washout period. Brains were collected from vehicle- and AM251-treated female rats 1 h after social interaction testing for analyses of EGR-1 protein expression as a measure of neural activation in the CA1, CA2, and CA3 subfields of the dorsal hippocampus and in the shell and core divisions of the NAc.

**Methods**

**Experiment 1**

**Animals**

Twenty-four male (Experiment 1A) and 24 female (Experiment 1B) rats (Charles River, Kingston, New York, USA) arrived at the Brock University Comparative Bioscience Facility on postnatal day (PND) 25. Upon arrival, rats were housed in same-sex pairs and allowed to acclimate to the facility for four days before the start of the experimental procedures. Rats were kept on a 12-hour light-dark cycle (lights on at 8:00
h) and were provided access to food and water *ad libitum*. All procedures were approved by the Brock University Institutional Animal Care Committee and were in accordance with the Canadian Council on Animal Care and National Institutes of Health guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1996). All efforts were made to minimize the number of animals used and their suffering.

**Weights**

All animals were weighed daily during the light phase for the 15 days of treatment (PND 30-44), and once more on PND 50 approximately 2 hours before the start of behavioural testing (8:00h) for both experiments 1 and 2.

**Drugs and injections**

The CB1 receptor antagonist / inverse agonist AM251 (Cayman Chemical, USA) was dissolved in a 1:1:18 dilution of DMSO, Tween-80, and 0.9% saline and administered i.p. at a concentration of 1.0 mg / kg. The vehicle was a 1:1:18 mixture of DMSO, Tween-80, and 0.9% saline in the absence of AM251. The dose of AM251 was chosen based on previous work from our lab (Simone et al., 2018). All injections were administered at a volume of 1.0 mL / kg on PND 30-44. The injection and treatment protocols were identical for experiments 1 and 2.

**Observations in the homecage**

Behaviour in the homecage was recorded 1 h (experiment 1) or 30 min (experiment 2) after injection for the first three days (PND 30-32) and the last three days (PND 42-44) of the injection protocol. A total of sixteen behaviours were observed and subsequently classified into the following categories based on Cirulli and colleagues (1996) as in our previous research (Cirulli et al., 1996; McCormick and Mathews, 2007;
Simone et al., 2018): Social Active (playing, allogrooming, sniffing cage partner), Social Inactive (immobile while in physical contact with cage partner), Non-Social Active (walking, rearing, eating, drinking), Non-Social Inactive (immobile and not in contact with cage partner), Digging (digging, burying), Self Directed (scratching, self-grooming), and Atypical (shaking, stretching on belly). An observation was made for each cage once per minute for 10 min, with each animal in the cage assigned one behaviour category during each observation for a total of 20 observations per cage (10 observations per rat X 2 rats per cage) per day. The total number of observations per cage per day for each behavioural category was averaged across the initial three days of treatment (PND 30-32) and the final three days of treatment (PND 42-44). To allow a degree of freedom in statistical analyses, scores for the Atypical category were excluded from all analyses. Behaviours were scored by an observer blind to experimental conditions.

**Social interaction test**

The 30 min social interaction test was adapted from Green et al., (2013) and involved three phases, allowing for measures of open field activity and interaction with a novel object, in addition to social interaction measures.

**Open field**

On PND 50 rats were tested for responses to a novel environment in an open field. The testing room contained four arenas, allowing for four animals (two pairs of cage partners) to be tested simultaneously. The testing arena consisted of white open-top melamine arenas (58 cm x 58 cm x 58 cm) illuminated indirectly by red light to attenuate anxiety related to bright illumination. The test session was 10 min in duration and occurred between 10:00 h and 12:00 h. Locomotor activity was recorded with a Sony
digital video camera mounted from the ceiling and connected to a computer tracking system (Smart; Panlab, Spain) that measured distance travelled in centimeters, as well as the percentage of time spent in the centre of the test arena (12 cm away from any wall), and the latency to enter the centre of the test arena. At the end of the 10 min open field test, rats remained in the testing arena for a subsequent 10 min novel object test.

**Novel object**

A novel object (a small black, plastic fan, 8 cm x 12 cm) was placed into a corner of the test arena, and the latency to approach, and the time spent interacting with the novel object were recorded. Interaction with the object was defined as any physical contact wherein the rat’s nose was directed at the object. After the 10 min test, the novel object was removed from the arena, and the rats remained in the arenas for subsequent social interaction testing.

**Social interaction**

Social interaction testing lasted 10 min and involved the placement of a novel rat (a Stimulus rat) into each testing arena occupied by a test rat. Test rats were paired with same-sex Stimulus rats that did not undergo experimental testing. Time spent in an interaction initiated by the Test rat and time spent in an interaction initiated by the Stimulus rat were scored. A social interaction was defined as any physical interaction between the Test rat and Stimulus rat, with the initiation of each interaction being assigned to whichever rat (Test or Stimulus) was the actor at the onset of the interaction. After the 10 min test, rats were removed from the arenas and placed back into their homecages in the housing room. Arenas were cleaned with Virox disinfectant after each test.
Experiment 2

Experiment 2 involved only female animals. All procedures were conducted as described in experiment 1 except for the measurement of behaviour after injection, which occurred 30 min after injection in this experiment (experiment 2) rather than 60 min after injection as in experiment 1, and with the inclusion of these additional tests.

Elevated plus maze

Rats were tested 24 h after the last AM251 injection (PND 45) for anxiety-like behaviours in an elevated plus maze. The apparatus consisted of two open and two closed arms extended from a common central platform 80 cm in height. The maze was constructed of grey plastic and was situated in the centre of the testing room. Rats were transported individually from the housing room to the testing room in an empty cage lined with paper towel, placed onto a closed arm of the maze, and left to explore the maze for 5 min. Testing took place under low illumination as we have previously reported anxiogenic effects of acute AM251 exposure in adult rats under these conditions. Testing occurred between 8:00 h and 11:00 h, and behaviour was recorded by an overhead camera. Behaviours scored by an observer blind to experimental condition were the time spent on the open arms, time spent on the closed arms, number of entries into an open arm, and number of entries into a closed arm. An arm entry was recorded when the two front paws of the animal were in the arm. The time spent on the open arms of the maze and the number of entries onto an open arm are the standard measures of anxiety-like behaviour in this test (Wall and Messier, 2001). The number of entries onto the closed arms of the maze is used as a measure of locomotor activity (Cruz et al., 1994; Rodgers and Dalvi, 1997). The maze was cleaned with Virox disinfectant between each test
To minimize effects of separation of cage partners on performance, cage partners were tested 2 h apart to allow recovery from any stress attributable to the cage partner's absence of approximately 7 min.

**EGR-1 immunohistochemistry**

One hour after social interaction testing (PND 50), rats were deeply anaesthetized by an overdose of sodium pentobarbital (150 mg / kg) and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4). Brains were removed from the skulls and post-fixed in a 30% sucrose and 4% paraformaldehyde solution until equilibrated. Coronal sections (40 µm; sections separated by 222 µm) were collected throughout the dorsal hippocampus and nucleus accumbens (NAc) and were stored in cryoprotectant at −20°C until the time of assay. Free-floating coronal sections were washed thoroughly in 0.1 M PBS, then in PBS-X (0.1 M PBS with 3% Triton X-100), and incubated at room temperature in 0.3% H₂O₂ in 0.1 M PBS-X solution for 30 min. Sections were then washed in PBS-X, blocked at room temperature in 10% goat serum (Sigma) for 1 h, and incubated at 4°C overnight in primary antibody (1:10,000; anti-EGR-1, rabbit mAb; 15F7; Cell Signaling Technology, Inc.) in PBS-X. The next day, sections were washed in PBS-X and then incubated for 2 h at room temperature in secondary antibody (biotinylated goat anti-rabbit IgG; 1:400; Vector Laboratories, Inc.). After another series of washes in PBS-X, sections were incubated in an avidin–biotin horseradish peroxidase complex (Vector Laboratories, Inc.) for 1.5 h at room temperature. Horseradish peroxidase was visualized with 3,3’-diaminobenzidine (DAB) in a 3 M sodium acetate buffer containing 0.05% H₂O₂ (Vector Laboratories, Inc.). After a final series of washes in PBS-X, sections were mounted on Superfrost Plus
slides (Fisher Scientific, Inc.) and were dried, dehydrated in increasing concentrations of ethanol (70%, 95%, 100%), placed in xylenes, and cover-slipped using Permount mounting medium (Fisher Scientific, Inc.). No immunoreactive (ir) cells were detected in control sections that were not treated with the primary antibody.

**Microscopy and cell counting**

Immuno-stained sections were analyzed using a Nikon Eclipse 80i microscope equipped with a digital camera (Nikon DXM1200F) and Nikon ACT-1 software. Sections were imaged at 400x magnification and a 250 µm² area in each hemisphere of the pyramidal layer of the CA1, CA2, and CA3 subfields of the dorsal hippocampus and in the shell and core subregions of the NAc were identified according to the atlas of Paxinos and Watson (Paxinos and Watson, 2005). EGR-1 ir cell counts were collected by a researcher blind to experimental condition. Dorsal hippocampal sections used for counting were within the coordinates of -2.64 mm and -3.24 mm from bregma. NAc sections that were used for counting were within the coordinates of 2.76 mm and 2.16 mm from bregma. The mean number of ir-cells per hemisphere per brain region per rat was used for analysis for dorsal CA1, CA2, and CA3 subfields and for NAc core and shell subregions.

**Statistical analyses**

Group differences were analyzed using independent samples t-tests. Time spent in social interactions was analyzed using mixed-model analysis of variance (ANOVA) with Individual (Test rat or Stimulus rat) as the within group factor and Drug (VEH or AM251) as the between group variable. Simple correlations and linear regression were used to investigate the relationship between neural activity and social behaviour.
Results

Experiment 1: Males

Weights

There were no differences in weights on the first day of adolescent treatment (PND 30) between VEH and AM251 males (p = 0.110; data not shown). The amount of weight gained from the first day of treatment (PND 30) to the last day of treatment (PND 44) was significantly reduced in AM251 compared with VEH males (t = 3.336 df = 20, p = 0.003; n = 11-12 / group) (see Fig. 4-1A).

Homecage behaviours

The MANOVA on behaviour during the first 3 days of treatment was significant (F= 85.107, p < 0.001, ηp² = 0.99; n = 6 cages / group). There was an effect of Drug such that AM251 males displayed significantly fewer Social Inactive behaviours (p = 0.001) and significantly more Self Directed behaviours (p < 0.001) than did VEH males, all other comparisons p > 0.230 (see Fig. 4-2A). The MANOVA on behaviour during the last 3 days of treatment was significant (F= 8.637, p = 0.016, ηp² = 0.912), and there was an effect of Drug such that AM251 males displayed significantly less Social Inactive (p = 0.023) behaviour and more Social Active (p = 0.038) and Self Directed behaviours (p = 0.001) than did VEH males. All other comparisons failed to show significance (p > 0.170; see Fig. 4-2A).

Social interaction test PND 50

Data pertaining to behaviour during the 10 min open field test were lost because of equipment failure.
There were no differences between VEH and AM251 males on the latency to approach, or time spent investigating, a novel object placed into the centre of the open field (ps > 0.866; n = 11-12 / group) (see Table. 4-1).

A mixed-model analysis of variance (ANOVA) with Individual (Test or Stimulus) as the within group factor and Drug (VEH or AM251) as the between group factor (n = 11-12 pairs/group) found that Test rats initiated more interactions than did Stimulus rats (F_{1,21} = 91.517, p < 0.001, \eta_p^2 = .813). There was no difference between VEH and AM251 males on time spent engaging in social interactions, nor was there an interaction between Drug and Individual (ps > 0.470) (see Fig. 4-3A).

**Experiment 1: Females**

**Weights**

There were no group differences in weight on the first day of adolescent treatment (PND 30) in female rats (p = 0.321; data not shown). Although AM251 females tended to gain less weight that did VEH females across the 15 days of treatment, the effect did not meet statistical significance (T = 1.756 df = 20, p = 0.094; n = 12 / group) (see Fig. 4-1B).

**Homecage behaviours**

The MANOVA on behaviour during the first 3 days of treatment was significant (F_{5,6} = 284.834, p < 0.001, \eta_p^2 = .997; n = 6 cages / group). There was an effect of Drug such that AM251 females displayed significantly less Social Inactive behaviour and significantly more Self Directed behaviours than did VEH females (ps < 0.001) (see Fig. 4-2B). All other comparisons showed no significant effect (ps > 0.063). The MANOVA for behaviour during the last 3 days of treatment was significant (F_{5,3} = 6.416, p = 0.030,
η_p^2 = .885), and there was an effect of Drug, such that AM251 females displayed significantly fewer Social Active (p = 0.016) and Alone Active (p < 0.001) behaviours and significantly more Self Directed behaviours (p = 0.006) than did VEH females. All other comparisons failed to show statistical significance (p > 0.350; see Fig. 4-2B).

**Social interaction test PND 50**

One VEH and one AM251 female were removed from the study for health reasons and, thus, are not included in the statistical analyses.

There were no differences between VEH and AM251 females on the time spent in the centre of the open field, nor on the total distance travelled during the 10 min open field test (ps > 0.150) (see Table 4-1).

There were no differences between VEH and AM251 females on the latency to approach, or time spent investigating, a novel object placed into the centre of the open field (ps > 0.642) (see Table 4-1).

A mixed-model ANOVA with rat (Test or Stimulus) as the within group factor and Drug (VEH or AM251) as the between group factor (n = 11 pairs / group) found that more time was spent in interactions initiated by Test rats than by Stimulus rats (F_{1,20} = 384.850, p < 0.001, η_p^2 = .591), and that the interaction between Drug group and individual was significant (F_{1,20} = 4.886, p = 0.039, η_p^2 = .196). Post hoc analysis indicated that among Test rats, AM251 initiated more time in social interactions than did VEH rats (p = 0.035). There were no differences among Stimulus rats in time spent initiating social interactions with VEH or AM251 rats (P = 0.408) (see Fig. 4-3B).

**Experiment 2 – Females**

All measures in experiment 2 were collected in female rats only.
**Weights**

There were no differences in weight between AM251 and VEH on the first day of experimental procedures on PND 30 (t = 0.497, df = 30, p = 0.623; data not shown). The weight gained across the 15 days of injection was greater for vehicle rats than for AM251 rats (t = 2.976, df = 30, p = 0.006); AM251 rats weighed less than vehicle rats on the last day of treatment on PND 44 (see Fig. 4-4).

**Homecage behaviours**

During the first three days of injection (PND 30-32), AM251 rats demonstrated fewer Inactive Social (t = 4.337, df = 14, p = 0.001) and more Self Directed behaviours (t = -12.528, df = 14, p < 0.001) than did vehicle rats. There was no effect of drug for any of the other behaviours scored (all p > 0.166) (see Fig. 4-5). During the last three days of injection (PND 42-44) AM251 rats displayed fewer Non-Social Inactive (t = 3.315, df = 14, p = 0.005) and Non-Social Active (t = 2.259, df = 14, p = 0.040) behaviours. AM251 exhibited more Self Directed behaviours than did VEH rats (t = -6.581, df = 14, p < 0.001). There was no effect of drug for any of the other behaviours scored (all p > 0.470) (see Fig. 4-5).

**Elevated plus maze test**

AM251 was without effect on any of the measures of behaviour in the elevated plus maze (all p > 0.249) (see Fig. 4-6).

**Social interaction test**

The data from four rats each from VEH and AM251 were lost because of equipment failure.
There were no differences between the AM251 and VEH groups for latency to enter the centre of the open field, time spent in the centre of the open field, or for distance travelled in the centre of the open field (all ps > 0.445). AM251 did not affect total distance travelled during the 10 min open field test (p = 0.717) (see Table 4-2).

There were no differences in latency to approach or in time spent investigating a novel object placed in the centre of the open field (ps > 0.590) (see Table 4-2).

A repeated measures analysis of variance (ANOVA) with Individual (Test rat vs. Stimulus rat) as the paired subject variable and Drug (VEH or AM251) as the between subject variable found a significant effect of Drug (F1,22 = 9.16, p = 0.006) and of Individual (F1,22 = 294.592, p > 0.001; Test rats initiated more social interactions than did Stimulus rats), and an Individual by Drug interaction (F1,22 = 54.983, p < 0.001). Independent sample t-tests conducted separately on Test and Stimulus rats indicated that among Test rats, females exposed to AM251 in adolescence initiated more time in social interactions that did VEH females (t = -6.931, df = 22, p < 0.001). Among Stimulus rats, those interacting with AM251 females spent less time initiating social interactions than did Stimulus females interacting with VEH females (t = 3.354, df = 22, p = 0.003) (see Fig. 4-7).

**EGR-1 cell counts**

AM251 females had a greater number of EGR-1 positive cell counts in the nucleus accumbens shell 1 h after the completion of social interaction testing than did vehicle rats (t = -2.924 df = 16, p = 0.010; see Fig. 4-8). There were no differences between AM251 and VEH females in the number of EGR-1 positive cell in the nucleus accumbens core (p = 0.461) or in any of the hippocampal subfields investigated (all p >
0.47) (see Fig. 4-8). We next examined the correlations between neural activity (as measured by the number of EGR-1 positive cell counts) and time in social interaction in the VEH and in the AM251 females (see Table 4-3, Fig. 4-9). We then used regression analysis to increase statistical power by analysing the relationships in the total sample while controlling for drug treatment. When simple correlations in each of the groups differed by more than .25 for a measure, the interaction was included in the second step of the regression after centering the data. The first regression analysis involved drug treatment and EGR-1 cell counts in the three hippocampal subfields (CA1, CA2, and CA3) as predictors of time spent in social interactions. The nucleus accumbens data set was not included as a predictor to keep the sample size as large as possible because of missing data for this measure. The model resulted in an adjusted $R^2$ of 0.79, and both drug treatment ($\beta = 0.875, p < 0.001$) and EGR-1 cell counts in the dorsal CA2 ($\beta = -0.440, p = 0.002$) emerged as unique predictors of individual differences in social interactions (see Table 4-4 for regression results).

We then conducted an exploratory analysis using the EGR-1 cell counts in the nucleus accumbens shell (NAcShell), Drug treatment, and EGR-1 cell counts in the CA2 as predictors of social interaction, which also allowed us to see if the relationship between EGR-1 in CA2 and social interactions remained in the smaller sample of rats. On the second step of the regression, the interaction of CA2 and Drug group and of NAcShell and Drug group were entered. The first step was significant ($F_{3,13} = 19.009, p < 0.001$) and resulted in an adjusted $R^2$ of 0.77. The change in F value from step 1 to step 2 was significant ($F_{2,11} = 4.977, p = 0.029$). In the second step, despite the reduced sample size and statistical power, the model resulted in an adjusted $R^2$ of 0.86 and CA2
remained a significant predictor ($\beta = -0.540, t = -2.665, p = 0.022$) and the interaction of NAcShell and Drug group was significant ($t = 2.858, p = 0.016$) (see Table 4-5 for regression results). To further investigate the significant interaction between Drug and NAcShell EGR-1 ir cell counts, we performed a simple slopes analysis on 1 S.D. above and 1 S.D. below the mean value of EGR-1 cell counts (see Fig. 4-10). Simple slopes analysis revealed that the difference between the regression lines for VEH and AM251 rats was significant at 1 S.D. below the mean ($\beta = 39.02, t = 2.20, p = 0.048$) and 1 S.D. above the mean ($\beta = 109.65, t = 7.72, p < 0.001$). Because AM251 rats spent greater time in social interactions at each point tested, we can interpret the main effect of Drug ($\beta = 74.82, t = 7.15, p < 0.001$). Simple slopes analysis indicated that the magnitude of difference between AM251 and VEH was greater when animals had higher EGR-1 cell counts in the NAc shell (see Fig. 4-10).

Discussion

The present findings highlight sex-specific contributions of adolescent endocannabinoid signalling to the normative development of social behaviours. Whereas repeated administration of the CB1 receptor antagonist AM251 (1 mg / kg) in adolescence (PND 30-44) increased social interactions in female rats on PND 50, there were no effects in males. Further, whereas EGR-1-positive cell counts in the nucleus accumbens shell emerged as a unique predictor of social interactions in AM251-treated females, cell counts in the dorsal hippocampal CA2 (dCA2) subfield emerged as a predictor of social interactions in both vehicle- and AM251-treated females. Our results demonstrate adolescent CB1 receptor antagonism-induced increases in social interactions in female rats that were associated with increased nucleus accumbens shell neural
activity, suggesting that the behavioural differences observed may be attributable to developmental alterations to the nucleus accumbens shell and its associated circuitry. Our results also provide further support for the dCA2 as a regulator of social interaction in rats.

**Sex-specific effects of AM251 on social interactions**

There is increasing evidence for the involvement of endocannabinoid signalling in the regulation and development of social behaviours in both humans (Wei et al., 2017) and rodents (Trezza et al., 2012; Trezza and Vanderschuren, 2008b; Wei et al., 2015). We have previously demonstrated sex-specific effects of CB1 receptor antagonism in adolescence on social behaviours in rats; repeated AM251 in adolescence increased social interactions in females with no effect in males when tested 48 h after the final AM251 exposure (Simone et al., 2018). As the half-life of AM251 is ~22 h in rats (McLaughlin et al., 2003), it is likely that our previous observations of increased social interactions in AM251-treated females after a 48 h drug washout period involved neurodevelopmental disruptions to social-regulatory networks stemming from repeated CB1 receptor antagonism. Nevertheless, we could not rule out the possibility that the observed effects were due to residual drug in the system. Thus, in the present study we increased the drug washout period from 2 to 5 days to allow for greater clearance of AM251 from the system. The effects of adolescent CB1 receptor antagonism persisted after the 5-day delay and were observed only in female rats, further supporting the hypothesis that CB1 receptor signalling in adolescence contributes to the development of social circuits in the brain, and thus to the development of social behaviours. Further, that the observed effects
on social interactions were specific to females suggests differential contributions of CB1 receptor signalling to social development in male and female rats.

There are limited studies into the role of CB1 receptor signalling in adolescent brain development, and most have involved only males (Carvalho et al., 2016; Cass et al., 2014; Renard et al., 2017; Renard et al., 2016b; Rubino et al., 2009b). Repeated exposure to CB1 receptor agonists in adolescence has been shown to alter the development of mesocorticolimbic networks in male rats leading to a state of dopaminergic hyperactivity along the prefrontal cortex-nucleus accumbens pathway (Renard et al., 2017). Adolescent CB1 receptor agonism also reduced spine density in the nucleus accumbens (Carvalho et al., 2016) and dentate gyrus of the hippocampus (Rubino et al., 2009b), and produced a functional downregulation of prefrontal cortical GABAergic transmission (Cass et al., 2014) in male rats. In females, exposure to AM251 in adolescence prevented developmental decreases in several markers of the glutamatergic system in the prefrontal cortex in adulthood (Rubino et al., 2015). Further, we have previously demonstrated that repeated AM251 treatment in adolescence decreased CB1 receptor expression in the dorsal hippocampus, and increased GAD67 expression (a marker of the GABAergic system) in the prefrontal cortex of female rats 24 h after their final drug exposure with no effects observed in males. Thus, when taken together, studies with CB1 receptor agonists and antagonists suggest that the contributions of the ECS to adolescent neurodevelopment are unique between the sexes.

The sex-specific nature of AM251 effects on social interactions are likely because of sex-specific neurodevelopmental alterations within social brain networks. Indeed, the development of the adolescent brain is sexually dimorphic, and differences in
development between males and females are often ascribed to differences in gonadal hormone signalling (Sisk and Zehr, 2005). Further, endocannabinoid signalling can regulate the release of gonadal hormones in both sexes (Gorzalka and Dang, 2012). Thus, disruptions to normative CB1 receptor signalling via administration of AM251 in adolescence may elicit sex-differences in brain and behaviour development via effects on gonadal hormone signalling. Another explanation for the observed sex-differences in the present study are sex-differences in the sensitivity to, and metabolism of, AM251. Female rats are often reported to have a greater sensitivity to the behavioural effects of cannabinoid drugs (Craft, 2005; Tseng and Craft, 2001; Tseng et al., 2004). The greater sensitivity to cannabinoid drugs in females has been attributed to the greater percentage of body fat in male compared with female rats, and thus to a greater percentage of drug being absorbed into adipose tissue in males (Tseng et al., 2004). Sex-differences in body fat composition are less of a factor in adolescent animals, however, and thus are unlikely to underlie the sex-specific effects observed in the present thesis. Further, our observations of behaviour in the homecage 30 min after AM251 administration demonstrate similar reductions in social behaviours and increases in Self Directed behaviours in both males and females while the drug is in the system. Thus, our results suggest that the dose of AM251 used in the present study produces comparable behavioural effects in the homecage in both sexes, and thus suggests that differences in drug sensitivity are not solely responsible for the observed effects on social behaviour.

**Associations between neural activity and social interactions in females**

In experiment 2 we sought to identify candidate brain regions that are involved in the AM251-induced increases in social interactions observed in female rats. One hour
after social interaction testing, we observed a greater number of EGR-1 ir cells in the nucleus accumbens shell of females previously exposed to AM251 compared with females previously exposed to vehicle, whereas there were no group differences observed in the nucleus accumbens core. Furthermore, EGR-1 ir cell counts in the nucleus accumbens shell were found to be a unique predictor of social behaviour in AM251 females, with no association found between cell counts and social interactions in vehicle females. While both the shell and core divisions of the nucleus accumbens contribute to social behaviours, there is evidence that their contributions are functionally distinct; whereas the nucleus accumbens core is implicated in the regulation of locomotor activity, the shell is highly implicated in social reward processing (Corbit et al., 2001; Deutch and Cameron, 1992; Ito et al., 2004; Zahm, 1999). Our finding of increased nucleus accumbens shell activity after social interaction testing is thus suggestive of a greater reward value of the interactions in AM251 compared with vehicle females. The observation that the increased social interactions were independent of changes in generalized anxiety-like behaviours (as measured by the EPM and OFT) or novelty-seeking behaviours (as measured by the novel object test) provides further support for increased social reward value in AM251 females. Furthermore, our results are in accordance with previous reports of adolescent CB1 receptor regulation of reward network development (Renard et al., 2017). Thus, it is likely that the increased sociality of AM251 females reflects greater reward value of social interactions resulting from altered neurodevelopment of mesocorticolimbic networks.

Although we found no effect of AM251 treatment on neural activity in the dorsal hippocampus, activity in the dCA2 emerged as a significant predictor of social
behaviours in both AM251 and vehicle females, in accordance with previous reports identifying dCA2 contributions to social regulation (Maaswinkel et al., 1996) (Hitti and Siegelbaum, 2014; Stevenson and Caldwell, 2014). Of note, the relationship between dCA2 activity (as measured by EGR-1 ir cell counts) and social interactions were negative for both groups, demonstrating an association between reduced dCA2 activity and greater social interactions. The negative relationship between dCA2 and social interactions may be explained by considering the greater dorsal hippocampal-ventral hippocampal-nucleus accumbens network that is involved in social behaviours. The dorsal CA2 projects to the CA1 division of the ventral hippocampus (vCA1), a region that regulates social avoidance via direct connectivity to the nucleus accumbens shell (Okuyama et al., 2016). Thus, reductions in the activity of the dCA2 may lead to reduced activation of the vCA1-nucleus accumbens shell pathway responsible for social avoidance, leading to increases in social approach. Nevertheless, this explanation is only speculative, and more work into the role of the dCA2 in social behaviour regulation is warranted.

**Conclusion**

Together, these results demonstrate endocannabinoid involvement in the development of social behaviours in female rats. Further, the increased sociality in AM251 females was independent of novelty-seeking or generalized anxiety-like behaviours and was associated with greater neural activation in the nucleus accumbens shell, and thus may involve increases in the reward value of social interactions. Further, we have provided support for a role of dorsal hippocampal CA2 activity in the regulation of social behaviours and demonstrate that greater dorsal hippocampal EGR-1 positive cell
counts are correlated with reduced social interactions, suggestive of an inhibitory influence of dCA2 signalling to social behaviour. Finally, our results shed new light on the developmental role of endocannabinoid signalling in adolescence and provide insight into the neural mechanisms underlying social behaviours and their development in adolescence.
Tables

Table 4-1. Means (including S.E.M.) for latency to approach, and time spent interacting with a novel object for male rats (left; n = 11-12 / group) and time spent in the centre, latency to approach the centre, and total distance travelled in the open field, and for latency to approach, and time spent interacting with a novel object for female rats (right; n = 11 / group) on PND 50 in experiment 1.

<table>
<thead>
<tr>
<th></th>
<th>Males Novel Object</th>
<th>Fema</th>
<th>les Novel Object</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Latency to approach (s)</td>
<td>Time spent interacting (s)</td>
<td>Total distance travelled (m)</td>
</tr>
<tr>
<td>VEH</td>
<td>86.41</td>
<td>144.56</td>
<td>47.79</td>
</tr>
<tr>
<td></td>
<td>(34.82)</td>
<td>(18.24)</td>
<td>(2.90)</td>
</tr>
<tr>
<td>AM251</td>
<td>88.52</td>
<td>149.61</td>
<td>49.71</td>
</tr>
<tr>
<td></td>
<td>(46.79)</td>
<td>(22.88)</td>
<td>(1.83)</td>
</tr>
</tbody>
</table>
Table 4-2. Means (including S.E.M.) for time spent in the centre, latency to approach the centre, and total distance travelled in the open field, and for latency to approach, and time spent interacting with a novel object for female rats on PND 50 in experiment 2 (n = 12 / group).

<table>
<thead>
<tr>
<th></th>
<th>Open Field</th>
<th>Novel Object</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total distance travelled (m)</td>
<td>Time in centre (s)</td>
</tr>
<tr>
<td>VEH</td>
<td>46.80</td>
<td>28.73</td>
</tr>
<tr>
<td></td>
<td>(1.42)</td>
<td>(6.86)</td>
</tr>
<tr>
<td>AM251</td>
<td>45.92</td>
<td>36.88</td>
</tr>
<tr>
<td></td>
<td>(1.96)</td>
<td>(15.53)</td>
</tr>
</tbody>
</table>
Table 4-3. Correlation table for time in social interactions initiated by the Test rat (TEST) and EGR-1 ir cell counts in the dorsal hippocampal CA1, CA2, and CA3 subdivisions and in the nucleus accumbens core (NAcCore) and shell (NAcShell) divisions for VEH females in experiment 2. Correlations accounting for more than 25% of the variance are in bold font. Values above the diagonal correspond to VEH rats and values below the diagonal correspond to AM251 rats.

<table>
<thead>
<tr>
<th></th>
<th>TEST</th>
<th>CA1</th>
<th>CA2</th>
<th>CA3</th>
<th>NAcCore</th>
<th>NAcShell</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEST</td>
<td>Pearson Correlation</td>
<td>1</td>
<td>.259</td>
<td>-.558</td>
<td>-.013</td>
<td>-.256</td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>.416</td>
<td>.074</td>
<td>.968</td>
<td>.506</td>
<td>.215</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>12</td>
<td>12</td>
<td>11</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>CA1</td>
<td>Pearson Correlation</td>
<td>.154</td>
<td>1</td>
<td>.649</td>
<td>.646</td>
<td>-.488</td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>.633</td>
<td>.031</td>
<td>.023</td>
<td>.182</td>
<td>.178</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>12</td>
<td>12</td>
<td>11</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>CA2</td>
<td>Pearson Correlation</td>
<td>-568</td>
<td>.357</td>
<td>1</td>
<td>.479</td>
<td>-.243</td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>.054</td>
<td>.254</td>
<td>.136</td>
<td>.562</td>
<td>.870</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>12</td>
<td>12</td>
<td>11</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>CA3</td>
<td>Pearson Correlation</td>
<td>.017</td>
<td>.727</td>
<td>.616</td>
<td>1</td>
<td>.004</td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>.959</td>
<td>.011</td>
<td>.044</td>
<td>.993</td>
<td>.731</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>NAcCore</td>
<td>Pearson Correlation</td>
<td>.485</td>
<td>.613</td>
<td>.030</td>
<td>.681</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>.186</td>
<td>.079</td>
<td>.940</td>
<td>.063</td>
<td>.251</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>NAcShell</td>
<td>Pearson Correlation</td>
<td>.500</td>
<td>.714</td>
<td>.269</td>
<td>.688</td>
<td>.807</td>
</tr>
<tr>
<td></td>
<td>Sig. (2-tailed)</td>
<td>.170</td>
<td>.031</td>
<td>.485</td>
<td>.059</td>
<td>.008</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>8</td>
<td>9</td>
</tr>
</tbody>
</table>
Table 4-4. Regression table predicting time in social interactions initiated by the Test rat from Drug group and EGR-1 ir cell counts in the dorsal hippocampal CA1, CA2, and CA3 subfields in experiment 2.

<table>
<thead>
<tr>
<th></th>
<th>B</th>
<th>Std. Error</th>
<th>Beta</th>
<th>t</th>
<th>Sig.</th>
<th>Partial</th>
<th>Part</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug</td>
<td>79.996</td>
<td>9.288</td>
<td>.875</td>
<td>8.612</td>
<td>&lt; .001</td>
<td>.902</td>
<td>.852</td>
</tr>
<tr>
<td>CA1</td>
<td>.629</td>
<td>.382</td>
<td>.219</td>
<td>1.645</td>
<td>.118</td>
<td>.371</td>
<td>.163</td>
</tr>
<tr>
<td>CA2</td>
<td>-1.288</td>
<td>.360</td>
<td>-.440</td>
<td>-3.581</td>
<td>.002*</td>
<td>-.656</td>
<td>-.354</td>
</tr>
<tr>
<td>CA3</td>
<td>.358</td>
<td>.799</td>
<td>.068</td>
<td>.449</td>
<td>.659</td>
<td>.108</td>
<td>.044</td>
</tr>
</tbody>
</table>

* Indicates significance, p < 0.05.
Table 4-5. Regression table predicting time in social interactions initiated by the Test rat from Drug group and EGR-1 ir cell counts in the dorsal hippocampal CA2 and nucleus accumbens shell (NAcShell) subfields in experiment 2.

<table>
<thead>
<tr>
<th></th>
<th>B</th>
<th>Std. Error</th>
<th>Beta</th>
<th>t</th>
<th>Sig.</th>
<th>Partial</th>
<th>Part</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug</td>
<td>74.823</td>
<td>10.464</td>
<td>.841</td>
<td>7.150</td>
<td>&lt; .001*</td>
<td>.907</td>
<td>.673</td>
</tr>
<tr>
<td>CA2</td>
<td>-1.450</td>
<td>.546</td>
<td>-.540</td>
<td>-2.655</td>
<td>.022*</td>
<td>-.625</td>
<td>-.250</td>
</tr>
<tr>
<td>NAcShell</td>
<td>-.434</td>
<td>.288</td>
<td>-.212</td>
<td>-1.503</td>
<td>.161</td>
<td>-.413</td>
<td>-.141</td>
</tr>
<tr>
<td>CA2 x Drug</td>
<td>.706</td>
<td>.628</td>
<td>.230</td>
<td>1.124</td>
<td>.285</td>
<td>.321</td>
<td>.106</td>
</tr>
<tr>
<td>NAcShell x Drug</td>
<td>1.422</td>
<td>.498</td>
<td>.408</td>
<td>2.858</td>
<td>.016*</td>
<td>.653</td>
<td>.269</td>
</tr>
</tbody>
</table>

* Indicates significance, p < 0.05.
Fig. 4-1. Mean (+/- S.E.M.) for weight gained across the 15 days of treatment (PND 30-44) in (A.) male and (B.) female rats in experiment 1. * compared with VEH, p < 0.05.
Fig. 4-2. Mean (+/- S.E.M) number of observations for each behavioural category for the first three days of treatment (PND 30-32) and for the last three days of treatment (PND 42-44) in (A.) male and (B.) female rats in experiment 1. * compared with VEH, p < 0.05.
Fig. 4-3. Mean (+/- S.E.M.) time spent in social interactions initiated by the Test rat or by the Stimulus rat on PND 50 for (A.) male and (B.) female rats on PND 50 in experiment 1. * compared with VEH, p < 0.05.
Fig. 4-4. Mean (+/- S.E.M.) weight gained across the adolescent treatment period (PND 30-44) for female rats in experiment 2. * compared with VEH, p < 0.01.
Fig. 4-5. Mean (+/- S.E.M) number of observations for each behavioural category for the first three days of treatment (PND 30-32) and for the last three days of treatment (PND 42-44) in female rats in experiment 2. * compared with VEH, p < 0.05.
Fig. 4-6. Mean (+/- S.E.M.) for behavioural measures in the elevated plus maze on PND 45 (24 h after final treatment) in female rats on PND 50 in experiment 2.
Fig. 4-7. Mean (+/- S.E.M.) time spent in social interactions initiated by the Test rat or Stimulus rat on PND 50 for females in experiment 2. * compared with VEH, p < 0.05.
Fig. 4-8. (A.) Mean (+/- S.E.M.) number of EGR-1 ir cell counts in the CA1, CA2, and CA3 subfields of the dorsal hippocampus and in the Core and Shell subdivisions of the nucleus accumbens for VEH and AM251 females 1 h after social interaction testing (experiment 2). (B.) Representative images of EGR-1 immunostained slices in the CA1, CA2, and CA3 subfields of the dorsal hippocampus and in the Core and Shell subdivisions of the nucleus accumbens.
Fig. 4-9. Correlations between time spent in social interactions initiated by the Test rat and EGR-1 ir cell counts in the dorsal hippocampal CA2 (A.) and nucleus accumbens shell (B.) for both VEH and AM251 female rats in experiment 2.
Fig. 4-10. Simple slopes analysis of the interaction between Drug and NAcShell EGR-1 ir cell counts and time spent in social interactions at LOW (1 S.D. below the mean) and HIGH (1 S.D. above the mean) cell counts for female rats in experiment 2. The magnitude of the difference between AM251 and VEH rats was greater when cell counts were high ($\beta = 109.65$) than when cell counts were low ($\beta = 39.02$). * compared with VEH, p < 0.05.
Chapter 5: Summary of main findings and general discussion.
Main summary

The findings from my thesis research support and extend previous literature into the developmental role of endocannabinoid signalling in adolescence and demonstrate sex-specific CB1 receptor regulation of adolescent development of brain and behaviour.

In chapter 2, I demonstrated that repeated CB1 receptor antagonism in adolescence (1 mg/kg AM251; postnatal days 30-44) increased social interactions, increased prefrontal cortex (PFC) expression of the GABAergic marker GAD67, and reduced dorsal hippocampal (DH) expression of CB1 receptor protein in female rats soon after (24-48 h) the final exposure, with no observed effects in males. Further, AM251 effects in females occurred irrespective of whether the drug was administered alone or in tandem with 1 h confinement stress. Thus, my findings suggest that adolescent female rats are more sensitive to the immediate developmental effects of CB1 receptor perturbations than are adolescent male rats.

In chapter 3, I demonstrated that the effects of adolescent CB1 receptor antagonism on PFC GAD67 and DH CB1 receptor expression in females were no longer evident when tested several weeks after the final drug exposure (PND 74). Conversely, adolescent AM251-treated males demonstrated reduced anxiety-like behaviour on PND 70; there was no effect of adolescent AM251 treatment on the same measure of anxiety in male rats when tested 24 h after the final exposure (chapter 2). Further, AM251 increased protein kinase A (PKA) activation only in male rats that underwent repeated adolescent confinement stress, whereas no differences were observed in adolescent males (chapter 2). Given that only modest long-term behavioural effects of AM251 were observed, and that in both adolescent and adult animals the AM251-induced changes in behaviour were
independent of stress exposures, I sought to further characterize the behavioural phenotype of adolescent AM251 rats of both sexes under non-stressed conditions only. To that end, adolescent male and female rats were treated with either vehicle or AM251 on PND 30-44, and were tested for contextual fear conditioning, recall, extinction, and reconditioning in adulthood (PND 78 onward). Adolescent AM251 increased contextual fear recall and reconditioning relative to vehicle in adult females, with no effects observed in adult males. Thus, the results from chapters 2 and 3 demonstrate that whereas effects of adolescent CB1 receptor antagonism tend to emerge with time in males, effects are present soon and long after cessation of treatment in females. Further, that effects of adolescent AM251 were specific to anxiety-like behaviours in males and to social interactions and conditioned fear in females suggests that there is a sex-specific regulation of neurodevelopment by the endocannabinoid system in adolescence.

In chapter 4, I sought to further investigate the increased social behaviours observed in AM251 females in chapter 2 by increasing the drug wash-out period from 2 to 5 days to better ensure the effects were not because of residual drug in the system. Additionally, I had previously tested male rats in social interactions with novel same-Drug Stimulus rats (e.g., AM251 Test rat paired with AM251 Stimulus rat), whereas females were paired with a novel rat naive to experimental manipulation (i.e., AM251 Test rat paired with a non-injected Stimulus rats). Thus, I repeated the social interaction test in both males and females while paired with an experiment-naïve Stimulus rat as to better compare effects between sexes. Consistent with chapter 2, AM251 increased time spent in social interactions relative to vehicle, with effects observed only in females. Given that I had reported reduced CB1 receptor expression in the DH of adolescent
AM251 females (chapter 2) and given the known role of both the DH and CB1 receptors in the regulation of social behaviours (File and Kenny, 1998; Maaswinkel et al., 1996; Stevenson and Caldwell, 2014; Trezza et al., 2012; Trezza and Vanderschuren, 2008a, 2009; Trezza and Vanderschuren, 2008b), I sought to determine the involvement of the DH in the AM251-induced increases in social behaviour. Further, exposure to a CB1 receptor agonist in adolescence reduced social interactions and altered the developmental of prefrontal-accumbens connectivity in adulthood (Renard et al., 2017), suggesting ECS contributions to social reward development in adolescence. Therefore, I also investigated the extent to which the observed effects involved the nucleus accumbens, a region critical for social reward processing (Trezza et al., 2011; Trezza et al., 2012). I repeated social interaction testing in females and collected brains 1 h after the end of behavioural testing for immuno-labelling of EGR-1 (the protein product of the immediate early gene zif268) as a measure of neural activity. Once again, prior AM251 treatment increased the amount of time spent in social interactions relative to vehicle. Further, greater NAc shell activation (as measured by the number of EGR-1 positive cell counts) was observed in AM251 females compared with vehicle females; no group differences in EGR-1 cell counts were observed for the NAc core or for any of the hippocampal subfields (CA1, CA2, CA3) investigated. A further exploration of the relationship between EGR-1 cell counts and social interaction behaviour revealed that whereas EGR-1 signalling in the nucleus accumbens shell is a unique predictor of social interactions in AM251 females, cell counts in the dCA2 predict behaviour in both vehicle and AM251 females. Thus, my findings suggest that disrupting normative adolescent endocannabinoid signalling via
repeated CB1 receptor antagonism alters the development of social reward systems in the brain, leading to dysregulated social behaviour.

Discussion

The protracted development of the adolescent brain renders it vulnerable to external influences such as physical and psychological stressors (Marco et al., 2011; McCormick and Green, 2013; McCormick et al., 2017), and drugs of abuse (Crews et al., 2007). Research with cannabinoid receptor agonists have exploited this phenomenon to demonstrate developmental consequences of perturbations to adolescent endocannabinoid signalling (O'Shea et al., 2006; O'Shea et al., 2004; Quinn et al., 2008b; Renard et al., 2017; Renard et al., 2016b; Rubino et al., 2015; Schneider and Koch, 2003; Schneider et al., 2008). That disruptions to adolescent endocannabinoid signalling via cannabinoid receptor activation produces long-term developmental consequences implicates the endocannabinoid system in normative adolescent development. Research into the contribution of endogenous cannabinoid signalling in adolescent brain and behaviour development is limited, however, with few studies investigating endogenous CB1 receptor signalling in this context. The following sections will discuss potential mechanisms underlying the developmental influences of CB1 receptor signalling, the utility of cannabinoid receptor agonists and antagonists in the investigation of endogenous cannabinoid signalling contributions to adolescent development, and future directions implicated from my thesis research findings.

Endocannabinoid regulation of adolescent development: Potential mechanisms

The expression of CB1 receptors, the predominant central cannabinoid receptor, in adolescence is dynamic, with peak expression occurring in the early-mid adolescent
period, followed by a steady decline to adult levels (Rodríguez de Fonseca et al., 1993; Rubino et al., 2015). The rise and fall of CB1 receptor expression across adolescence is mirrored by fluctuations in other major neurotransmitter systems, such as the glutamatergic and GABAergic systems (Caballero et al., 2014; Rubino et al., 2015). CB1 receptors, in the most general sense, act as inhibitors of cell to cell communication, a process critical for the establishment and maintenance of appropriate synaptic connections (Lin and Koleske, 2010). That CB1 receptor expression increases across early- to mid-adolescence concomitant with decreases in glutamate (Rubino et al., 2015) and GABA (Caballero et al., 2014) receptor expression suggests that CB1 receptor signalling may be involved in excitatory and inhibitory synapse formation and maintenance during this period. Indeed, endocannabinoid signalling at CB1 receptors is a process known to regulate neuronal connectivity via regulation of intercellular communication (Berghuis et al., 2007). As CB1 receptor signalling is inhibitory to synaptic activity, direct receptor antagonism is expected to result in dis-inhibition of the synapse, and thus, to greater communication between pre- and post-synaptic neurons. The greater cell to cell communication associated with CB1 receptor inhibition is expected to result in reduced synaptic pruning, an idea that is supported in the literature (Rubino et al., 2015). Thus, given the extensive reorganization of corticolimbic synapses in adolescence, and the suspected role of endogenous CB1 receptor activity in promoting synaptic pruning, my findings of developmental consequences of adolescent CB1 receptor antagonism likely involve effects on neural network development.

Although CB1 receptor influences on intercellular communication and synaptic organization represent a likely mechanism for understanding the developmental role of
adolescent endocannabinoid signalling, influences on intracellular signalling pathways are also likely involved. CB1 receptor activation is known to regulate the activity of several intracellular signalling pathways involved in cellular growth, differentiation, and activity. In particular, CB1 receptor activity is associated with increases in the activation of the extracellular-signal regulated kinase-1 and -2 (ERK1/2) (Dalton and Howlett, 2012; Dalton et al., 2009; Dalton et al., 2013) and phosphatidylinositol-3-kinase / protein kinase B (PI3K/Akt) (Gomez del Pulgar et al., 2000; Ozaita et al., 2007; Puighermanal et al., 2009; Sanchez, 2003) pathways, and decreases in the activation of the cyclic adenosine monophosphate-dependent protein kinase A (PKA) pathway (Dalton et al., 2009; Howlett, 2004; Howlett et al., 2010).

CB1 receptor inhibition of PKA is attributed to the direct inhibition of membrane-bound adenylyl cyclase, and thus to reductions in the PKA activator cAMP, by the alpha subunit of the CB1 associated G protein (Gα). CB1 regulation of ERK1/2 and Akt activity is not as well characterized, though there are several mechanisms that have been proposed. For instance, PKA activity has been shown to be inhibitory to ERK1/2 activation via the PKA-mediated phosphorylation, and subsequent inhibition, of the ERK1/2 activator Raf1 (Scotter et al., 2009). Thus, CB1 receptor-mediated reductions in PKA activity lead to dis-inhibition of Raf1 and to subsequent activations of ERK1/2 (Scotter et al., 2009). Alternatively, CB1 receptor activation can increase synthesis and intracellular release of ceramide via actions at the tumor necrosis factor receptor (TNFR) (Galve-Roperh et al., 2002); ceramide elicits increases in ERK1/2 activity via activational effects on Raf1 (Scotter et al., 2009). CB1 receptors have also been suggested to directly activate ERK1/2 via Gα interactions with Raf1 (Scotter et al., 2009). Similarly, the effects
of CB1 receptor signalling on Akt activation are thought to occur through direct interactions between Gβγ subunits and the upstream Akt activator phosphatidylinositol-3-kinase (PI3K) (Díaz-Alonso et al., 2012). Although the general consensus within the literature is of a pro-activational influence of CB1 receptor signalling on ERK1/2 and Akt (Dalton and Howlett, 2012; Dalton et al., 2009; Dalton et al., 2013; Díaz-Alonso et al., 2012; Galve-Roperh et al., 2002; Gomez del Pulgar et al., 2000; Howlett, 2002, 2004; Howlett et al., 2010; Ozaita et al., 2007; Puighermanal et al., 2009; Sanchez, 2003; Scotter et al., 2009), there is evidence for cannabinoid-mediated inhibition of both pathways (Ellert-Miklaszewska et al., 2005). Further, there are sex-differences in the expression and functional contributions of ERK1/2 and Akt that are thought to involve differential regulation by gonadal hormones (Borrás et al., 2005; Giachini et al., 2010; Hunter and Korzick, 2005; Kousteni et al., 2001; Matsuda et al., 2015; Stewart and O’Brien, 2004; Ter Horst et al., 2009; Zhu et al., 2009). Thus, although the regulation of intracellular signalling by CB1 receptors represents a potential mechanism underling endocannabinoid influences on neurodevelopment, this process likely involves complex cell, region, and sex-specific interactions between the systems. Nevertheless, disruptions to CB1 receptor regulation of synaptic activity and coupling to downstream intracellular signalling pathways are likely mechanisms involved in the developmental effects of adolescent CB1 receptor antagonism observed in the present thesis, and may also explain, to a degree, the sex-specificity of the observed effects.

Sex differences in endocannabinoid biology and pharmacology

To date, most rodent studies into the behavioural and pharmacological effects of CB1 receptor signalling have involved males, with little known in females (Fattore and
Fratta, 2010). Nevertheless, sex-differences in behavioural responses to cannabinoid drugs have been reported across a variety of measures, and in general, females tend to be more sensitive and require lower doses than those used in males to achieve similar effects. For instance, CB1 receptor agonists ($\Delta^9$-THC, 11-OH-$\Delta^9$THC, CP55,940) had greater potency and efficacy in a behavioural model of nociception in adult female rats compared with adult male rats (Tseng and Craft, 2001); the greater antinociceptive effects of CP55,940 in females were also evident when rats were tested in adolescence (postnatal day 40) (Romero et al., 2002). Similarly, there are greater locomotor effects of cannabinoid drugs observed in female rodents, though the direction of effects (i.e., locomotor enhancing versus reducing effects) are inconsistent, with $\Delta^9$-THC eliciting greater locomotor activity in female than male mice (Wiley et al., 2011), and the CB1 receptor agonists $\Delta^9$-THC, 11-OH-$\Delta^9$THC, and CP55,940 eliciting greater catalepsy in female compared with male rats (Cohn et al., 1972; Tseng and Craft, 2001; Tseng et al., 2004).

The greater sensitivity of females to cannabinoid drugs can be explained, in part, by sex-differences in pharmacodynamics and pharmacokinetics. For instance, there are sex-differences in the hepatic metabolism of cannabinoids, with female rats preferentially metabolizing $\Delta^9$-THC into the more potent 11-OH-$\Delta^9$THC compared with male rats; the conversion of $\Delta^9$-THC to 11-OH-$\Delta^9$THC depends on the enzyme CYP2C6 in females and CYP2C11 in males (Narimatsu et al., 1992; Narimatsu et al., 1991). Further, sex-differences in cannabinoid drug bio-availability have been reported, and are attributed to the greater body fat percentage, and thus greater absorption of drug into adipose tissue, of male compared with female rodents (Tseng et al., 2004). In addition to differences in
drug disposition, sex-differences in various components of the endocannabinoid system have been reported across several brain regions. Whereas greater CB1 protein expression and receptor binding has been observed in male rats (de Fonseca et al., 1993; Gonzalez et al., 2000; Marco et al., 2007; Reich et al., 2009), greater CB1 receptor-mediated G-protein activity was observed in female rats (Mateos et al., 2011). Sex-differences in endocannabinoid biology and physiology are largely attributed to differences in gonadal hormone signalling, as neural endocannabinoid content and CB1 receptor density haven been shown to fluctuate in a region-dependent manner as a function of estrous cycle phase in female rats (Bradshaw et al., 2006; de Fonseca et al., 1993; Gonzalez et al., 2000). Additionally, CB1 receptor signalling can reduce circulating concentrations of androgens and estrogens via central inhibition of luteinizing hormone (LH) and gonadotropin releasing hormone (GnRH) (Gorzalka and Dang, 2012). Thus, the sexual dimorphism of the endocannabinoid system likely involves a combination of differences in the metabolism and distribution of cannabinoids, and bidirectional interactions between endocannabinoid signalling and gonadal hormone systems. Consequently, sex-differences in in the absorption, distribution, and sensitivity to AM251, as well as sex-differences in CB1 receptor coupling to downstream effectors may explain the sex-specific developmental effects on brain and behaviour observed in the present thesis.

**Pharmacological tools for the investigation of endocannabinoid signalling**

Most of what is known regarding CB1 receptor contributions to adolescent development stems from studies with cannabinoid receptor agonists. In particular, most studies of CB1 receptor agonism in adolescence address questions concerning the long-term developmental impacts of adolescent exposure to cannabis and cannabinoid drugs.
Indeed, cannabis is one of the most widely used drugs globally (Gowing et al., 2015) and in Canada, there is a growing movement towards greater access to medical and recreational cannabis that has led to the implementation of the Access to Cannabis for Medical Purposes Regulations (S.O.R./2016-230) in 2016, and the recent enactment of the Cannabis Act (Bill C-45) by the Canadian federal government in 2018. The largest demographic of cannabis users in Canada are adolescents (prevalence rate of 22% in Canadians aged 15-19) and young adults (prevalence rate of 26% in Canadians aged 20-24), and cannabis is the most commonly used drug by Canadian youth aged 15 to 24 (CTADS, 2013). Further, a comparison of 29 of the world’s advanced economies found Canada to have the highest rate of adolescent cannabis use per capita (Unicef Office of Research, 2013). Thus, the importance of understanding cannabis effects on the adolescent brain cannot be understated. Such studies of the consequences of perturbed CB1 signalling in adolescence have also shed light on the contributions of the endocannabinoid system to normative adolescent development.

Behaviourally, adolescent exposure to CB1 receptor agonists is most commonly associated with altered spatial and contextual learning and memory functions. Exposure to CB1 receptor agonists in adolescence led to reductions in novel object recognition and spatial location in male and female rats (O'Shea et al., 2006; O'Shea et al., 2004; Quinn et al., 2008a; Renard et al., 2013; Schneider and Koch, 2003; Schneider et al., 2008). The dorsal hippocampus (DH) is a brain region that is highly implicated in spatial and contextual processing (Fanselow and Dong, 2010; Frankland et al., 1998; Gold and Kesner, 2005), and in the present thesis the DH was the only brain region investigated to show a sensitivity to adolescent CB1 receptor blockade under basal conditions (chapters
2 and 3). In chapter 2 of the present thesis, I demonstrated that repeated antagonism of CB1 receptors in adolescence reduced CB1 receptor expression in the DH of female rats, whereas in chapter 3 I found that adolescent exposure to AM251 in combination with confinement stress increased PKA activity in the DH of adult male rats. Further, in chapter 3 I also demonstrated that adolescent CB1 receptor blockade increased contextual fear recall and reconditioning in adult female rats; contextual fear conditioning is critically dependent on the DH (Maren, 2001; McEown and Treit, 2010; Spiacci et al., 2016; Wang et al., 2013; Zhang et al., 2014). Thus, my findings and the findings of others implicate the dorsal hippocampus as a sensitive region for the developmental effects of disrupted CB1 receptor signalling.

In addition to DH-dependent spatial and contextual tasks, adolescent CB1 receptor agonism effects on social behaviours are well-documented (O'Shea et al., 2006; O'Shea et al., 2004; Renard et al., 2017; Schneider et al., 2008). In both male and female rats, repeated exposure to a CB1 receptor agonist in adolescence led to reduced social behaviours soon and long after cessation of treatment. Further, in one study, CB1 receptor agonist-induced social impairments were associated with hyper-dopaminergic activity along the mesocorticolimbic reward pathway, implicating CB1 receptor regulation of social reward development in adolescence (Renard et al., 2017). In chapter 2, I demonstrated that adolescent CB1 receptor blockade increases sociality in female rats only, and in chapter 4 I extended that finding by demonstrating that the increased sociality of AM251 females was associated with greater neural activation of the nucleus accumbens shell, a region critically implicated in social reward processing (Trezza et al., 2011; Trezza et al., 2012). Thus, that my findings with a CB1 receptor antagonist were
opposite to those reported with CB1 receptor agonists (O'Shea et al., 2006; O'Shea et al., 2004; Renard et al., 2017; Schneider et al., 2008) further supports a social-inhibitory developmental role of endocannabinoid signalling in adolescence. It should be noted, however, that whereas effects on social behaviour after adolescent CB1 receptor agonism were evident in both sexes, my observations with an antagonist showed effects were specific to females (chapters 2 and 4). The discrepancy in results is likely because of the on-demand nature of endocannabinoid signalling and the differences in signalling properties between CB1 receptor agonists and antagonists (Di Marzo, 2009). Whereas a systemically administered CB1 receptor agonist will activate receptors indiscriminately throughout the organism, a CB1 receptor antagonist will inhibit signalling only at those receptors for which endocannabinoids are being actively released, thereby preserving the spatio-temporal specificity of endogenous cannabinoid signalling. Thus, although studies with agonists provide valuable information regarding the susceptibility of the adolescent brain to drugs such as cannabis, as well as of the potential of CB1 receptors to contribute to neurodevelopmental processes, they are limited in their ability to discern the contributions of endogenous signalling. Studies with antagonists provide more accurate information as to the functional involvement of CB1 receptors to adolescent development.

Despite the considerable evidence implicating CB1 receptor involvement in normative adolescent development (Bambico et al., 2010; Cass et al., 2014; Kevin et al., 2017; O'Shea et al., 2006; O'Shea et al., 2004; Quinn et al., 2008a; Renard et al., 2013; Renard et al., 2017; Renard et al., 2016b; Rubino and Parolaro, 2008; Rubino et al., 2015; Rubino et al., 2009a; Schneider and Koch, 2003; Schneider et al., 2008), investigations
into the endogenous contributions of the ECS are lacking. Before the writing of this thesis, only two studies had reported on the developmental consequences of CB1 receptor blockade in adolescence (Lee et al., 2015; Rubino et al., 2015). Despite the paucity of information regarding endogenous cannabinoid signalling in adolescence, the available evidence supports a role for this system. In female rats exposed daily (PND 35-45) to the highly-selective CB1 receptor antagonist / inverse agonist AM251 (0.5 mg / kg), prefrontal cortical expression of the glutamatergic markers GluN2A, GluA1, and PSD95 were elevated relative to rats treated with vehicle over the same period (Rubino et al., 2015). The increased expression of glutamatergic synapses in AM251 females relative to vehicle-treated females is in accordance with the hypothesis that intercellular communication is critical for the maintenance of synapses (Chechik et al., 1999; Chen and Tonegawa, 1997), and suggests that blockade of CB1 receptor signalling in adolescence increases cell to cell communication within the PFC, thereby reducing the extent of synaptic pruning that is characteristic of the adolescent period (Blakemore, 2008; Crews et al., 2007; Rubino et al., 2015). The PFC is a critical brain region for the regulation of socioemotional behaviours and neuroendocrine stress responses. Consequently, the findings reported by Rubino and colleagues (2015) would suggest that PFC-dependent behaviours would be affected in AM251 females, however, behaviour was not assessed in that study. Thus, in chapter 2 and 3 of the present thesis, I investigated the extent to which adolescent AM251 exposure (1 mg / kg; PND 30-44) would affect anxiety-like and social behaviours, and neuroendocrine stress responses. I found that whereas the effects of adolescent AM251 were absent for anxiety-like behaviour and baseline and stress-induced CORT concentrations (an index of stress
responses), they were evident for social behaviours in female. Although social behaviours involve PFC signalling, I observed no drug effect on any of the neural protein measures in the PFC, suggesting that the effects on social behaviour involved alternate brain regions. Likewise, although male rats exposed to AM251 in adolescence were found to have an anxiolytic phenotype in adulthood compared to vehicle treated males (chapter 3), I observed no differences in PFC neural protein measures in males. Thus, the effects of adolescent AM251 on social behaviours in females and anxiety-like behaviours in males are likely mediated by regions other than the PFC. Consistent with its involvement in social regulation, I observed reduced dorsal hippocampal CB1 receptor expression in female rats 24 h after their final exposure to AM251 (chapter 2). Nevertheless, follow up studies (chapter 4) determined that the effect of AM251 on social behaviour in female rats was independent of dorsal hippocampal signalling. The effects of adolescent AM251 exposure on social behaviour were, however, associated with increased nucleus accumbens shell activity, and signalling in this region was found to be a unique predictor of social behaviour in AM251 females. Thus, whereas studies with CB1 receptor agonists identified the endogenous cannabinoid system as a potential regulator of adolescent development, they are not able to disambiguate the specific contributions, nor the extent to which these contributions differ between sex. Alternatively, my thesis research findings have identified a highly sex-specific role of CB1 receptor signalling in adolescence in normative brain and behaviour development. Whereas CB1 receptor signalling contributions to anxiety-like behaviour appear to be greater for male rats, contributions to social and fear-related behaviours appear to be greater for female rats.

Limitations and future directions
The extent to which developmental contributions of adolescent CB1 receptor signalling involve regulation of excitatory versus inhibitory neurotransmission is not clear. The available evidence suggests that effects of CB1 receptor inhibition on glutamatergic (Rubino et al., 2015) and GABAergic (Renard et al., 2017); chapter 2) signalling are evident soon after cessation of treatment and persist into adulthood. To investigate the extent to which the AM251-induced increases in social interactions in female rats involved altered GABAergic signalling, I co-labelled cells in the dorsal hippocampus and nucleus accumbens for EGR-1 and glutamic acid decarboxylase 67 (GAD67, a marker of GABAergic neurons). Although I was able to determine that neural activity (as measured by EGR-1 ir cell counts) in the nucleus accumbens shell predicts social interactions in AM251 females, I was unable to obtain reliable staining for GAD67, and thus could not draw conclusions regarding the cell-type responsible for the observed effects. Future studies into the specific cell-types involved in the observed behavioural changes will help to shed light on the developmental mechanisms underlying CB1 receptor regulation of adolescent behaviour.

The effects of adolescent AM251 on behaviour in the present thesis were consistently sex-specific (e.g., effects present in one sex but not the other). Although this is likely a reflection of sex-specific neurodevelopmental processes in adolescent mammals, we cannot rule out the potential of sex-differences in sensitivity to AM251. Replication of these findings with the addition of alternate drug doses, as well as with the inclusion of measures pertaining to the pharmacokinetics and pharmacodynamics of AM251 in each sex would provide insight into the extent to which the observed effects are because of dose / differential sensitivities to the drug, or because of sex-specific endogenous
regulation of development by CB1 receptors. Further, although considered a highly-selective CB1 receptor antagonist, AM251 also possesses inverse agonist properties via disruptions to constitutive receptor activity. Therefore effects of AM251 may extend beyond the blockade of endogenous ligands (e.g., AEA, 2-AG) and instead may involve inverse agonism of CB1 and the blockade of ligand-independent receptor activity. The recent availability of neutral CB1 receptor antagonists (i.e., antagonists devoid of inverse agonistic properties) provide a means of disambiguating the effects of endogenous cannabinoid antagonism from CB1 receptor inverse agonism (Salamone et al., 2007; Sink et al., 2010a; Sink et al., 2010b). Alternatively, studies employing adolescent administration of endocannabinoid hydrolysis and reuptake inhibitors (thereby increasing the synaptic concentrations of AEA and 2-AG only at synapses where they are being actively synthesized and released) would not only confirm the available evidence from CB1 receptor antagonists, but also would allow problems related to inverse agonism and receptor constitutive activity to be circumvented.

Finally, although my research has demonstrated a role of CB1 receptor signalling in adolescent development, the underlying cellular and molecular mechanisms are not clear. Future investigations into the interactions between the ECS and gonadal hormone systems in adolescence, as well as the influence of adolescent CB1 receptor signalling on intracellular signalling, are warranted.

**Conclusions**

The more modest effects reported in models of adolescent CB1 receptor antagonism versus agonism likely stem from the on-demand nature of endocannabinoid signalling. Administration of cannabinoid receptor agonists is presumed to elicit CB1
receptor activation throughout the whole organism, and thus observed effects of agonists may be attributable to activity in brain areas and / or specific cell populations that would otherwise be absent of CB1 receptor signalling. Conversely, antagonism of CB1 receptors presumably only blocks signalling at synapses where endocannabinoids are being actively released, providing insights into the potential mechanisms and circuits that are regulated by endogenous cannabinoid signalling in adolescence. Using a rat model of adolescent CB1 receptor antagonism, my thesis research demonstrates for the first time the involvement of endogenous CB1 receptor signalling in the development of social behaviours in female rats, and implicates CB1 receptor regulation of mesocortical reward pathways as a likely substrate for these effects. The findings from my research provide further support for adolescence as a sensitive period of development, and highlight tonic endogenous CB1 receptor signalling as a key mediator of normative adolescent development.
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Appendix A

Animal Care Committee (ACC)
Chair – Fiona Hunter, PhD 905.688.5550 ext. 3394
Clinical Veterinarians – Dr. Alistair Ker and Dr. Susan Warren
Animal Care Committee Coordinator – Dale Carlson, RMLAT 905.688.5550 ext 5820

Date: Feb 1, 2016

Dear Dr. McCormick and Mr. Simone,

Your “Animal Use Protocol (AUP)” entitled:

Investigating the effects of chronic CB1 receptor blockade during adolescence on hypothalamic-pituitary-adrenal axis function, and anxiety-like and social behaviours in male and female Long-Evans rats.

has been approved by the Animal Care Committee.

This approval expires in one year on the last day of the month.
The number for this project is AUP # 16 - 01 - 01.
This number must be indicated when ordering animals for this project.

ANIMALS APPROVED: 288 male and 288 female long evans rats

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures, as described in this protocol, are familiar with the contents of this document.

Fiona Hunter, Chair of ACC

THIS PROTOCOL IS IN EFFECT FOR A PERIOD OF ONE YEAR ONLY
AND IS SUBJECT TO POST APPROVAL MONITORING.

ALL UNEXPECTED MORTALITIES MUST BE REPORTED TO ANIMAL CARE SERVICES STAFF IMMEDIATELY.
Appendix B

AUP Renewal Application Teaching and Research Page 4 of 6

AUP No. 16 01 01

Renewal 1 of 3

Original Title of Project:
Investigating the effects of repeated CBR receptor blockade during adolescence on hypothalamic-pituitary-adrenal axis function and anxiety-like behaviours in male and female Long Evans rats

SECTION IV PRINCIPAL INVESTIGATOR, TECHNICIAN AND/OR STUDENT INVESTIGATOR DECLARATION

Signatures reflect commitment to abide by the following principles for the duration of the animal project proposed:

a. All animals entered into this project will be treated in a humane manner, in accordance with the principles and guidelines of the CCAC as are stated in the “Guide to the Care and Use of Experimental Animals”.

b. This renewal accurately describes all proposed animal use. It will be kept current and will be modified only after obtaining approval of the Animal Care Committee (ACC).

c. All procedures will be carried out by well-trained and experienced personnel who are competent in the use of recognized techniques.

d. All procedures, which may cause pain or discomfort have been technically improved and/or minimized in such a way so that the expected results will be obtained with a minimum of discomfort to the animals.

e. The protocol number assigned by the ACC to this submission will be used when ordering animals and these animals will be used only for the project described.

Cheryl McCormick 20/1/2017
Principal Investigator's Name and Signature

Alternate Investigator's Name and Signature

Jonathan Simone 20/1/2017
Lead Technician/Student Investigator's Name and Signature (if applicable)

Technician/Student's Name and Signature (if applicable)

Technician/Student's Name and Signature (if applicable)

As Departmental Chair, I agree to fund animal-related expenses incurred by this teaching protocol up to a maximum amount of $1

Departmental Chair's Name and Signature (Teaching Renewals Only)

ACC APPROVAL

Jean Hampson 24/1/2017

Acting ACC Chair's Name and Signature

Comments: