Role of the Endocannabinoid System in Extinction of Learned
Behaviours Motivated by Opioid-Induced Reward and Aversion in Rats

By

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ROLE OF THE ENDOCANNABINOID SYSTEM IN EXTINCTION OF LEARNED BEHAVIOURS MOTIVATED BY OPIOID-INDUCED REWARD AND AVERSION IN RATS

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ABSTRACT

Recent evidence suggesting that the endogenous cannabinoid (ECB) system can be selectively manipulated to facilitate or impair the extinction of learned behaviours — specifically regarding drug-induced aversive memories — has important consequences for research on opiate withdrawal and abstinence. Data presented here support and expand previous findings that the ECB system has an important function in the extinction of aversively motivated behaviors and is mediated by i) an increase in available endogenous CB₁ receptor agonists, primarily anandamide, and ii) the exogenous CB₁ receptor agonist Δ⁹-THC, in a manner that is dependent upon both the dose and route of administration. Experiments demonstrated that the fatty acid amide hydrolase (FAAH) inhibitor, URB597, which blocks deactivation of endogenous CB₁ ligands, such as anandamide, significantly facilitated extinction of naloxone-precipitated morphine withdrawal-induced conditioned cue aversion, whereas the CB₁ receptor antagonist/inverse agonist SR141716 significantly impaired extinction. Several experiments demonstrated that neither the CB₁ antagonist AM251 nor the FAAH
inhibitor URB597 had any effect on extinction learning for morphine-induced conditioned cue preference. A method was developed for analysing cannabinoid levels in blood by liquid chromatography/mass spectrometry (LC/MS) to compare bioavailable levels of Δ⁹-THC and its primary psychoactive metabolite. Experiments were designed to meet three primary objectives: 1) to provide further support for the role of the ECB system in the extinction of aversively-motivated behaviours, 2) to compare bioavailable levels of Δ⁹-THC and its primary psychoactive metabolite, 11-OH-Δ⁹-THC, after pulmonary and parenteral administration, and 3) to demonstrate that the route of administration of Δ⁹-THC can have a significant impact on whether or not it facilitates or impairs extinction learning. Results showed that inhaled Δ⁹-THC dose- and time-dependently facilitated rates of extinction learning of the conditioned aversion whereas injected Δ⁹-THC significantly impaired extinction. These data suggest that the route of administration of Δ⁹-THC has important consequences for its resulting pharmacokinetic and behavioural effects, specifically, that pulmonary exposure facilitates, whereas parenteral exposure impairs, rates of extinction learning for conditioned cue aversion. Thus, pulmonary administration of Δ⁹-THC may prove more beneficial for pharmacological potentiation of extinction learning for aversive memories, such as those supporting drug-craving/seeking in opiate withdrawal-syndrome.
DECLARATION OF WORK PERFORMED

Chapter 2 is based on a report by Manwell et al. (2009) published in *Pharmacology, Biochemistry and Behavior* in collaboration with Elham Satvat, Stefan Lang, Craig Allen, and supervisors Dr. Francesco Leri and Dr. Linda Parker. Experiment 2.1 was conducted by Craig Allen, Experiment 2.2a was conducted by Elham Satvat, Experiment 2.2d was conducted by Stefan Lang, and Experiments 2.2b, 2.2c and Experiment 2.3 were all conducted by Laurie Manwell; data analyses and writing of the manuscript was done by Laurie Manwell and Linda Parker.

Chapter 3 is based on a manuscript prepared by Laurie Manwell in collaboration with Drs. Armen Charachoglyan, Dyanne Brewer and Paul Mallet. All experiments were conducted by Laurie Manwell with training and supervision provided by Drs. Charachoglyan and Brewer at the Mass Spectrometry Facility and under the supervision of Dr. Mallet at Wilfrid Laurier University; all data analysis was performed by Laurie Manwell and the manuscript was written by Laurie Manwell. The manuscript was reviewed by Dr. Masoud Jelokhani-Niakari in the Department of Chemistry also at Wilfrid Laurier University.

Chapter 4 is based on a manuscript prepared by Laurie Manwell in collaboration with Dr. Paul Mallet. All experiments were conducted by Laurie Manwell.
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The world is truly a magnificent place – for those with infinite curiosity and imagination, it is a vast and fascinating playground for discovery that is free to all, unrivaled in resources, and unrestricted by bureaucracy. It holds endless secrets waiting to be discovered, some elegant and illuminating, others seemingly disorganized and perplexing, some that are deceptive or efficiently brutal, and even those that are ultimately self-annihilating. I have learned something valuable from all of them, that I hope to share with you, and I wish to thank those who helped me discover them.

Thank you to Drs. Paul Mallet and Elena Choleris for being excellent supervisors, I will be ever grateful for your guidance, support and wisdom. Paul and Elena, thank you for giving me the resources and freedom to run my experiments with great independence, but still being there 24/7 for emergencies and reassurance. You are the role models I hope to be one day for my students. Many wonderful thanks to Drs. Linda Parker, Cheryl Limebeer, Bettina Kalisch, Francesco Leri, Boyer Winters, Armen Charachoglyan, Dyanne Brewer, Brandi Ormerod, Malcolm Maden, Lance de-Haven-Smith, Matthew Witt, Steve Sider, and Olga Sutherland for the fantastic opportunities and research experience I gained while working with you along the way. Thank you to all of my wonderful labmates over the years, especially Lorman Ip for being there the whole way, as a colleague and dear friend – thank you for all of the deep conversations about life while dissecting sheep brains, going to conferences, sparring at the club, and drinking homemade wine, not all at once, of course! Thank you to my family and friends for your support: Gramma, Mom, Dad, and Cara, Derek, Jallaina and Sarah, and Donna, Denise and Bob, I love you all and couldn’t have done it without you! Thank you for everything.
For Patricia and Dennis Murphy

Magnum Opus

Imagine for a Moment…
That you are without Sight -
How would I teach you what blue is?
Or what the sign of the cross means?
Or what can lurk in a shadow?
Why the blood from white men and brown men
run red together from the streets of New York to Baghdad.
But you See, so you cannot Imagine.

Imagine for a Moment…
That you are without Hearing -
How would I teach you what D minor is?
Or what the echo of an empty well means?
Or what is lost in translation?
Why the people’s protests here are silent
while mothers wail over their children’s bodies worlds away.
But you Hear, so you cannot Imagine.

Imagine for a Moment…
That you are without Taste -
How would I teach you what chocolate is?
Or what it means to eat bitter?
Or what is exchanged when tongues twine or clash?
Why victory smells sweet while vomit spews from cowards.
But you Taste, so you cannot Imagine.

Imagine for a Moment…
That you are without Feeling -
How would I teach you what cashmere is?
Or what havoc instinct wreaks on the gut?
Or what truths empathy can betray to a leper?
Why men pillage and plunder one another to feel alive.
But you Feel so you cannot Imagine.

Imagine for a Moment…
That you are without Consciousness -
How would I teach you what a mirror is?
Or what it means to fear life but not death?
Or what gods wait to be found in a prison?
Why Plato’s puppeteers will never unchain their slaves.
But you Think so you cannot Imagine.

Now let me imagine for a Moment…
That I am You -
How would I learn what Imagination is?
Or what ignorances seize the soul?
Or what the universe tells us when it bends the rules of space and time?
Why I cannot Teach you what you Know.
But I can Imagine so I cannot be You -

And the Moment is always in Passing…In this Magnificent Place.
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LIST OF ABBREVIATIONS

\( \Delta^9 \)-THC: \( \Delta^9 \)-tetrahydrocannabinol

11-OH-\( \Delta^9 \)-THC: 11-hydroxy-\( \Delta^9 \)-tetrahydrocannabinol

2-AG: \( sn \)-2-arachidonoyleglycerol

2-HPBCD: 2-hydroxypropyl-\( \beta \)-cyclodextrin

5-HT: serotonin, serotonergic

ACE: adverse childhood experience

AEA: N-arachidonylethanolamide, anadamide

APCI: atmospheric pressure chemical ionization

BBB: blood-brain barrier

\( \beta \)-END: \( \beta \)-endorphin

BLAC: basolateral amygdala complex

CB\(_1\): cannabinoid receptor subtype 1

CBD: cannabidiol

CBR: cannabinoid receptor

CPA: conditioned place aversion (also conditioned cue (floor) aversion, CCA)

CPP: conditioned place preference (also conditioned cue (floor) preference, CCP)

CeA: central amygdala

CNS: central nervous system

COOH-\( \Delta^9 \)-THC: 11-nor-9-carboxy-\( \Delta^9 \)-tetrahydrocannabinol

CR: conditioned response

CRF: corticotropin releasing factor

CS: conditioned stimulus
CV: coefficient of variation
DA: dopamine
D2: DA receptor subtype 2
DAG: diacylglycerol
DAGL: diacylglycerol lipase
DI: deuterated internal standards
DSM-IV-TR: Diagnostic and Statistical Manual of Mental Disorders, 4th Ed. Revised
ECB: endogenous cannabinoid
EDTA: ethylenediaminetetraacetic acid
EOS: endogenous opioid system
ESI: electrospray ionization
FAAH: fatty acid amide hydrolase
GABA: gamma-aminobutyric acid
GC/MS: gas chromatography/mass spectrometry
GLU: glutamine, glutamaterigic
GPCR: G-protein coupled receptor
HIV: human immunodeficiency virus
HPA: hypothalamic pituitary axis
HPLC: high performance liquid chromatography
ICON: Interagency Committee on Neurotoxicity
IPI: injection-to-placement interval
IR: immunoreactivity
IUD: injection drug user
LC: liquid chromatography
LC/MS: liquid chromatography/mass spectrometry
LLE: liquid-liquid extraction
LOD: limit of detection
LOQ: limit of quantification
LTD: long-term depression
LTP: long-term potentiation
MDS: mesocorticolimbic dopamine system
MS/MS: tandem (triple-quadrupole) mass spectrometry
Nacc: nucleus accumbens
NAPE: N-arachidonoyl phosphatidylethanolamine
NE: norepinephrine
NMDA: N-methyl-D-aspartate
PET: positron emission tomography
PFC: prefrontal cortex
PI: phosphatidylinositol
PLC: phospholipase C
PNS: peripheral nervous system
PTSD: post-traumatic stress disorder
RBC: red blood cell
Si: silica
SPE: solid phase extraction
SUD: substance use disorder
TLC: thin layer chromatography
UNODC: United Nations Office on Drugs and Crime
UR: unconditioned response
US: unconditioned stimulus
VTA: ventral tegmental area
WHO: World Health Organization
“In short, the life-foundational opioid/love/pleasure/pain relief apparatus provides the entry point for narcotic substances into our brains. The less effective our own internal chemical happiness system is, the more driven we are to seek joy or relief through drug-taking or through other compulsions we perceive as rewarding.

The very essence of the opiate high was expressed by a twenty-seven-year-old sex trade worker. She had HIV and has since died. “The first time I did heroin,” she said to me, “it felt like a warm soft hug.” In that phrase she told her life story and summed up the psychological and chemical cravings of all substance-dependent addicts.”

- Dr. Gabor Maté (2008, p. 157)

_In The Realm of Hungry Ghosts: Close Encounters With Addiction_
CHAPTER 1
General Introduction

1.1. Estimates of Drug Use Worldwide: Opioids and Cannabinoids

According to the 2011 World Drug Report (UNODC, 2011), in 2009, an estimated 149 to 272 million persons (3.3% to 6.1% of the population) between the ages of 15 to 64 worldwide used illicit drugs at least once; of those, at least 15 to 39 million are estimated to be heavy or problematic drug users. Although the prevalence varies greatly from region to region, injecting drug users (IDUs) are estimated worldwide to number between 11 and 21 million; of those, between 0.6 and 6.6 million IUDs are estimated to be infected with the human immunodeficiency virus (HIV) (UNODC, 2009). Regarding treatment, opiates continue to present as the world’s greatest drug problem: “More users die each year from problems related to heroin use, and more are forced to seek treatments for addiction, than for any other illicit drug […] opiates are also the most costly in terms of treatment, medical care, and, arguably, drug-related violence” (UNODC, 2010, p. 37).

Record opium poppy (*Papaver somniferum*) production peaked between 2007 and 2008 in the major cultivating countries, Afghanistan, Myanmar, and Lao People’s Democratic Republic (UNODC, 2009). In 2008, in Afghanistan alone, estimates of opium production ranged from 6 330 to 9 308 metric tons (mt); approximately 60% of which was converted into morphine and heroin within the country for export, and the remainder exported as opium (UNODC, 2009). While less precise, 2008 estimates of worldwide marijuana production (indoor and outdoor) — derived from the hemp plant (*Cannabis sativa*) — range from 13 000 to 66 000 mt for the plant and 2 200 to 9 900 mt for its resin (UNODC, 2009). Although 15 to 21 million people were estimated to have...
used opiates at least once in the past year in 2007, in the same period, an estimated 143 to 190 million people used cannabis — a population approximately 9 times greater (UNODC, 2009). Surprisingly, in North America, there has been nearly a decade of decline in cannabis use, stabilizing in 2007/2008 to a level still above 10% of the populations in both the US and Canada (UNODC, 2009). (For an extended discussion on the social aetiology of drug addiction and treatment implications please refer to Appendix A: Towards a Global Humanitarian Perspective on Drug Addiction.)

1.2. Neurobiology of Drug Use, Abuse and Dependence

The Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR; American Psychiatric Association, 2000) classifies a multitude of maladaptive behaviors arising from drug use as Substance-Related Disorders, distinguishing between different types of use: substance use, substance abuse, and substance dependence. Substance (or chemical or drug) abuse and dependence are chronic relapsing disorders in which drug use can lead to abuse and/or chemical dependence, but does not necessarily progress through all three stages. Substance abuse is classified by the DMS-IV-TR as a behavioural disorder — in which the behaviour is intentional and voluntary — and that demonstrates a maladaptive pattern of use leading to impairment or distress, as diagnosed by the presentation of one or more of the following criteria (within a 12-month period): a) recurrent use resulting in failure to meet major obligations, b) recurrent use that is physically hazardous, c) recurrent drug-related legal problems, and/or d) continued use in spite of social or interpersonal problems, and these symptoms have never met the criteria for chemical dependence (DSM-IV-TR, 2000; as summarized by Erickson, 2007). Substance dependence is classified as a pathological condition — in which the behaviour is unintended and control is impaired — and that presents with three or more
of the following (within a 12-month period): a) tolerance to the drug’s effects, b) withdrawal in the absence of the drug, c) drug use is more than intended, d) inability to control drug use, e) effort is expended to obtain the drug, f) important activities are replaced by drug use, and/or g) use of the drug continues in spite of the presence of persistent physical or psychological problems (DSM-IV-TR, 2000; as summarized by Erickson, 2007). Two types of dependence are designated — physiological (including either withdrawal or tolerance) or psychological (not including withdrawal or tolerance) — and they can be dissociated. Physical dependence occurs when the body requires the presence of the chemical for continued functioning, whereas psychological dependence occurs when the person believes that they require the drug for continued functioning (Erickson, 2007).

There are many theories on the transitions from drug exposure and use to drug abuse and drug dependence; since the essential parts of neural reward circuits are subcortical — being located below the area of the neocortex and thus consciousness — drug dependence develops without conscious recognition. The effects of drugs on neural projections to the frontal lobes are what eventually lead to the impaired control over drug use, through a reduction of cortical decision-making functions (Erickson, 2003, 2007). Explanations that involve neurobiological mechanisms have largely focused on changes that lead to dysregulation of neurotransmitters in the mesocorticolimbic dopamine (DA) system (MDS) (Erickson, 2007). In general, typical drugs of abuse, including opioids, cannabinoids, alcohol, nicotine, and psychostimulants, that are self-administered by both animals and humans in experimental studies result in elevated dopamine (DA) activity — particularly in the nucleus accumbens (Nacc) shell — which is believed to underlie their reinforcing effects (Di Chiara et al., 2004; Self, 2004; Trigo et al., 2010). For example, in the rat brain, connections within the MDS, originating in the ventral
tegmental area (VTA), include the following, as shown in Figure 1 (adapted from Camí and Farré, 2003, Figure 4, p. 982): a) neurons in the VTA have projections that synapse in the Nacc, amygdala, and prefrontal cortex (PFC), b) neurons in the PFC have glutamatergic (GLU) projections to the Nacc and VTA, and c) projections from the gamma-aminobutyric acid (GABA) neurons of the Nacc have projections that synapse in the Nacc and PFC. The opioid interneurons in this neural reward circuit thus modulate GABA-inhibitory actions on the VTA and affect the activation of norepinephrine (NE) neurons in the locus ceruleus. In addition, there are neurons in the raphe nucleus that have serotonergic (5-HT) projections which synapse in the VTA and Nacc.
Figure 1.1. Neural reward circuits important in the reinforcing effects of drugs of abuse. As shown in the rat brain, mesocorticolimbic dopamine (DA) systems (MDS) originating in the ventral tegmental area include projections from cell bodies of the ventral tegmental area to the nucleus accumbens, amygdala, and prefrontal cortex; glutamatergic (GLU) projections from the prefrontal cortex to the nucleus accumbens and the ventral tegmental area; and projections from the γ-aminobutyric acid (GABA) neurons of the nucleus accumbens to the prefrontal cortex. Opioid interneurons modulate the GABA-inhibitory action on the ventral tegmental area and influence the firing of norepinephrine (NE) neurons in the locus ceruleus. Serotonergic (5-HT) projections from the raphe nucleus extend to the ventral tegmental area and the nucleus accumbens. The figure shows the proposed sites of action of the various drugs of abuse in these circuits. *Reproduced with permission from Camí and Farré (2003, Figure 4, p. 982.), Copyright Massachusetts Medical Society.
1.3. Neurotoxicology of Cannabinoids and Opioids

Neurotoxicity, as defined by the Interagency Committee on Neurotoxicology (ICON), refers to “any adverse effect on the structure or function of the central and/or peripheral nervous system by a biological, chemical, or physical agent and may result from direct or indirect actions or reflect permanent or reversible changes in the nervous system” (c.f. Scallet, 1991). Neurotoxicological effects of cannabinoids and opioids are well known, particularly in terms of habituation, dependence, and withdrawal phenomena (reviewed in Koob and Le Moal, 2006). Of critical importance is that there is some degree of neuroadaptation to the presence of the drug and a corresponding neuroreadaptation in the absence of the drug, and thus the effects on behaviour reveal some degree of dysregulation of normal functioning (Scallet, 1991; Erickson, 2007).

In general, both marijuana and opium are classified as analgesics and euphoriant, and marijuana also as a hallucinogen and anxiolytic (Kobayashi et al., 1999). Cannabinoids and opioids have similar pharmacological effects, such as antinociception, sedation, catalepsy, and hypothermia (Bloom and Dewey, 1978; Bhargava and Matwyshyn, 1980; Narimatsu et al., 1987; Fuentes et al., 1999; Pontieri et al., 2001a/b); after chronic exposure, both agents result in tolerance to their analgesic and hypothermic effects, leading to the development of physical dependence (Pertwee, 1988; Bhargava, 1991; Tsou et al., 1995; Aceto et al., 1996; Rubino et al., 1997). Cannabinoids and opioids are often used together in the treatment of pain because of their synergistic effects on the regulation of noxious stimuli (Welch, 1993; Welch and Eads, 1999; Cichewicz, 2004).
More than 400 different constituents of cannabis have been identified, at least 60 of which are phytocannabinoids (Turner et al., 1980a/b). Phytocannabinoids are compounds with a similar C_{21} aromatic hydrocarbon structure derived from *Cannabis sativa* (Koob and Le Moal, 2006); a small proportion are classified as psychoactive, meaning that these compounds cross the blood-brain barrier (BBB), acting directly at the level of the central nervous system (CNS), and alter brain function in ways that affect consciousness, mood, perception and behavior (Segal and Duffy, 1999). Of these, the primary psychoactive cannabinoid is Δ⁹-tetrahydrocannabinol (Δ⁹-THC), an aromatic terpenoid that exerts its effects in the central nervous system via binding to cannabinoid receptors (CBRs) in the brain (Mechoulam, 1970; Dewey, 1986; Devane et al., 1988). Pulmonary administration (inhalation) of cannabis produces the greatest bioavailable levels of Δ⁹-THC, with dose-dependent blood levels peaking within minutes and subjective effects occurring almost immediately; in comparison, subjective effects are delayed from 30 to 120 min after both parenteral (injected) and enteral (oral) administration, which result in significantly lower peak blood levels due to considerable first-pass liver metabolism, where it is degraded by enzymes of the cytochrome P450 system (Matsunaga et al., 1995; reviewed in Grotenhermen, 2003, and Koob and Le Moal, 2006). During metabolism, hydroxylation of Δ⁹-THC produces 11-OH-Δ⁹-THC, which is also psychoactive; although the elimination half-life for Δ⁹-THC is estimated within the range of 20-60 h, the elimination half-life for 11-OH-Δ⁹-THC is much longer, lasting up to 5-6 days (reviewed in Grotenhermen, 2003, and Koob and Le Moal, 2006).

Tolerance to the effects of Δ⁹-THC has been experimentally demonstrated in both animals and in humans; pharmacodynamic tolerance is largely attributed to brain neuroadaptation, as opposed to pharmacokinetic changes, such as changes in disposition, absorption, or metabolism of the parent compound (Hunt and Jones, 1980; Maykut, 1985;
Tolerance that develops rapidly after a few doses diminishes quickly, whereas tolerance to large doses persists, even after drug use has stopped. Cessation after chronic marijuana use can produce a withdrawal syndrome in humans — with a typical onset of 1-3 days post-cessation — including symptoms of irritability, restlessness, mild agitation, sleep electroencephalography (EEG) disturbance, insomnia, nausea and cramping (O’Brien, 1996). Psychological and physical dependence have been reported in both animals and humans after repeated administration of marijuana (Kobayashi et al., 1999; Koob and Le Moal, 2006).

More than 20 alkaloids are present in opium; the most potent is morphine, composing approximately 10% of opium, then thebaine (paramorphine) and codeine (Reisine and Pasternak, 1996; Koob and Le Moal, 2006). Heroin (3-6-diacetylmorphine) is a semi-synthetic derivative of morphine; after intravenous injection or inhalation, blood levels peak within 1-5 min and after crossing the blood-brain barrier it is rapidly metabolized to 6-monoacetylmorphine and then more slowly to morphine (Koob and Le Moal, 2006). Another almost equipotent synthetic derivative of morphine is oxycodone administered orally; however, when administered via the parenteral route, oxycodone is 20-30 times more potent than morphine (Koob and Le Moal, 2006). In addition to analgesia, morphine induces a pleasurable state of euphoria and indifference to environmental stimuli; it is also reported to blunt ‘emotional pain’ (Koob and Le Moal, 2006). The development of tolerance with repeated exposure is a characteristic feature of opiates; after cessation, it is followed by a characteristic withdrawal syndrome, including vomiting, diarrhea, tremors, insomnia, increased temperature and blood pressure, and dysphoria (Koob and Le Moal, 2006). Opiate dependence is both psychological and physical — which are dissociable — increasing the risk of overdose and death (Siegel et al., 1982; Koob and Le Moal, 2006).
1.4. Endogenous Systems Involving Opioids and Cannabinoids

There is an important relationship between drugs of abuse derived from natural sources and their corresponding endogenous systems, for example, opioid receptors (ORs) and cannabinoid receptors (CBRs) and their respective endogenous peptide ligands, which are distributed widely throughout the central (CNS) and peripheral (PNS) nervous systems (Trigo et al., 2010; Howlett et al., 2004). Membrane receptors for opiates were first described in 1973 (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973) followed in the 1990s by the molecular characterization of the three primary types, μ, δ, and κ (Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993; Meng et al., 1993; Thompson et al., 1993; Yasuda et al., 1993). Opioid receptors are 7-transmembrane-domain receptors coupled to G_{i/o} proteins (GPCRs); when activated by their corresponding ligands, they initiate signaling cascades leading to a) the inhibition of both 3'-5'-cyclic adenosine monophosphate (cAMP) production and voltage-gated Ca^{2+} channels, b) increases in intracellular Ca^{2+}, and c) stimulation of K+ channels and activation of the mitogen-activated protein (MAP) kinase pathway (reviewed in Law et al., 2000). Endogenous opioid receptor ligands include the following: a) β-endorphin, which has a greater binding affinity for μ- than κ- or δ-opioid receptors, b) met- and leu-enkaphalins, which have greater affinity for δ- than μ-opioid receptors, c) dynorphins, which are putative κ-opioid receptor ligands, and d) endomorphin-1 and -2, which are putative μ-selective opioid receptor ligands (Trigo et al., 2010). Opioid receptors and their endogenous ligands are distributed in brain regions known to be involved in motivation and reward processes (reviewed in Mansour et al., 1995, and Trigo et al., 2010).

Δ^9-THC was first isolated from marijuana in 1964 (Gaoni and Mechoulam, 1964), but it was not until the 1990’s that the endogenous receptors for Δ^9-THC in the brain and
spinal cord were identified. Originally, two main types of CBRs, both of which are GPCRs, were identified — CB-subtype1 \([\text{CB}_1]\) and CB-subtype2 \([\text{CB}_2]\) (Howlett et al., 1990; Matsuda et al., 1990; Munro et al., 1993; Gérard et al., 1991; Glass et al., 1997) — with further research on the CBRs ultimately leading to the discovery of their two main endogenous agonists, the lipid neuromodulators N-arachidonylethanolamide (anandamide or AEA) (Devane et al., 1992) and \(\text{sn-2}\)-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995). AEA is synthesized from membrane lipids in consecutive steps, by two main enzymes: phosphatidylethanolamine is converted by N-acyl transferase to N-arachidonoyl phosphatidylethanolamine (NAPE), which is then converted by phospholipase D to anandamide (Freund et al., 2003; refer to Figure 1.2). Similarly, 2-AG is synthesized in a two-step manner: phosphatidylinositol (PI) is converted by phospholipase C (PLC) to diacylglycerol (DAG), which is then converted by diacylglycerol lipase (DAGL) to 2-AG (Freund et al., 2003; refer to Figure 1.2).
Figure 1.2. Main pathways of biosynthesis and degradation of two endocannabinoids, AEA and 2-arachidonoylglycerol.

*Reproduced with permission from Masanobu Kano from Hashimotodani, Ohno-Shosaku, and Kano (2007, Figure 1, p. 128), Endocannabinoids and synaptic function in the CNS, The Neuroscientist, 13: 127-137, Copyright by Sage.
AEA and 2-AG exert their effects primarily through a CB₁-mediated pathway in the CNS and through a CB₂-mediated pathway in the PNS. Endogenous cannabinoids (ECBs) act as retrograde synaptic messengers, traveling backward across the synapse to bind to presynaptic CBRs, inhibiting the release of inhibitory (e.g., GABA) or excitatory (e.g., glutamate) neurotransmitters (reviewed in Hashimotodani et al., 2007). Within the cell, AEA and 2-AG are deactivated by fatty acid amide hydrolase (FAAH) (Ueda et al., 2000; Vandervoode and Lambert, 2005). URB597 and OL-135, both FAAH-inhibitors, and VDM11, an AEA uptake inhibitor and putative FAAH inhibitor, have been shown to increase endogenous AEA and 2-AG (Kathuria at al., 2003; Fegley et al., 2005; Vandevooorde and Fowler, 2005; Bortolato et al., 2007). Whereas CB₂ is expressed in the PNS in the immune system, CB₁ is found predominantly within the CNS and is distributed throughout the brain and spinal cord (e.g., neocortex, hippocampus, basal ganglia, and cerebellum) (see Figures 1.3-1.4 adapted from Moldrich and Wenger, 2000, Figures 1-2, p. 1737-8), specifically in regions associated with motor coordination, learning and memory, emotion regulation, and the dopamine reward system (Howlett et al., 1990, 2004; Hashimotodani et al., 2007).

*Reprinted from Moldrich and Wenger (2000, Figure 1, p. 1737), Localization of the CB₁ cannabinoid receptor in the rat brain, Peptides, 21, 1735-1742, with permission from Elsevier.*
Figure 1.4. Schematic drawing of different forebrain areas to show the presence of cannabinoid receptor immunoreactivity (CB\textsubscript{1}-IR). The numbers indicate the distance from the interaural line (according to Paxinos-Watson atlas). Only structures expressing CB\textsubscript{1}-IR are labeled. (See Figure 1.3 for abbreviations.)

*Reprinted from Moldrich and Wenger (2000, Figure 2, p. 1738), Localization of the CB1 cannabinoid receptor in the rat brain, Peptides, 21, 1735-1742, with permission from Elsevier.
1.5. Role of Opioid and Cannabinoid Systems in Learning, Memory and Behaviour

Related to Drug Use, Abuse and Dependence

Research on the behavioural and molecular correlates of learning and memory demonstrate significant roles for both the endogenous opioid system (EOS) and ECB system. The EOS is involved in modulating the neurobiological and behavioural effects of many drugs of abuse. Opioid receptors and their endogenous ligands are abundantly expressed in regions of the brain implicated in reward learning and memory and related motivational processes; specifically, they are present in the Nacc, PFC, amygdala, hypothalamus, and VTA, where they modulate neural circuits in the MDS reward pathway (Delfs et al., 1994; Mansour et al., 1993, 1994, 1995; see Figure 1.5 adapted from Trigo et al., 2010, Figure 1, p. 185). DA release is facilitated by opioids through a) activation of μ- and δ-opioid receptors in the Nacc, and b) reduced GABA-inhibition by activation of μ-opioid receptors in the VTA; μ-opioid receptors are located primarily on GABA interneurons within this circuit (reviewed in Trigo et al., 2010).

Regarding interaction with EOS receptors, μ-opioid receptor agonists may possess the greatest potential for abuse and dependence, in both humans and animals; for example, μ-opioid receptor agonists, such as morphine, are self-administered by humans and experimental animals and μ-opioid receptor antagonists, such as naloxone, result in dysphoria in humans and conditioned place aversions in experimental animals (reviewed in Tschentke, 2007, and Trigo et al., 2010).

Within the limbic system there exist numerous regions of anatomical overlap between μ-opioid and CB1 receptors, both in coupling of function and interactions that are bidirectional (Trigo et al., 2010). For example, there is some evidence of allosteric
modulation between the EOS and ECB systems: a) *in vivo* behavioural studies in animals have shown that naloxone blocks the effects of Δ⁹-THC and the CB₁ antagonist/inverse agonist SR141716 blocks the effects of morphine (Solinas and Goldberg, 2005), b) *in vitro* molecular studies show that Δ⁹-THC and cannabidiol (CBD) are allosteric modulators at both μ- and δ-opioid receptors but that SR141716 is not (Kathmann et al., 2006), and c) combined studies indicate that, in the Nacc, μ and CB₁ receptors putatively form heterodimers which control GABA and glutamate release (Schoffelmeer et al., 2006).
Figure 1.5. Schematic representation of the possible mechanisms involved in the modulation of the reward circuit by the endogenous opioid system (EOS). Enkephalinergic interneurons are present in the ventral tegmental area (VTA) and nucleus accumbens (Nacc). Endogenous enkephalins released in the VTA might act on μ-opioid receptors located on presynaptic GABAergic interneurons, inhibiting this GABAergic neurotransmission which facilitates dopamine (DA) release in the Nacc. Enkephalins may also facilitate DA release by activating μ- and δ-opioid receptors in the Nacc. β-Endorphin released in the Nacc and VTA from the projecting neurons of the arcuate nucleus (arcN) of the hypothalamus might facilitate DA release through activation of μ-opioid receptors in these two mesolimbic structures. K-Opioid receptors and their endogenous dynorphin ligands are highly expressed in the Nacc and also present in the VTA. The activation of the dynorphin/κ-opioid receptor system might serve as a feedback mechanism to counteract the high levels of DA released by drugs of abuse, opposing the effects of drugs on the reward circuitry. PFC: prefrontal cortex; VTA: ventral tegmental area; Nacc: nucleus accumbens; arcN: arcuate nucleus; DA: dopamine; Glu: glutamate; β-END: β-endorphin; ENK: enkaphalin; DYN: dynorphin. *Reprinted from Trigo, Martín-Garcia, Berrendero, and Maldonado (2010, Figure 1, p. 185), The endogenous opioid system: A common substrate in drug addiction, Drug and Alcohol Dependence, 108: 183-194, with permission from Elsevier.
To date, many studies have documented the important role of the ECB system in brain regions associated with learning, memory, and behaviour, and specifically related it to drug-induced reward processes. Evidence from animal studies indicates that cannabinoids have long-lasting behavioural and neurochemical effects, particularly in the MDS (Pistis et al., 2004). In the Nacc, a region involved in reward behaviour (Rolls and Williams, 1987; Fibiger and Philips, 1988; Hernandez and Hobel, 1988; Young et al., 1992; Kolb et al., 2006), ECB mediated long-term depression (ECB-LTD) at excitatory synapses (ECB-LTD(e)) can be blocked by both CB₁ antagonists (Robbe et al., 2002) and Δ⁹-THC (possibly by a functional tolerance of CB₁) (Hoffman et al., 2003; Mato et al., 2004). It has been suggested that ECB-LTD(e) in the Nacc may be an underlying mechanism of behaviours regulated by motivation and reward, such as relapse to drug addiction (Hashimotodani et al., 2007). Indeed, subchronic cannabinoid administration can produce behavioural sensitization — such as greater locomotor responsiveness, both non-stereotyped (i.e., exploratory locomotion and sniffing) and stereotyped (i.e., repetitive gnawing and licking and confined sniffing, all similar to behaviours associated with morphine sensitization) — (Cadoni et al., 2001; Rubino et al., 2001, 2003) and cross-sensitization to other drugs of abuse, in particular opiates in a context-independent manner (e.g., induced in the homecage and observed in test environment) (Cadoni et al., 2001; Pontieri et al., 2001a, 2001b). Furthermore, during development, the effects of Δ⁹-THC exposure appear to have more long-term residual effects than for exposure later in life, such as impairments in sensorimotor gaiting behaviour (i.e., prepulse inhibition of an acoustic startle response), object recognition memory, and operant responding (Schneider and Koch, 2003); exposure to CB₁ agonists has been reported to impair DA neuron responsiveness in the mesoaccumbens region, with exposure during adolescence, and not adulthood, resulting in long-lasting cross-tolerance to morphine, cocaine and
amphetamine (Pistis et al., 2004). This is supported by both *in vivo* and *in vitro* studies demonstrating that excitatory synapses in the Nacc are very sensitive to inhibition induced by cannabinoids (Hoffman and Lupica, 2001; Pistis et al., 2002; Robbe et al., 2001).

One proposed mechanism underlying multidrug cross-sensitization and tolerance is related to the very similar inhibitory G-protein systems and effectors common to CB<sub>1</sub>, μ-opioid, and DA receptor subtype 2 (D2); it is possible that shared intracellular signaling cascades activated by these GPCRs might be dysregulated by subchronic stimulation of CB<sub>1</sub> receptors (Yao et al., 2003; Pistis et al., 2004). Both endogenous and exogenous cannabinoids can modulate excitatory inputs to DA neurons in the VTA (Melis et al., 2004) leading to functional alterations in DA neuronal activity; in addition, ECBs which are released in response to D2 stimulation can reduce glutamatergic inputs to DA neurons (Melis et al., 2004a/b). Thus, it is conceivable that DA neuronal activity, affected by chronic cannabinoid exposure, may in turn disrupt ECB signaling leading to dysregulation of short- and long-term synaptic inputs (Pistis et al., 2004).

In addition to the reinforcing effects of acute and prolonged exposure, Δ<sup>9</sup>-THC produces a withdrawal syndrome in humans and animals, with many different areas of evidence suggesting that the EOS is involved in this process. In rodents, both spontaneous and precipitated withdrawal from Δ<sup>9</sup>-THC produces characteristic signs that are also similar to opioid withdrawal signs, such as body and paw tremors, wet dog shakes, head shakes, teeth chattering, salivation, diarrhea, drooping eyelids (ptosis), compulsive grooming behaviours, and raised skin hairs (piloerection) (Rodriguez de Fonseca et al., 1997; reviewed in Koob and LeMoal, 2006). There is also evidence of a reciprocal relationship between the EOS and ECB systems in both withdrawal syndromes; for example, in rodents chronically treated with morphine, opioid withdrawal
signs can be precipitated by the CB₁ antagonist, SR141716A, and reciprocally, in rodents chronically treated with cannabinoids, withdrawal signs can be precipitated by the opioid antagonist naloxone (Kaymakcalan et al., 1977; Navarro et al., 1998).

In addition to these somatic signs of withdrawal, various motivational effects of spontaneous and precipitated cannabinoid withdrawal in chronically treated rodents have been reported, such as suppressed operant responding for food rewards (Beardsley and Martin, 2000) and increased anxiogenic behaviours in the defensive withdrawal test (e.g., greater time spent in hiding inside a chamber) (Rodriguez de Fonseca et al., 1997), the latter of which were also notably associated with increased levels of corticotropin releasing factor (CRF) and Fos in the central amygdala (CeA) and other stress-responsive brain regions receiving CeA projections (Rodriguez de Fonseca et al., 1997). Koob and LeMoal (2006) propose that the motivational effects of cannabinoid withdrawal arise from concurrent changes in the MDS and the hypothalamic pituitary axis (HPA) stress response system; specifically, there is a decrease in MDS activity accompanied by an increase in pituitary adrenal corticotropin-releasing factor (CRF) in the extended amygdala and associated brain areas during withdrawal. This is supported by evidence that precipitated withdrawal from cannabinoids results in decreased activity in dopamine neurons (in the VTA) (Diana et al., 1998a/b), reduced dopamine levels (in the Nacc) (Tanda et al., 1999), and the blockade of anxiety-like behaviours (induced by withdrawal) by co-administration of a CRF antagonist (α-helical CRF) (Rodriguez de Fonseca et al., 1997) (also reviewed in Koob and LeMoal, 2006). Thus, it is important to consider how different somatic and motivational signs of dependence and withdrawal may arise during exposure to opioids and cannabinoids in various learning paradigms.
1.6. Phases of Learning: Evidence that Extinction is Not Unlearning

After an association or response has been acquired and established by classical (Pavlovian) or instrumental (Skinnerian) conditioning, it can be reversed through the process of extinction. Pavlovian learning paradigms are based on the pairing of a conditional stimulus (CS) (e.g., tone or light) with an unconditional stimulus (US) (e.g., food or footshock) possessing inherent motivational properties (e.g., rewarding or aversive) that elicit an unconditional response (UR) (e.g., salivation or freezing behaviour) which, after successful association, becomes a conditioned response (CR) and can be elicited by the CS alone (CS-). Extinction is observed as a decline in the intensity and frequency of the CR when the CS is repeatedly presented in the absence of the US (see Domjan, 2003, for review). Studies of extinction strongly suggest that it is neither forgetting nor unlearning, processes which imply that the decline or loss of the CR reflects a reversal of the plasticity involved in acquisition. Instead, mounting research shows that extinction is the acquisition of new learning that mediates previous learning, acting as a form of inhibition, and is accompanied by additional plasticity (Bouton, 1988, 2000, 2002; Bouton and Swartzenruber, 1991; Falls, 1998; Rescorla, 2001; Kitazawa, 2002; see Quirk and Mueller, 2008, and Myers and Davis, 2002, for reviews). Furthermore, as with all types of learning, extinction progresses through three distinct phases: acquisition, consolidation, and retrieval (see Quirk and Mueller, 2008, for review), and is also subject to a subsequent period of reconsolidation during which memories are vulnerable to eradication by numerous mechanisms that can prevent them from being successfully established and enduring (see Quirk and Mueller, 2008, for review). The result is that the new learning and the current meaning are determined largely by the current context (Bouton, 2002). Several related phenomena indicate that the original association or response that is learned can be reversed, but not erased
completely, as observed by spontaneous recovery, reinstatement, renewal, and reacquisition of learning (reviewed in Bouton, 2002, Table 1).

Spontaneous recovery is the most common and widely demonstrated recovery effect after extinction has occurred (Pavlov, 1927; Bouton, 2002; Domjan, 2003). Both classically conditioned (Brooks and Bouton, 1993; Robbins, 1990; Rosas and Bouton, 1996) and instrumentally conditioned (Devenport, 1998; Rescorla, 1997) responses that are subsequently extinguished will reappear after the passage of time and a greater time delay will tend to produce a stronger response recovery. For example, extinguished instrumental responses for food reward were recovered at a greater rate after a time delay of 7 days compared to testing after only 24 h (Rescorla, 1996). However, retrieval cues for extinction can attenuate spontaneous recovery (Brooks and Bouton, 1993), thus it has been proposed that it is the failure to retrieve information related to extinction that promotes recovery of the conditioned response (Bouton, 2002).
Table 1.1

Four Context and Extinction Phenomena That May Provide Mechanisms of Relapse

<table>
<thead>
<tr>
<th>Phenomenon</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reinstatement</td>
<td>Recovery of behaviour that occurs when the subject is exposed to the US after extinction. Strongly controlled by contextual conditioning produced when the US is presented, hence the phenomenon is strongest when the CS is tested in the context in which the US has occurred.</td>
</tr>
<tr>
<td>Renewal</td>
<td>Recovery of extinguished behaviour that can occur when the context is changed after extinction. Most often observed when the subject is returned to the original context of conditioning, but it also depends in part on mere removal from the extinction context.</td>
</tr>
<tr>
<td>Spontaneous recovery</td>
<td>Recovery of responding that occurs when the CS is tested after time has passed following the conclusion of extinction.</td>
</tr>
<tr>
<td>Reacquisition</td>
<td>Recovery of responding that occurs when the CS is paired with the US (or reinforce) again after extinction. Often rapid, especially when cues in the background cues continue to retrieve extinction.</td>
</tr>
</tbody>
</table>

*Reprinted from Bouton, M.E. (2002, Table 1, p. 977), Context, ambiguity, and unlearning: Sources of relapse after behavioral extinction, Biological Psychiatry, 52: 976-986, with permission from Elsevier and author.*
Through the process of reinstatement, the extinguished behaviour reappears after the subject is exposed to the US alone. For example, after a classically conditioned fear response to a tone (by previous pairings with footshock) is successfully extinguished by repeatedly presenting the tone without the shock, merely exposing the subject to the footshock in a separate session 24 h later reproduces the fear response (e.g., Pavlov, 1927; Rescorla and Heth, 1975; Delamater, 1997). Reinstatement can occur for an extinguished instrumentally conditioned response as well, as demonstrated when successfully extinguished cocaine-reinforced self-administration responses were subsequently increased by exposure to amphetamine or morphine alone (de Witt and Stewart, 1981). The strength of reinstatement can be influenced by the strength of the association of the contextual cues with the US (Bouton and King, 1983; Bouton, 1984) and extinguished behaviours may be particularly vulnerable to increasingly ambiguous contextual cues, thus reinstatement is more robust when the conditioned stimulus is tested in the same context wherein the US initially occurred (Bouton, 2002).

Renewal of the extinguished behaviour occurs when the extinguished response reappears when the subject is tested in the original context. For example, a subject’s response which is conditioned in context A, then extinguished in context B, will reappear when the subject is reintroduced to context A, but not context B. Bouton and King (1983) demonstrated this type of renewal in a study of conditioned fear and conditioned suppression of behaviour. Rats that were conditioned to lever press for food reward in one of two chambers were also conditioned to fear a tone previously paired with footshock in the same chamber — subsequent presentations of the tone resulted in suppressed lever pressing for the food reward. Each chamber contained contextual cues
that were distinctly different and conditioned responses were then extinguished in either
the same chamber (as conditioning) or in the other chamber. Rats showed similar rates of
conditioned suppression at the beginning of extinction trials (regardless of the
congruency of conditioning and extinction contexts), and conditioned suppression of
lever pressing by the tone was successfully extinguished in both groups. However, in a
series of test trials, rats that were given extinction trials in the same chamber in which
they were conditioned showed less renewal of conditioned suppression than rats given
extinction trials in a different chamber than that of conditioning. Thus, the more
incongruent the test context is with the extinction context, the greater the renewal effect
when the original context is restored.

Reacquisition occurs when, following extinction training, the conditioned
stimulus is once again paired with the reinforcer and the conditioned response reappears.
The rate of reconditioning is usually more rapid than the rate of the original conditioning,
however it can be slower when extinction cues are present and interfere with recovery of
the association. For example, reacquisition is faster for reconditioning of an extinguished
eye-blink response in rabbits than when the conditioned stimulus was novel (Napier et
al., 1992), but reacquisition can be significantly slower for fear conditioning (Bouton,
1986; Bouton and Swartzenruber, 1989), learned taste aversion (Hart et al., 1995; Calton
et al., 1996), and is greatly impeded by an increasing number of extinction trials (Bouton,
2002).

The occurrence of spontaneous recovery, reinstatement, renewal, and
reacquisition, after successful extinction, all indicate that learning is not destroyed by the
extinction process; instead, it is available for extended periods of time, at varying
degrees, depending upon the context (Bouton, 2002). These phenomena have been proposed to be the underlying mechanisms of relapse (Bouton 2002). Evidence also suggests the involvement of endocannabinoids in regulating the relapse phenomenon.

1.7. Uncovering Phenomena and Relapse to Drug-seeking for Drugs of Abuse

These phenomena — spontaneous recovery, reinstatement, renewal, and reacquisition — have been proposed to be the underlying mechanisms of relapse to drug-seeking behaviour (Bouton, 2002). However, within the behavioural learning theory framework, the reoccurrence of extinguished behaviour should be considered with a clear distinction between ‘lapse’ and ‘relapse.’ In terms of learning, ‘lapse’ represents the first occurrence of the extinguished behaviour after successful extinction that can affect subsequent behaviour and ‘relapse,’ which is differentially defined as a return to pre-extinction levels of conditioned responding (Marlatt and Gordon, 1985; Bradley, 1989; cited in Leri and Stewart, 2002).

Many animal models studying relapse to craving and drug-seeking during disintoxication (e.g., removal of the state of intoxication or dependence on intoxicating agents) use the extinction/reinstatement paradigm (see Fattore et al., 2007 for review). There are two main types of animal models of drug abuse and relapse: classical conditioning (e.g., conditioned cue preference (CCP), also termed conditioned place preference (CPP), and conditioned cue aversion (CCA), also termed conditioned place aversion) (Mucha et al., 1982) and instrumental/operant conditioning (e.g., self-administration) (Stewart and De Wit, 1987). In CCP/CPP, an animal is conditioned to expect a drug in a particular context (e.g., chamber) and/or in the presence of specific
cues (e.g., tone), whereas in operant conditioning, an animal is trained to self-administer a drug (e.g., by lever-pressing or nose-poking). After the behavioural association with the drug is learned, the drug is then withheld and the animal continues to respond to seek and/or receive the drug until eventually the association between the behaviour and the drug is extinguished and the animal ceases to respond (reviewed in Stewart, 2003). Drug seeking/taking behaviour is reinstated in three main ways (reviewed in Fattore et al., 2007):

1) **Drug-induced reinstatement or cross-reinstatement**: priming the animal with the same drug (e.g., via single injection) or priming with a different drug (Parker and McDonald, 2000; Mueller et al., 2002; Shaham et al., 2003; Leri and Rizos, 2005);

2) **Context/Cue-induced reinstatement**: a conditioned stimulus associated with drug taking is re-presented without the drug reinforcer (CS-) (e.g., repeated pairing of the drug with discriminative stimuli such as a chamber, tone, or light, which then alone can elicit drug-seeking/taking post-extinction training) (Ciccocioppo et al., 2001; Leri and Rizos, 2005; Parker et al., 2006);

3) **Stress-induced reinstatement**: relapse provoked by experiencing acute/chronic stress (e.g., elicited by mild footshock, food-deprivation, predator odour) (Shaham et al., 2000; Stewart, 2000; Shalev et al., 2002; Kalivas and McFarland, 2003).

Relapse in drug addiction is a significant problem, even after patients have abstained for long periods of time, for many types of addictive substances, including alcohol, nicotine, opioids, and psychostimulants (Bozarth, 1987; Chornock et al., 1992;
Hodgson et al., 1979; Jaffee et al., 1989; Ludwig and Wickler, 1974). Challenging the prevailing view that periods of acute withdrawal pose the greatest risk for relapse are studies showing that enduring changes in neural circuitry, resulting from repeated exposure to drugs of abuse, actually increase vulnerability to relapse in the long-term (reviewed in Stewart, 2003). In fact, several brain systems have been implicated in drug-induced relapse, including the ECB system. Recent studies of the ECB system have shown significant involvement of CBRs (e.g., CB₁) in the process of extinction as evidenced by observed levels of reinstatement. For example, both drug-induced and cue-induced reinstatement of drug-seeking behaviour involve the processes of excitation or inhibition of CB₁, and stimulation of CB₁ elicits relapse to cannabis, cocaine, alcohol, heroin, and methamphetamine, an effect which can be significantly attenuated by pretreatment with the CB₁ antagonist SR141716A (reviewed in Fattore et al., 2007). Similarly, priming rats with CB₁ receptor agonists results in reinstatement of extinguished operant behaviour (Yamamoto et al., 2004) and relapse to drug-seeking behaviour can be elicited by exposure to SR141716A alone (Le Foll and Goldberg, 2005; De Vries and Schoffelmeer, 2005).

1.8. Experimental Factors Affecting Drug-Induced Conditioned Behaviour

Commonly abused drugs typically have reinforcing effects that can be measured in studies of classical and operant conditioning. In fact, Δ⁹-THC has been demonstrated to produce both CPP and CPA in both mice and rats and there are some reports of synthetic cannabinoid self-administration in rodents; however, these results are highly dependent upon the dose and conditions of the experiment and not always replicable
(reviewed in Murray and Bevins, 2010, and Panlilio et al., 2010). In particular, the establishment of either a conditioned preference or aversion to cannabinoids can vary with the type of conditioning apparatus used and any preexisting biases associated with it, the number and length of conditioning trials, administration-to-placement intervals, drug exposure prior to training, and the dose regimen (reviewed in Murray and Bevins, 2010, Table 1, p. 270). In addition, species’ metabolic rates can significantly alter the dose range that reliably produce CPP and CPA; for example, mice typically have a faster rate of drug metabolism (Lemberger et al., 1973) and the doses required to produce conditioned associations are often higher than in rats, sometimes on the magnitude of ten times (reviewed in Murray and Bevins, 2010).
Table 1.2.a Δ⁹-THC Place Conditioning Study Details: MICE.

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Table 1.2.b. Δ⁹-THC Place Conditioning Study Details: RATS.

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In general, lower doses of Δ⁹-THC tend to produce null results or CPP, whereas higher doses tend to induce CPA; however, there is a significant degree of variability across studies in both rats and mice (reviewed in Murray and Bevins, 2010). For example, Braida et al. (2004) found CPP induced by Δ⁹-THC at doses between 0.075 to 0.75 mg/kg in Wistar rats; both SR141716A and naloxone pretreatment abolished this effect. In addition, Braida et al. (2004) found no effect of Δ⁹-THC for doses between 1 and 3 mg/kg, but a conditioned aversion at 6 mg/kg. However, Parker and Gillies (1995) demonstrated that Δ⁹-THC induced CPA at 0.75 and 1.5 mg/kg (i.p.), but had no effect at 0.2 or 0.4 mg/kg, in both Lewis and Sprague-Dawley rats. Similarly, Cheer et al. (2000) reported Δ⁹-THC-induced CPA at 1.5 mg/kg (i.p.); however, the CB₁ antagonist/inverse agonist SR141716A produced a CPP at 0.25, 0.5, 2, and 3 mg/kg (i.p.) in Lister hooded rats. Mallet and Beninger (1998) showed Δ⁹-THC-induced CPA at 1.0 and 1.5 mg/kg (i.p.), but not 0.1, 0.5, 2.0, 4.0 or 8.0 mg/kg; locomotor activity was decreased at doses of 1.0, 1.5, 2.0 and 4.0 mg/kg (i.p.) in Wistar rats.

Some of these discrepancies can be attributed to temporal conditions of the experiments, such as time intervals between training sessions and the injection-to-placement interval (IPI) (reviewed in Murray and Bevins, 2010, Table 1, p. 270). In general, increasing the time between sessions resulted in lower dosages producing CPP and higher dosages producing CPA; these effects are typically a result of the biphasic effects of Δ⁹-THC metabolism (reviewed in Murray and Bevins, 2010). For example, when training session intervals were 24 h, both 2 and 4 mg/kg Δ⁹-THC produced CPP but 1 mg/kg had no effect; in comparison, 48 h training session intervals shifted the dose effect curve such that 1 mg/kg Δ⁹-THC now produced a CPP and 2 and 4 mg/kg
produced CPA (Lepore et al., 1995). Lepore et al. (1995, p. 2076) reported that this “abrupt step-up dose-effect function” found with Δ⁹-THC is consistent with their studies on brain stimulation reward thresholds demonstrating a “rebound anhedonic or dysphoric state” that follows acute Δ⁹-THC exposure. For example, acute administration of 1 mg/kg Δ⁹-THC was sufficient to reduce reward thresholds in the brain, but then was followed by an increase in reward thresholds (a rebound shift) that lasted up to 48 h (consistent with Lepore et al.’s unpublished observations) (Lepore et al., 1995). Thus, when the between session intervals increased from 24 to 48 h, the “wash-out” time also increased allowing the brain stimulation reward thresholds to return to baseline (pre-Δ⁹-THC) levels which resulted in 1 mg/kg Δ⁹-THC producing CPP (Lepore et al., 1995). This explanation is also consistent with the explanation that the higher dosages of 2 and 4 mg/kg may have been sufficient to surpass the negative affective state produced by the prior Δ⁹-THC administration when the between session interval was 24 h, and thus producing CPP, but insufficient when it was extended to 48 h, thus producing CPA. It is possible that the higher dosages produced greater rebound shifts in which the negative affective states lasted longer and that a period of 48 to 72 h would be required to return to pre- Δ⁹-THC affective states after higher dosages (Lepore et al., 1995). These biphasic effects demonstrated for Δ⁹-THC are similar to those of nicotine, which also produces CPP at lower dosages and CPA at higher dosages (Fudala et al., 1985; Jorenby et al., 1990).

In Lepore et al.’s (1995) studies, only the between session intervals were manipulated and the IPI was held constant; in contrast, manipulating the IPI could potentially vary the extent of the CPP/CPA. For example, altering the IPI can create a different temporal relationship between the CS (compartment context/cue) and the US
(drug); in general, the strongest associations are produced when the CS onset precedes the US onset and there is some degree of temporal overlap between them (Murray and Bevins, 2010). In fact, the length of the IPI may also interact with the route of administration of the drug to further affect the development of CPP/CPA. This is particularly relevant to studies with Δ⁹-THC, which may be delivered through parenteral (injection) or pulmonary (inhaled) administration, because the time required for Δ⁹-THC and its primary psychoactive metabolite, 11-OH-Δ⁹-THC, to reach peak blood and brain levels depends on the route of administration (e.g., a few seconds to minutes after inhalation versus up to 20 minutes or longer after intraperitoneal injection) (reviewed in Koob and Le Moal, 2006). Thus, it is important to consider the pharmacokinetic effects of drugs in conditioning studies, particularly the temporal associations of drug peak effects with exposure to conditioning cues and contexts, and how the route of administration of a drug can interact with the IPI to alter the strength of drug-induced conditioned associations.

1.9. Route of Administration: Differential Effects of Parenteral and Pulmonary Cannabinoid Exposure on Learning and Memory

All of the experimental factors affecting CPP and CPA induced by cannabinoid exposure reviewed in Murray and Bevins (2010) involved only one route of administration (e.g., intraperitoneal). More importantly, studying the effects of cannabinoids in animal models to better understand the development of drug use, dependence and abuse in human, should account for the fact that cannabis is typically inhaled by human users, either by combustion (smoking) or vapourization. However, few
studies have directly compared the effects of route of administration for cannabinoids on learning and memory. Recent evidence suggests $\Delta^9$-THC may produce differential effects on behaviour and memory depending upon the route of administration (Naef et al., 2003, 2004; Niyuhire et al., 2007; Ford et al., 2009; Manwell and Mallet, 2009). For example, Niyuhire et al. (2007) reported that, while injected $\Delta^9$-THC (1, 3, 10 mg/kg) dose-dependently disrupted both acquisition and recall of the platform location in the Morris water maze task, inhaled smoke from marijuana (50, 100, and 200 mg) only impaired performance at the highest dose (estimated to have 4.2 mg $\Delta^9$-THC before burning). Although Niyuhire et al. (2007) suggested that previous pharmacokinetic studies (e.g., Wilson et al., 2006) indicated that brain levels of $\Delta^9$-THC from 1 mg/kg intravenous exposure may be roughly equivalent to smoke from 200 mg marijuana, they did not directly quantify bioavailable levels of $\Delta^9$-THC. Finally, combustion of cannabis releases many compounds in addition to $\Delta^9$-THC, including toxins and carcinogens (e.g., anthrocyclines, nitrosamines, polycyclic aromatic hydrocarbons, terpenes, and vinyl chloride) (Sarafian et al., 1999; Zhang et al., 1999; Roth et al., 2001; Hashibe et al., 2005; Voirin et al., 2006; Aldington et al., 2008; Berthiller et al., 2008; also reviewed in Reece, 2009). In comparison, vapourization of pure $\Delta^9$-THC provides a more accurate assessment of the direct effects of $\Delta^9$-THC and its primary psychoactive metabolite, $\text{11-OH-}\Delta^9$-THC. Accordingly, studies in animals need to also account for actual bioavailable levels of $\Delta^9$-THC and its primary psychoactive metabolite 11-OH-$\Delta^9$-THC, in blood after injection and inhalation of pure $\Delta^9$-THC, not just marijuana smoke containing unknown quantities of $\Delta^9$-THC and numerous other toxicants.
1.10. Rationale and the Experiments

The underlying mechanisms involved in ECB-mediated extinction learning require further investigation, primarily the effects of CB₁ activation or blockade on the extinction of CPP and CPA induced by opiates. The following sections specifically address the ECB system’s involvement in extinction learning primarily for drug-induced conditioned behaviour in the extinction/reinstatement paradigm. It is hypothesized that the ECB system can be selectively manipulated, through activation or blockade of CB₁, to facilitate or impair rates of extinction for opiate-induced CPP and CPA. It is also hypothesized that these manipulations will demonstrate dose-dependent effects. Chapter 2 presents a series of experiments evaluating the potential of the FAAH inhibitor, URB597, to facilitate extinction rates of CPP and CPA induced by opiates in rats. Chapter 3 reports a series of experiments designed to quantify levels of primary psychoactive cannabinoids in biological tissues after exposure to Δ⁹-THC via different routes of administration, specifically parenteral (liquid injection) and pulmonary (vapour inhalation) administration in rats; the purpose being to directly compare the effects of different routes of administration of Δ⁹-THC on the extinction of CPA. This required the design of a simple, inexpensive, and reliable method of analyzing levels of Δ⁹-THC and its primary psychoactive metabolite, 11-OH-Δ⁹-THC, in small amounts of whole blood from rodents administered Δ⁹-THC either via injection or vapour inhalation. Chapter 4 presents a final series of experiments, based on those in Chapters 2 and 3, that compare the pharmacokinetic and behavioural effects of pulmonary and parenteral Δ⁹-THC exposure, particularly on the potential of Δ⁹-THC to alter the extinction rates of an opiate-induced CPA in rats.
CHAPTER 2

2.1. Endocannabinoid System and Extinction Learning: Effects on Extinction

Learning for Conditioned Cue Preference and Aversion

Chapter 2 is based on a report by Manwell et al. (2009) published in Pharmacology, Biochemistry and Behavior in collaboration with Elham Satvat, Stefan Lang, Craig Allen, and supervisors Dr. Francesco Leri and Dr. Linda Parker. Experiment 2.1 was conducted by Craig Allen, Experiment 2.2a was conducted by Elham Satvat, Experiment 2.2d was conducted by Stefan Lang, and Experiments 2.2b, 2.2c and Experiment 2.3 were all conducted by Laurie Manwell; data analyses and writing of the manuscript was done by Laurie Manwell and Linda Parker.

Reprinted from Manwell, Satvat, Lang, Allen, Leri, and Parker (2009), FAAH inhibitor, URB597, promotes extinction and CB₁ antagonist, SR141716, inhibits extinction of conditioned aversion produced by naloxone-precipitated morphine withdrawal, but not extinction of conditioned preference produced by morphine in rats, Pharmacology, Biochemistry and Behavior, 94: 154-162, with permission from Elsevier and author.
2.2. Abstract

Converging evidence suggests that the endogenous cannabinoid (ECB) system is involved in extinction of learned behaviours. Using operant and classical conditioning procedures, the potential of the fatty acid amide hydrolase (FAAH) inhibitor, URB597, and the CB₁ antagonist/inverse agonist, SR141716, to promote and inhibit (respectively) extinction of learned responses previously motivated by either rewarding or aversive stimuli was investigated. In the operant conditioning procedure (Expt. 2.1), rats previously trained to lever press for sucrose reward were administered URB597 (0.3 mg/kg) or the CB₁ antagonist/inverse agonist SR141716 (2.5 mg/kg) prior to each of three extinction trials. In the conditioned floor preference procedure (Expts 2.2a–d), rats trained to associate morphine with one of two distinctive floors were administered one of several doses of the CB₁ antagonist/inverse agonist, AM251 (Expt 2.2a) or URB597 (Expt 2.2b and 2.2d) prior to each extinction/test trial wherein a choice of both floors was presented and prior to forced exposure to each floor (Expt 2.2c). In the conditioned floor aversion procedure (Expt. 2.3), rats trained to associate a naloxone-precipitated morphine withdrawal with a floor cue were administered URB597 or SR141716 prior to each of 24 extinction/testing trials. URB597 did not promote and SR141716 did not reduce extinction rates for sucrose reward-induced operant responding (Expt. 2.1) or morphine-induced conditioned floor preference (Expts. 2.2a–d). In contrast, URB597 facilitated, whereas SR141716 impaired, extinction of the conditioned floor aversion (Expt. 2.3). These data support previous reports that the ECB system selectively facilitates extinction of aversive memories. URB597 may prove useful in targeting extinction of aversively motivated behaviours.
2.3. Introduction

Considerable evidence indicates that the endogenous cannabinoid (ECB) system is involved in extinction learning of aversively motivated learned behaviors (Marsicano et al., 2002; Varvel and Lichtman, 2002). Marsicano et al. (2002) reported that CB₁ knockout mice and wild-type mice administered the CB₁ inverse agonist/antagonist, SR141716 (Rimonabant), showed impaired extinction in classical auditory fear-conditioning tests, with unaffected memory acquisition and consolidation. Using the Morris water maze task, Varvel and Lichtman (2002) reported that CB₁ knockout mice and wild-type mice exhibited identical acquisition rates in learning to swim to a fixed platform; however, the CB₁-deficient mice demonstrated considerable deficits during a reversal task in which the location of the hidden platform was moved to the opposite side of the tank. Since animals deficient in CB₁ receptor activity show impairments in suppressing previously learned behaviors, cannabinoid agonists would be expected to facilitate extinction of learned behaviors in non-deficient animals. Indeed, Pamplona et al. (2006) recently reported that the potent cannabinoid receptor agonist WIN 55,212-2 facilitated extinction of contextual fear memory and spatial memory in rats, whereas an ethyl derivative of SR141716 significantly impaired extinction. These results suggest that the ECB system modulates extinction of aversively motivated learned responses.

With the recent discovery of drugs that enhance endogenous cannabinoids by blocking their reuptake (e.g., AM404) or by inhibiting the enzyme (fatty acid amide hydrolase [FAAH]) that deactivates anandamide (e.g., OL135 and URB597), the effect of prolonging anandamide's activity during extinction learning has also been investigated (Chhatwal et al., 2005; Varvel et al., 2007). Chhatwal et al. (2005) reported that
pretreatment with AM404 selectively facilitated extinction of fear potentiated startle in rats, an effect that was reversed by SR141716 pretreatment. Varvel et al. (2007) reported that mice deficient in FAAH, either by genetic deletion (FAAH −/−) or by pharmacological inhibition with OL135, displayed both faster acquisition and extinction of spatial memory tested in the Morris water maze; SR141716 reversed the effect of OL135 during both task phases.

In fact, recent evidence suggests that the ECB system may not regulate extinction of reward-based learning (e.g., Lutz, 2007). Neither CB₁-deficient mice (Hölter et al., 2005) nor wild-type mice treated with SR141716 (Niyuhire et al., 2007) displayed a deficit in extinction of operant responding reinforced with food. In comparison, SR141716-treated mice displayed impaired extinction of conditioned freezing and passive avoidance (Niyuhire et al., 2007). It was also reported that SR141716 disrupted extinction learning in an aversive, but not appetitive, Barnes maze conditioning task (Harloe et al., 2008). Therefore, unlike the recent evidence that the partial N-methyl-D-aspartate (NMDA) glutamate receptor agonist, D-cyloserine, promotes extinction of both positive and aversive based learning (e.g. Paolone et al., 2009), the ECB system may be more selective for aversive memories. Further research on the role of the ECB system in the extinction of other forms of reward-based learning and aversion learning is warranted.

The present experiments evaluated the potential of FAAH inhibition (by URB597) and CB₁ inverse agonism/antagonism (by SR141716 [Experiments 2.1 and 2.3] and AM251 [Experiment 2.2a]) to facilitate and inhibit (respectively) extinction of operant responding for sucrose (Experiment 2.1), morphine-induced conditioned floor preference (Experiments 2.2a–d) and naloxone-precipitated morphine withdrawal-
induced conditioned floor aversion (Experiment 2.3). The effect of endocannabinoid manipulation on extinction of a conditioned floor preference (Experiments 2.2a-d) was evaluated in four subexperiments. In Experiments 2.2a and 2.2b various doses of AM251 (0, 1, 3 or 8 mg/kg) and URB597 (0.0, 0.03, 0.1 and 0.3 mg/kg) failed to modify extinction of morphine conditioned floor preferences when administered prior to each extinction/test trial. In Experiment 2.2c, URB597 also failed to modify extinction of morphine conditioned floor preferences when administered prior to extinction trials with forced confinement to each of the floors. In each of Experiments 2.2a–c, the conditioned preference among the vehicle treated rats extinguished rapidly; conditioned preferences are less resistant to extinction than are conditioned aversions (e.g., Parker and McDonald, 2000). Therefore, Experiment 2.2d, evaluated the potential of 0.3 mg/kg URB597 to promote extinction of a more robust conditioned floor preference according to a procedure recently described by Paolone et al. (2009). Again, URB597 failed to promote extinction of a conditioned preference.

Experiment 2.3 evaluated the potential of URB597 and SR141716 to modify the rate of extinction of a conditioned floor aversion. Although naloxone alone produces only a weak conditioned place aversion (Mucha and Iverson, 1984; Parker and Rennie, 1992), it produces a profound conditioned place aversion when preceded by an injection of morphine (Parker and Joshi, 1998). In fact, naloxone-precipitated morphine withdrawal-induced conditioned place aversions occur even when naloxone (1 mg/kg) is administered 24–48 h after an injection of morphine (20 mg/kg) in a single conditioning trial (Parker et al., 2002). If the ECB system selectively facilitates extinction of aversively motivated learned responses, then it should more effectively enhance the speed
of extinction of the conditioned floor aversion than the conditioned preference or operant responding for sucrose.

2. 4. Materials and Method

2.4.1. Subjects

The subjects in Experiment 2.1 and 2.2a were Sprague–Dawley rats and in Experiments 2.2b–d, and 2.3 were male Long–Evans rats. The change in the strain to Long–Evans rats was aimed at producing a robust conditioned preference/aversion (e.g., Paolone et al., 2009). The animals were group-housed in shoebox cages in the colony room at an ambient temperature of 21°C with a 12/12 light dark schedule (lights off at 8 AM) and were maintained on an ad libitum schedule of food (except Experiment 2.1 in which they were maintained at 85% body weight) and water. All procedures adhered to the guidelines of the Canadian Council of Animal Care and were approved by the Animal Care Committee of University of Guelph.

2.4.2. Drugs

Morphine (Sigma) was prepared in physiological saline (Sigma) at concentrations of either 10 (Expts 2.2a–d) or 20 (Expt. 2.3) mg/ml and administered subcutaneously (s.c.) in a volume of 1 ml/kg at 10 min prior to conditioning. Naloxone (Sigma) was prepared in physiological saline in a concentration of 1 mg/ml and administered (s.c.) in a volume of 1 ml/kg at 10 min prior to conditioning (Expt. 2.3). URB597 (Cayman Chemicals) was prepared in 2-hydroxypropyl-β-cyclodextrin (2-HPBCD, 45%; Sigma) at a concentration of 0.3 mg/kg (Expts. 2.1, 2.2d and 2.3) administrated at a volume of 1 ml/kg intraperitoneally (i. p.) at 2 h prior to extinction testing. Previous work has shown that 0.3 mg/kg URB597 produces neither a place preference nor a place aversion (Gobbi
et al., 2005), although it augments the hypothermic effects of exogenously administered anandamide (Fegley et al., 2005), produces anxiolytic effects (Kathuria et al., 2003; Scherma et al., 2007), antidepressant effects (Gobbi et al., 2005), and anti-nausea effects (Cross-Mellor et al., 2007; Rock et al., 2008). URB597 has been shown to produce a slow and reliable accumulation of anandamide in the brain with a maximal effect at 2 h post-injection (Fegley et al., 2005; Gobbi et al., 2005; Kathuria et al., 2003). Experiments 2.2b–c employed various doses of URB597 as indicated. SR141716 (Sequoia Research, UK) was prepared in 45% 2-HPBCD at a concentration of 1 mg/ml and administered (i.p.) in a volume of 2.5 ml/kg at 30 min prior to extinction testing (Expts. 2.1 and 2.3). AM251 (Cayman Chemicals; Expt 2.2a) was prepared in a vehicle of 1 ml ethanol/1 ml Cremaphor (Sigma)/18 ml saline at concentrations of 1, 3 and 8 mg/ml and administered at a volume of 1 ml/kg.

2.4.3. Apparatus

In Experiment 2.1, 16 Plexiglas operant chambers (model ENV-008CT, Med Associates, Lafayette, IN) were used, each enclosed in larger sound-attenuating plywood chambers model ENV-018M, Med Associates). Each operant box had a retractable lever located 8 cm above the floor of the box and 5 cm from the magazine feeder. Two lights (28 V) were on during testing, one located on the same wall as the lever, the other on the opposing wall. Each chamber was also equipped with a hopper mounted on the exterior of the operant chamber that would deliver sucrose pellets (45 mg dustless Precision Pellets; Bio-Serv, Frenchtown, NJ) into the magazine feeder.

In Experiments 2.2 and 2.3, the conditioning apparatus used consisted of a black Plexiglas rectangular box (60×25×25 cm³) with a wiremesh lid (as previously described
in Parker et al., 2004). During conditioning trials, the tactile cues on both sides of the box were identical. During pretest and choice tests, one side of the chamber had a metal hole floor and the other side a metal bar floor (counterbalanced), and the intersection of the two floors was defined as a neutral zone (9×25 cm). The amount of time (sec) each rat spent on each of the floors was recorded and subsequently analyzed by the Noldus Etho-Vision videotaping system (Noldus Information Technology, Sterling, VA). Pretests did not indicate a significant difference between seconds spent on the metal hole or bar floors indicating that the apparatus provides an unbiased test of conditioned preference and aversion.

2.4.4 Procedure

2.4.4.1. Experiment 2.1: Effect of URB597 and SR141716 on Extinction of Operant Responding Motivated by Sucrose

The rats had been extensively trained to lever press for sucrose as part of a previous experiment in which some animals received cocaine. The previous drug groups were counterbalanced across treatment groups; analysis showed that there was no effect of the counterbalancing variable on the behavior reported here. A stable baseline of reinforced responding was established over 5 days of testing on a fixed ratio 25 (1 pellet for every 25 responses) schedule of reinforcement. Each day, rats received one 1 h long session consisting of 5 min acclimatization to the chamber without the lights or levers present, followed by 1 h of testing starting with house and key lights turning on and insertion of the lever.

Beginning the following day, rats (matched by baseline responding) were administered (i.p.) URB597 (n=14; 0.3 mg/kg) at 2 h or Vehicle (n=13) at 30 min or
SR141716 (n=13; 2.5 mg/kg) at 30 min, prior to each extinction trial. Extinction conditions were identical to baseline but no sucrose was delivered. Each animal received 3 extinction sessions over 3 days with each session 1 h long.

2.4.4.2. Experiment 2.2: Effect of AM251 or URB597 on Extinction of Morphine-induced Conditioned Preference

2.4.4.2.1. Experiment 2.2a. All rats were administered a 10 min pretest and the amount of time spent on each floor was measured. They were assigned to groups matched on the basis of their pretest score. The rats received 4 cycles of conditioning trials. During conditioning cycles all rats were injected with morphine or saline 10 min prior to being placed into the box with a distinctive floor for 30 min. Thus, each conditioning cycle consisted of one morphine trial and one saline trial separated by 24 h. Each of the cycles of conditioning was separated by 48–72 h. The order of the morphine trial within a cycle and the floor paired with morphine were counterbalanced. Forty-eight h after the fourth conditioning cycle, the rats were given a 15 min test. The rats were given two additional conditioning trial cycles following the test. Forty-eight h after the sixth conditioning cycle, the rats were given repeated 15 min extinction choice test trials, each separated by 24 h. Thirty min prior to each extinction trial, the rats (n=10/group) were injected (ip) with vehicle (1 ml/kg), or 1, 3 or 8 mg/kg AM251. The trials continued until the conditioned preference had extinguished; that is there was no significant difference in preference for the morphine-paired floor and the saline-paired floor.

2.4.4.2.2. Experiment 2.2b. The rats were treated as in Experiment 2.2a during conditioning except that they received 4 conditioning trial cycles. The 10 min extinction/test trials began 72 h after the final conditioning cycle. Two hours prior to each
extinction/test trial, the rats were injected (1 ml/kg, ip) with vehicle (n=18), 0.03 mg/kg URB597 (n=18), 0.1 mg/kg URB597 (n=15), 0.3 mg/kg URB597 (n=18). Every 48 h, the rats continued to receive this treatment until the conditioned preference had extinguished.

**2.4.4.2.3. Experiment 2.2c.** The conditioning procedure for Experiment 2.2c was identical to that of 2.2b, except the rats received 6 conditioning trial cycles. Unlike Experiments 2.2a and 2.2b, rats were given forced exposure to the floor cues during extinction training, rather than extinction by testing. Forty-eight h following the final conditioning trial, the rats received the extinction manipulation in one of 4 conditions: No Extinction (n=10), Vehicle (n=9), 0.1 mg/kg URB597 (n=10) or 0.3 mg/kg URB597 (n=10). Rats in the No Extinction group remained in their home cage during the extinction manipulation. Rats in the remaining groups were injected with the appropriate solution 2 h prior to the extinction manipulation on each trial. The extinction manipulation included a cycle of two trials, during which rats were treated exactly as they were during the conditioning trials, but received saline injections on both trials. In a counterbalanced order, on one trial the rats were placed on the morphine-paired floor for 30 min and on the other trial they were placed on the saline-paired floor for 30 min. Forty-eight h after each cycle of extinction training, the rats were given a 20 min drug-free preference test trial; they were injected with saline and placed in the test chamber and the preference for the morphine or the saline floor was assessed. This procedure of extinction/testing continued until the groups no long demonstrated a conditioned preference.

**2.4.4.2.4. Experiment 2.2d.** In Experiment 2.2d the procedures of conditioning and testing were modified to ensure a robust conditioned preference that was resistant to
extinction by using some procedures described by Paolone et al. (2009). The rats received 8 cycles of conditioning trials in a similar manner as Experiments 2.2a–c, however, only those rats which developed a preference continued to extinction testing. To test for the initial strength of the conditioned preference, all rats received a 10 min drug-free choice test 24 h after the last conditioning trial. The rats were considered not to have developed a conditioned preference if the difference in time spent on the morphine-paired floor and the saline-paired floor was less than 45 s and were then removed from the experiment (e.g., Paolone et al., 2009). The extinction trials began 96 h after this test. On each of 10 extinction trials separated by 72–96 h to attenuate the development of extinction (e.g., Mueller et al., 2002; Paolone et al., 2009), the rats were injected (ip.) with 0.3 mg/kg URB597 (n=8) or Vehicle (n=9) 2 h prior to a 10 min test trial. One week after the final extinction test trial, the rats received a test trial following an injection of saline and on the following day a test trial following an injection of morphine (2.5 mg/kg) in a reinstatement test. This procedure evaluated the effect of prior treatment with URB597 during extinction training on subsequent morphine-induced reinstatement of the floor preference.

2.4.4.3. Experiment 2.3: Effect of URB597 and SR141716 on Naloxone-precipitated Morphine Withdrawal-induced Conditioned Aversion

The rats were assigned to groups matched on the basis of a 20 min pretest score. They were given two conditioning trial cycles (separated by 72 h), each consisting of a 3 day schedule separated by 24 h (as previously described by Parker and Joshi, 1998). For each conditioning cycle, on Day 1 the rats were injected with saline (s.c.) at 10 min prior to placement in the chamber with a distinctive floor for 30 min. On Day 2, they were
given an injection (s.c.) of 20 mg/kg morphine in their home cages. On Day 3, they were given an injection (s.c.) of naloxone (1 mg/kg) at 10 min prior to placement in the chamber with the opposite distinctive floor (as on Day 1) for 30 min. Naloxone-alone controls were not included in the present experiment because previous work (e.g., McDonald et al., 1997; Parker and Joshi, 1998; Parker et al., 2002) demonstrated that the conditioned aversion produced by naloxone alone is greatly strengthened by pretreatment with morphine 24–48 h prior to the conditioning trial. For all extinction testing trials, which began 72 h after the final conditioning cycle, rats were administered (i.p.) 0.3 mg/kg URB597 (n=8) at 2 h, or vehicle (n=8) at 30 min or 2.5 mg/kg SR141716 (n=8) at 30 min, prior to a test trial which lasted for 20 min.

A 20 min drug-free conditioned aversion test was administered 72 h following the final extinction trial. One week later, the potential of naloxone-precipitated morphine withdrawal to reinstate the extinguished floor aversion was evaluated. On Day 1, rats were injected (s.c.) with 1 ml/kg saline 10 min prior to a 20 min choice test. On Day 2, they were injected (s.c.) with 20 mg/kg morphine in their home cage. On Day 3, the rats were injected (s.c.) with 1 mg/kg naloxone 10 min prior to a choice test to determine the effect of prior extinction treatments on reinstatement of the conditioned aversion.

2.5. Data Analysis

In Experiment 2.1, the number of responses during each 10 min interval on each of 3 extinction days were entered into a mixed factors ANOVA with the extinction treatment as a between groups factor and 10-min interval and extinction day as a within group factor. In Experiment 2.2 the mean seconds spent on the drug-paired floor and on the saline-paired floor during extinction by repeated test trials (Expts. 2.2a, b, d) or
during each preference test that followed forced extinction (Expt. 2.2c) were entered into a mixed factors ANOVA with extinction treatment as the between groups factor and the floor and trial (Expts. 2.2a, b and d) as within groups factors. Because conditioned aversions are more resistant to extinction than conditioned preferences (e.g., Parker and McDonald, 2000), in Experiment 2.3 the data for the 24 extinction trials was analyzed as blocks of 4 trials in 2×6 (floors×blocks of trials) repeated measures ANOVAs for each extinction condition. The data for the reinstatement trials were analyzed as mixed factors ANOVAs for each trial. Bonferroni post hoc comparison tests were conducted as appropriate. In each experiment, the time spent in the intersecting center zone was also evaluated revealing no differences among groups in Experiments 2.2 and 2.3. Statistical significance was defined as p < 0.05.

2.6. Results

2.6.1. Experiment 2.1: Effect of URB597 and SR141716 on Extinction of Operant Responding for Sucrose Reward

URB597 pretreatment during extinction training did not modify the rate of extinction of operant responding for sucrose reward. In comparison, SR141716 pretreatment suppressed operant responding during extinction. Fig. 2.1 presents the mean number of responses across six 10 min intervals on each of three test trials (separated by 48 h). The mixed factors ANOVA revealed significant effects of drug, F(2, 37)=7.8; p<0.001, day, F(2, 74)=103.7; p<0.001, interval, F(5, 185)=176.3; p<0.001, drug by day F(4, 74)=3.9; p<0.01; drug by interval, F(10, 185)=3.45; p<0.01; day by interval, F(10, 370)=34.6; p<0.001 and a drug by day by interval interaction, F(20, 370)=2.0; p<0.01). The triple interaction was analyzed as separate drug by interval mixed factors ANOVAs
for each Day. On Day 1 there was a significant effect of drug, F(2, 37)=6.9; p<0.01, interval, F(10, 185)=3.8; p<0.001 and a drug by interval interaction, F(5, 185)=133.9; p<0.001. Overall, SR141716 suppressed operant responding relative to URB597 and Vehicle (p's<0.05) which was most pronounced during the first 10 min of responding (p's<0.05). On Days 2 and 3 there was a significant effect of drug (p's<0.025) with SR141716 showing greater suppression overall than vehicle (p<0.05) and Intervals (p's<0.001) with responding decreasing across the intervals. The operant responding extinguished within the session and between sessions, but the speed was not affected by pretreatment with URB597.
Fig. 2.1. Experiment 2.1. Mean (+sem) number of lever-pressing responses for sucrose pellets during extinction for rats pretreated with Vehicle (n=13), 0.3 mg/kg URB-597 (n=14) or 2.5 mg/kg SR141716 (n=13).
2.6.2. Experiment 2.2: Effect of AM251 and URB597 on Extinction of Morphine-induced Conditioned Preference

2.6.2.1. Experiment 2.2a

AM251 did not modify the progression of extinction of a morphine-induced conditioned preference in Experiment 2.2a. Fig. 2.2 presents the mean (+sem) seconds that the rats spent on the morphine-paired floor and on the saline-paired floor following the pretreatment with the vehicle or one of several doses of AM251 during each extinction/test trial. The mixed factors ANOVA revealed only a significant effect of trial, F(2, 72)=3.5; p<.05 and a significant trial by floor interaction, F(2, 72)=4.2; p<.025, but no effects of pretreatment drug. Across all pretreatment drug conditions, the rats displayed a significant preference for the morphine-paired floor only on the first (p<.01) and the second (p<.05) extinction trial, but not on the third. Additionally, on the drug-free test after 4 conditioning trials, the rats spent significantly (p<.001) more time on the morphine-paired floor (Mean=498 s) than on the saline-paired floor (Mean=330 s).
Fig. 2.2. Experiment 2.2a: Mean (+sem) seconds spent on the morphine-paired floor and on the saline-paired floor during each extinction/test trial following 6 cycles of conditioning. Thirty min prior to each 15-min extinction/test trial, rats were administered Vehicle (n=10), 1 mg/kg AM251 (n=10), 3 mg/kg AM251 (n=10), or 8 mg/kg AM251 (n=10).
2.6.2.2. Experiment 2.2b

The FAAH inhibitor, URB597, did not promote the extinction of a morphine-induced conditioned preference. Fig. 2.3 presents the mean (+sem) seconds that the rats spent on the morphine- and the saline-paired floors during each extinction/test trial following 4 cycles of conditioning trials. The mixed factors ANOVA revealed significant effects of conditioning floor, F(1, 130)=13.3; p<.001, and an extinction drug by conditioning floor by trial interaction, F(6, 130)=2.3; p<.05. To evaluate the 3 way interaction, the data for each extinction trial was entered into a 4 by 2 repeated measures ANOVA. These analyses revealed a significant effect of conditioning floor on extinction trial 1, F(1, 65)=15.9; p<.001, and trial 2, F(1,65)=11.3; p<.001, but not on trial 3. The extinction drug by floor effect was not significant on any trial, largest F(3, 65)=1.9. Since the conditioned preference extinguished after only two test trials, it is conceivable that the rats displayed within session extinction across the 10 min trials. To assess changes in the preference among the groups within the session, the mean number of seconds spent in the drug-paired floor and the saline-paired floor during the first 5 min and the second 5 min of the test were included as an additional factor in the 4 (drug) by 2 (floor) mixed factor ANOVA for each extinction/test session. These additional analyses revealed the same pattern as the overall analyses for each trial; that is only the drug floor effect was significant and the strength of the floor preference did not change across test time or pretreatment group.
Fig. 2.3. Experiment 2.2b: Mean (+sem) seconds spent on the morphine-paired floor and on the saline-paired floor during each extinction/test trial following 4 cycles of conditioning. Two hours prior to each 10-min extinction/test trial, rats were administered Vehicle (n=18), 0.03 mg/kg URB597 (n=18), 0.10 URB597 (n=15) or 0.30 URB597 (n=18).
2.6.2.3. Experiment 2.2c

URB597 also did not promote extinction of a morphine-induced conditioned preference when administered during confinement on the morphine-paired and saline-paired floor. As is depicted in Fig. 2.4, there was only a significant effect of floor following one cycle of extinction training, $F(1, 35)=10.4; p<0.01$, and following two cycles of extinction training, $F(1, 35)=5.2; p<0.05$, but not following three cycles of extinction training. There were no significant interactions on any test trial.
Fig. 2.4. Experiment 2.2c: Mean (+sem) seconds spent on the morphine-paired floor and on the saline-paired floor during each 20-min test trial that followed an extinction cycle during which rats were confined on the morphine-paired floor on one day and on the saline-paired floor on the other day. Two hours prior to each extinction day, rats were injected with Vehicle (n=9), 0.1 mg/kg URB597 (n=10) or 0.3 mg/kg URB597 (n=10). An additional group of rats (No Extinction; n=10) remained in their home cage during the extinction sessions.
2.6.2.4. Experiment 2.2d

A more robust conditioned preference was produced in Experiment 2.2d than Experiments 2.2a–c, but URB597 did not promote extinction of a morphine-induced conditioned preference across 10 extinction trials. Following 8 conditioning trial cycles, rats displayed an overall preference for the morphine-paired floor, t(17)=8.6; p<0.001 in the initial preference test. Fig. 2.5 displays the mean (+sem) seconds spent on the morphine-paired floor and the saline-paired floor for the rats pretreated with Vehicle or URB597 at 2 h prior to each extinction trial. The mixed factor analysis revealed only a significant effect of conditioning floor, F(1, 135)=9.4; p<0.01, and a significant conditioning floor by trial interaction, F(9, 135)=2.0; p<0.05, but no effect of extinction treatment. Rats displayed significantly (p<0.05) greater preference for the morphine-paired floor than the saline-paired floor on extinction trials 1–3 and 5–6, but not on trial 4 (p<0.10) or trials 7–10 (p's>0.10).

A morphine prime reinstated the extinguished morphine-induced conditioned preference, but prior treatment with URB597 during extinction did not affect the strength of reinstatement, as can be seen in Fig. 2.6. The mixed factors ANOVA revealed only significant effects of conditioning floor, F(1, 15)=16.4; p<0.001, and a conditioning floor by trial interaction, F(1, 15)=12.9; p<0.01. On the morphine reinstatement test trial, but not on the saline reinstatement test trial, rats spent more time on the morphine-paired floor than on the saline paired floor (p<0.01); however, prior treatment with URB597 during extinction did not influence the strength of reinstatement.
**Fig. 2.5.** Experiment 2.2d. Mean (+sem) seconds spent on the morphine-paired floor and on the saline-paired floor during each of 10 extinction test trials in Experiment 2.2d. Groups were treated with vehicle (n=9) or URB597 (n=8) 2 hours prior to each extinction test trial. Asterisks indicate that rats spent more time on the morphine-paired floor than the saline-paired floor irrespective of pretreatment drug (*=p<0.05; **=p<0.01).
Fig. 2.6. Mean (+sem) seconds spent on the morphine-paired floor and on the saline-paired floor during the saline and morphine reinstatement tests for rats that had been treated with URB597 (n=8) or VEH (n=9) during extinction in Experiment 2.2d.
2.6.3. Experiment 2.3: Effect of URB597 and SR141716 on Extinction of a Naloxone-precipitated Morphine Withdrawal-induced Conditioned Aversion

Fig. 2.7 presents the extinction data depicted as 6 blocks of 4 trials each. Compared to the vehicle, it is clear that URB597 facilitated extinction and SR141716 impaired extinction. There was a significant floor by block of trials interaction for each extinction group: Vehicle (F(1, 24)=3.55; p<0.001), URB597 (F(1, 24)=1.71; p=0.028), and SR141716 (F(1, 24)=2.75; p<0.001). During Block 1 (extinction tests 1–4), all three groups showed a significant aversion to the withdrawal-paired floor in comparison to the saline-paired floor (all p's<0.01). During Blocks 2–4 (extinction tests 5–16) only the vehicle and SR141716 groups retained the aversion (p's<0.05). During Block 5 (extinction tests 17–20), only the SR141716 group retained the aversion (p<0.01). There was no significant floor effect for any group during Block 6 (extinction tests 21–24).

In the post-extinction, drug-free test 72 h after the last extinction trial, the mixed factor ANOVA revealed no significant effects, indicating that extinction was maintained in the absence of the treatment drugs. Previous treatment with either URB597 or SR141716 during extinction training did not modify the strength of subsequent reinstatement of the naloxone-precipitated morphine withdrawal-induced conditioned aversion following extinction training, as can be seen in Fig. 2.8. Although the rats maintained extinction of the conditioned floor aversion following a saline prime, F(1, 24)=0.5, they did display a reactivated aversion following the naloxone-precipitated morphine withdrawal prime, F(1, 24)=4.3, p=0.05. However, the prior extinction pretreatments did not modify the strength of the reinstated floor aversion as indicated by no significant interactions (Saline test, F(2, 24)=1.1; Withdrawal test, F(1, 24)=0.3).
Fig. 2.7. Experiment 2.3. Mean (+sem) seconds spent on the naloxone-precipitated morphine withdrawal-paired floor and on the saline-paired floor during each block of 4 trials for rats pretreated with vehicle (n=8), URB597 (n=8) or SR141716 (n=8) group. Asterisks indicate that rats spent less time on the withdrawal-paired floor than the saline-paired floor during block of trials (*=p<0.05; **=p<0.01).
Fig. 2.8. Mean (+sem) seconds spent on the withdrawal-paired floor and on the saline-paired floor during the saline and naloxone-precipitated morphine withdrawal reinstatement tests for rats that had been treated with VEH (n=8), URB597 (n=8) or SR141716 (n=8) during extinction in Experiment 2.3.
2.7. Discussion

The data from Experiments 2.1–2.3 are consistent with an emerging consensus in the literature that manipulations of the ECB system selectively modify extinction of aversive, but not appetitive, learning (e.g. Lutz, 2007; Harloe et al., 2008, Hölter et al., 2005; Niyuhire et al., 2007). Administration of the FAAH inhibitor and indirect CB₁ agonist, URB597, promoted the extinction of a naloxone-precipitated morphine withdrawal-induced conditioned aversion (Expt. 2.3), but did not affect extinction of a morphine-induced conditioned preference (Expts. 2.2a–d) nor sucrose-motivated operant responding (Expt. 2.1). Additionally, administration of the CB₁ inverse agonist/antagonist SR141716 interfered with the extinction of the conditioned aversion (Expt. 2.3), but did not interfere with extinction of responding motivated by sucrose reward, nor did AM251 interfere with extinction of a morphine-induced place preference (Expt 2.2a). In fact, SR141716 suppressed operant responding during extinction, suggesting either a motivational or locomotor suppressant effect.

Four experiments evaluated the potential of CB₁ antagonist/inverse agonist, AM251 (in Experiment 2.2a) or URB597 (in Experiments 2.2b–d), to modify the rate of extinction of a morphine-induced conditioned floor preference. In Experiments 2.2a–c, manipulation of the ECB system occurred on the first extinction trial (as in Experiment 2.3), both during extinction by testing (Expts 2.2a-b) and during extinction by forced exposure (Expt 2.2c). Because the conditioned preference extinguished rapidly in Experiments 2.2a–c, Experiment 2.2d was designed to ensure more robust resistance to extinction of the conditioned preference. In Experiment 2.2d, the number of conditioning trials was increased to 8, the duration of the extinction/test trials was 10 min and they were spaced by 72–96 h (e.g., Mueller et al., 2002; Paolone et al., 2009). Additionally, only rats that displayed a conditioned preference greater than 45 s in a probe test trial...
following conditioning were continued into extinction (e.g., Paolone et al., 2009). With these modifications, resistance to extinction was enhanced in Experiment 2.2d. Regardless of the method of extinction, manipulations of the ECB system had no effect on the speed of extinction of the conditioned floor preference.

Instead, it appeared that the failure of manipulations of the ECB system in Experiments 2.2a–d to affect extinction of a morphine induced conditioned preference may have been a function of the appetitive nature of the task. This hypothesis was then tested by evaluating the potential of URB597 to promote, and SR141716 to interfere with, the extinction of a conditioned floor aversion produced by an aversive stimulus, naloxone-precipitated morphine withdrawal (Experiment 2.3). In fact, URB597 facilitated extinction of the conditioned aversion in comparison to vehicle alone, whereas SR141716 prolonged the aversion. These effects were not apparent during the first block of test trials, but emerged over repeated testing suggesting an effect on extinction, but not retrieval of the memory. These results are consistent with others (Harloe et al., 2008; Niyuhire et al., 2007) showing a selective effect of manipulation of the ECB system on learning motivated by an aversive stimulus, but not on learning motivated by a rewarding stimulus. Due to known interactions between the ECB and opioid systems (e.g., Navarro et al., 1998; Solinas and Goldberg, 2005), it is important to extend the generality of this finding to extinction of a conditioned floor aversion produced by other aversive drugs, such as lithium chloride.

It has been well established that extinction is not unlearning, but instead is new inhibitory learning that interferes with the originally learned response (e.g., Bouton, 2002). Indeed, simply presenting a priming dose of the unconditioned stimulus (US) drug prior to a test for a previously extinguished place preference (Parker and McDonald, 2000; Mueller et al., 2002) or a place aversion (Parker and McDonald, 2000) reinstates
the learned response. Both a morphine prime (Experiment 2.2d) and a naloxone-precipitated morphine withdrawal prime (Experiment 2.3) reinstated the conditioned floor preference and aversion respectively. However, prior treatment with neither URB597 nor SR141716 during extinction modified the strength of subsequent reinstatement by a drug prime.

The selective effects of manipulation of the ECB system on extinction of the conditioned floor aversion may be related to the known anxiolytic effects of URB597 (e.g., Kathuria et al., 2003) and anxiogenic effects of SR141716 (e.g., Navarro et al., 1998; Mitchell and Morris, 2007). That is, maintenance of conditioned aversive associations may be promoted by conditioned fear and/or anxiety responses experienced at the time of testing. It is less likely that the hedonic effects of URB597 or SR141716 influenced their ability to modify extinction of the conditioned aversion, because previous investigations have shown that URB597 produces neither a conditioned place preference nor aversion (Gobbi et al., 2005) and SR141716 generally produces neither a conditioned place preference nor aversion (Chaperon et al., 1998; Mas-Nieto et al., 2001; Navarro et al., 2001; Li et al., 2008, but see Sañudo-Peña et al., 1997).

Our data support and expand previous findings suggesting that the ECB system functions primarily in the extinction of aversively motivated behaviors and is mediated by an increase in available anandamide. Marsicano et al. (2002) reported that impaired extinction — but not acquisition and consolidation — of fear memory by the ECB system was mediated by elevated anandamide specifically in the basolateral amygdala (BLA). Although ECB levels were not measured in the present experiments, the facilitation of extinction of the conditioned aversion produced by URB597 may also be mediated by elevated anandamide in the BLA, an area of the brain that has been implicated in the
expression of conditioned place aversion learning (e.g., Zanoveli et al., 2007; Lucas et al., 2008). The finding that pretreatment with SR141716 interfered with the extinction of the conditioned aversion suggests that the effects are CB₁-mediated; however, future experiments that evaluate the potential of a CB₁ antagonist/inverse agonist to reverse the effects of URB597 are warranted to ensure a CB₁ mechanism of action.

The results reported here complement previous reports using different behavioural procedures (Chhatwal et al., 2005; Harloe et al., 2008; Hölt et al., 2005; Marsicano et al., 2002; Niyuhire et al., 2007; Pamplona et al., 2006; Varvel and Lichtman, 2002; Varvel et al., 2007), each suggesting that activation of the ECB system selectively facilitates extinction of aversively motivated behaviours. Converging lines of evidence suggest that manipulation of the ECB system may be a promising therapeutic target to promote extinction of aversive memories, such as those experienced by people suffering from posttraumatic stress disorder.
3.1. Quantification of Primary and Secondary Psychoactive Cannabinoids in Blood

Chapter 3 is based on a manuscript prepared by Laurie Manwell in collaboration with Drs. Armen Charachoglyan, Dyanne Brewer and Paul Mallet. All experiments were conducted by Laurie Manwell with training and supervision provided by Drs. Charachoglyan and Brewer at the Mass Spectrometry Facility and under the supervision of Dr. Mallet at Wilfrid Laurier University; all data analysis was performed by Laurie Manwell and the manuscript was written by Laurie Manwell. The manuscript was reviewed by Dr. Masoud Jelokhani-Niakari in the Department of Chemistry also at Wilfrid Laurier University.

*Title*: “Quantification of Primary and Secondary Psychoactive Cannabinoids in Whole Blood and Plasma by Solid-Phase Extraction and Liquid-Chromatography/Mass Spectrometry.”
3.2. Abstract

A validated, sensitive, and specific application for the extraction, identification, and quantification of the primary psychoactive component of Cannabis sativa, Δ⁹-tetrahydrocannabinol (Δ⁹-THC), and its psychoactive metabolite, 11-hydroxy-Δ⁹-tetrahydrocannabinol (11-OH- Δ⁹-THC), in whole blood and plasma was developed using liquid-liquid (LLE) and solid-phase (SPE) extraction followed by liquid chromatographic separation and combined atmospheric pressure chemical and electrospray ionization mass spectrometry detection. Mean recoveries for Δ⁹-THC and 11-OH-Δ⁹-THC were 88% and 91% respectively and the limits of quantification (LOQ) and detection (LOD) were 5 ng/ml and 2 ng/ml respectively. This method was successfully employed in pharmacokinetic assays comparing biological levels of Δ⁹-THC and 11-OH-Δ⁹-THC after Δ⁹-THC was administered to rats either pulmonally (1, 5, and 10 mg of inhaled vapour) or parenterally (1.0, 1.5, and 2.0 mg/kg injected intraperitoneally). This method has high specificity, sensitivity, and selectivity for detecting psychoactive cannabinoids in whole blood and plasma, using a minimized protocol for extraction, detection, and quantification that produces an efficient pharmacokinetic analysis for the purposes of comparing biological levels of psychoactive cannabinoids in studies of animal behaviour.

Keywords: Δ⁹-tetrahydrocannabinol (THC); 11-hydroxy-Δ⁹-tetrahydrocannabinol (11-OH-THC); whole blood; plasma; liquid-chromatography/mass spectrometry (LC/MS); pulmonary administration; rat

Abbreviations: Limit of quantification (LOQ); limit of detection (LOD); liquid-liquid extraction (LLE); solid-phase extraction (SPE); electrospray ionization (ESI); atmospheric pressure chemical ionization (APCI); selected ion monitoring (SIM); deuterated internal standards (DIs); coefficient of variation (CV); high performance liquid-chromatography (HPLC)
3.3. Introduction

Although there are numerous analytical methods for the determination of cannabinoids in biological fluids, most involve complicated methods of extraction and purification followed by expensive methods of detection and quantification. In whole blood, serum and/or plasma, \( \Delta^9 \)-tetrahydrocannabinol (\( \Delta^9 \)-THC) — the primary psychoactive component of *Cannabis sativa* (marijuana plant) — and its metabolites, 11-hydroxy-\( \Delta^9 \)-tetrahydrocannabinol (11-OH-\( \Delta^9 \)-THC) (also psychoactive) and 11-nor-9-carboxy-\( \Delta^9 \)-tetrahydrocannabinol (COOH-\( \Delta^9 \)-THC), can be analyzed by liquid-liquid extraction (LLE) and/or solid-phase extraction (SPE) followed by gas-chromatography mass spectrometry (GC/MS) detection (refer to Table 3.1). However, GC/MS has several limitations. For example, it requires sophisticated sample preparation, there are problems associated with sample volatility and the potential for thermal degradation of samples, and there are particular extraction and separation requirements (McMaster, 2005). GC/MS requires labor-intensive derivatization of samples, which may vary depending upon whether or not analytes are thermolabile or non-volatile, and can increase analysis time, cost, and handling errors (McMaster, 2005). In addition, GC/MS often necessitates additional splitting of fractions containing non-acidic \( \Delta^9 \)-THC and 11-OH-\( \Delta^9 \)-THC from acidic COOH-\( \Delta^9 \)-THC (Weller et al. 2000; Giroud et al., 2001; Kauert et al., 2007; Steinmeyer et al., 2002; Naef et al., 2004; Thomas et al., 2007). Since \( \Delta^9 \)-THC, 11-OH-\( \Delta^9 \)-THC, and COOH-\( \Delta^9 \)-THC differ significantly in terms of acidity and polarity, they can be extracted in the same fraction, as demonstrated by Feng et al. (2000), with immunoaffinity extraction using a broad cannabinoid cross-reacting antibody (e.g.,
Marine Antibody 11A6) followed by GC/MS. This can be quite expensive, in terms of both resources and time.

Other methods of cannabinoid extraction (from blood, plasma, or serum) and separation by chromatography use LLE and/or SPE followed by some variation of liquid chromatography (LC), including thin-layer chromatography (TLC) (Agurell et al., 1970; Gill and Jones, 1972; Fehr and Kalant, 1974; Just et al., 1974) and high-performance liquid chromatography (HPLC) followed by tandem (triple-quadrupole) mass spectrometry (MS/MS) (Maralikova and Weinmann, 2004; Coulter et al., 2008; Jagerdeo et al., 2009; Sergi et al., 2009) (refer to Table 3.1). Although LC/MS/MS has greater sensitivity and specificity over TLC, triple-quadrupole mass analyzers are far more expensive than single-quadrupole MS detectors.

When used in combination with atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), LC/MS can provide a significant degree of specificity and sensitivity (Valiveti and Stinchcomb, 2004; Teixeira et al., 2007) (refer to Table 3.1). For example, both Valiveti and Stinchcomb (2004) and Teixeira et al. (2007) reported assays for Δ⁹-THC, 11-OH-Δ⁹-THC, and COOH-Δ⁹-THC with improved efficiency by combining a simple extraction method and LC/MS detection; the limits of quantification (LOQ) and detection (LOD) were, respectively, 5 ng/ml and 2 ng/ml for Δ⁹-THC, 11-OH-Δ⁹-THC, and COOH-Δ⁹-THC (Valiveti and Stinchcomb, 2004) and 0.5 ng/ml and 2 ng/ml for Δ⁹-THC and 11-OH-Δ⁹-THC but ≥ 20 ng/ml for COOH-Δ⁹-THC (Teixeria et al., 2007). However, in both studies, Δ⁹-THC was not administered directly to subjects; rather, all three cannabinoids were added directly to either rat or guinea pig plasma, not whole blood, (Valiveti and Sinchcomb, 2004) or drug-free human blood (Valiveti and
Furthermore, when the method developed by Valiveti and Stinchcomb (2004) was applied to a pharmacokinetic study of cannabinoids in animals administered an intravenous dose of Δ⁹-THC, deuterated internal standards (DIs) (e.g., Δ⁹-THC-D₃, 11-OH-Δ⁹-THC-D₃, and COOH-Δ⁹-THC-D₃), typically used to calibrate recovery efficiencies, were not employed (Valiveti and Stinchcomb, 2004). In fact, most studies putatively measuring recovered cannabinoid levels from biological fluids, typically add DIs only to plasma and/or serum fractions, and not whole blood (e.g., Feng et al., 2000; Weller et al., 2000; Giroud et al., 2001; Steinmeyer et al., 2002; Maralikova and Weinmann, 2004; Sergi et al., 2009; Valiveti et al., 2004; Kauert et al., 2007); and many are added after the sample has been flash-frozen and thawed (Giroud et al., 2001; Naef et al., 2003; Steinmeyer et al., 2002; Kauert et al., 2007) (refer to Table 3.1). If the main purpose of a study is to assess biological levels of Δ⁹-THC and its metabolites in humans and/or animals after ingestion, then DIs must be added immediately and directly to whole biological samples to assess the accuracy of extraction recovery; otherwise, amounts inevitably lost during post-collection handling — including freezing, storage, thawing, mixing, and fractionation — will be unaccounted for, thus affecting overall quantification (e.g., Johnson et al., 1984; Christophersen, 1986; Goodall and Basteyns, 1995; Moody et al., 1999; Giroud et al., 2001; Toennes and Kauert, 2001; Skopp and Pötsch, 2002; Skopp et al., 2002).

Here, we directly compared extraction methods of the analytes and DIs in whole biological and fractionated samples: in whole blood, red blood cell (RBC) fractions, and plasma fractions. In addition, we administered Δ⁹-THC to animals, both parenterally and pulmonally, to directly compare cannabinoid recovery levels; for future applications to
animal behavior studies, we were only interested in the psychoactive cannabinoids $\Delta^9$-THC and its metabolite $11\text{-OH-}\Delta^9$-THC (as described in detail in Chapter 4). Although most studies evaluating the behavioural effects of $\Delta^9$-THC in animals employ various routes of injection, cannabis is typically smoked by human users. Indeed, the vast majority of animal research on the pharmacological and behavioural effects of $\Delta^9$-THC and its metabolites employ the administration of $\Delta^9$-THC parenterally (e.g., intraperitoneal i.p. or intravenous injection i.v.), whereas the most common route of administration for human users is pulmonary (e.g., smoking or vapourizing marijuana). In addition to $\Delta^9$-THC, combustion releases many more cannabinoids and other compounds, including toxins and carcinogens from cannabis. In comparison, vapourization of pure $\Delta^9$-THC provides a more accurate assessment of the direct effects of $\Delta^9$-THC and its primary psychoactive metabolite, $11\text{-OH-}\Delta^9$-THC. Preliminary data from Mallet and colleagues suggest that $\Delta^9$-THC may produce differential behavioural effects depending upon its route of administration (Ford et al., 2009; Manwell and Mallet, 2009). However, the comparability of biological levels of $\Delta^9$-THC administered parenterally or pulmonally is unknown. The present study introduces a sensitive, reliable, and inexpensive method of analyzing levels of $\Delta^9$-THC and its primary psychoactive metabolite, $11\text{-OH-}\Delta^9$-THC, in small amounts of whole blood from rodents following $\Delta^9$-THC administration via injection or vapour inhalation.
Table 3.1

Summary of Pharmacokinetic Characterization Methods of Δ⁹-THC and Its Primary Metabolites in Whole Blood, Plasma or Serum

<table>
<thead>
<tr>
<th>Authors</th>
<th>Source</th>
<th>Extraction Method</th>
<th>LC or GC Column Type</th>
<th>Detection Method</th>
<th>Cannabinoids</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coulter et al. (2008)</td>
<td>Whole blood; human</td>
<td>SPE Cerex® Polycom THC (10-15 μm) HPLC C18 Zorbax Extend (2.1 x 50 mm x 1.8 μm)</td>
<td>LC/MS/MS Agilent 1200 LC pump; 6410 triple-quadrupole MS; ESI pos. mode</td>
<td>Δ⁹-THC</td>
<td>∆⁹-THC</td>
<td>Mean recovery (%)</td>
</tr>
<tr>
<td>Feng et al. (2000)</td>
<td>Plasma; human</td>
<td>Immuno-affinity with THC antibody (Marine Antibody 11A6; Roche) SPE Vac Elut BSTFA/TMCS derivatized; HP 5890 GC DB-5MS column (25 m x 0.2 mm i.d., 0.33 μm)</td>
<td>GC-MS HP 5890 GC HP 5970 MSD; El mode; SIM</td>
<td>∆⁹-THC</td>
<td>∆⁹-THC</td>
<td>Mean recovery (%)</td>
</tr>
<tr>
<td>Giroud et al. (2001)</td>
<td>Whole blood, serum, plasma; human (living or post-mortem)</td>
<td>SPE SPEC C18 AR discs (30 mg of sorbent)</td>
<td>TMAH/DMSO derivatized with idomethane; HP-5MS 30 m column (0.25 mm i.d. x 0.25 μm)</td>
<td>GC-MS Agilent 6890 series II; HP 5973 quadrupole MS; split mode</td>
<td>∆⁹-THC</td>
<td>∆⁹-THC</td>
</tr>
<tr>
<td>Jagerdeo et al. (2009)</td>
<td>Whole blood; human</td>
<td>⁶SPE (8 types) C2, CN, C18, C18-EC, SH, GP, C8, C8-EC</td>
<td>⁶HPLC columns Allure Biphenyl, Gemini C₁₈-Phenyl Synergi Polar RP, *Xterra MS C₁₈</td>
<td>LC/MS/MS Spark Holland Symbiosis; Applied Biosystems 4000Qtrap; ESI pos. mode</td>
<td>∆⁹-THC</td>
<td>∆⁹-THC</td>
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<td>Kauert et al. (2007)</td>
<td>Serum; human</td>
<td>SPE Strata C18</td>
<td>TBAH/DMSO idomethane derivatized; factorFour VF-5 MS capillary column (30 m x 0.25 mm i.d., 0.25 μm)</td>
<td>Ion-trap GC-MS/MS Finnigan PolarisQ GC-MSn; Trace GC</td>
<td>∆⁹-THC</td>
<td>∆⁹-THC</td>
</tr>
</tbody>
</table>

Mean recovery (%), LOQ (ng/ml), LOD (ng/ml), %CV
### Table 3.1 Continued

Summary of Pharmacokinetic Characterization Methods of Δ⁹-THC and Its Primary Metabolites in Whole Blood, Plasma or Serum

<table>
<thead>
<tr>
<th>Authors</th>
<th>Source</th>
<th>Extraction Method</th>
<th>LC or GC Column Type</th>
<th>Detection Method</th>
<th>Cannabinoids</th>
<th>Results</th>
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<tr>
<td>Maralikova and Weinmann</td>
<td>Plasma; human</td>
<td>SPE Zymark RapidTrace</td>
<td>HPLC Luna 3u PhenylHexyl (50 x 2 mm i.d., 3 μm)</td>
<td>LC/MS/MS</td>
<td>Δ⁹-THC</td>
<td>Mean recovery (%)</td>
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<td>API 3000 triple-quadrupole MS with Turbo IonSpray interface and LINAC collision cell; Series 200 LC Plus; ESI pos. mode</td>
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<td>11-OH-Δ⁹-THC</td>
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<td>COOH-Δ⁹-THC</td>
<td>95 – 104</td>
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<td>Naef et al. (2003)</td>
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<td>DB-5MS column</td>
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<td>(25 m x 0.2 mm i.d., 0.33 μm)</td>
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<tr>
<td>Sergi et al. (2009)</td>
<td>Plasma; human</td>
<td>Simple protein precipitation; poly-(tetra-fluoroethylene) filter 0.45 μm from Alltech</td>
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<td>2695 Alliance System; Waters ZQ 2000 MS; ESI; SIM pos. and neg. modes</td>
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<td>Sergi et al. (2009)</td>
<td>Plasma; human</td>
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<td>Mean recovery (%)</td>
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<td>Mean recovery (%)</td>
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<td>LC or GC Column Type</td>
<td>Detection Method</td>
<td>Cannabinoids</td>
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<td>Mean recovery (%)</td>
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<td>Thomas et al. (2007)</td>
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<td>Plasma; rat, guinea pig</td>
<td>LLE acetonitrile/ethyl acetate (1:1, v/v)</td>
<td>HPLC C18 column Waters Symmetry (2.1 x 150 mm, 5 μm)</td>
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<td>SPE Baker C18 Column</td>
<td>MSTFA derivatized; DB-1 column (30 m x 0.25 mm i.d., 0.25 μm)</td>
<td>Ion-trap GC-MS/MS Finnigan GCQ-GC</td>
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<td>74 – 82</td>
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<td>COOH-∆(^9)-THC</td>
<td>90 – 96</td>
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</table>

\(^a\) LOD: limit of detection  \(^b\) LOQ: limit of quantification  \(^c\) %CV: coefficient of variation

SPE types: C2 (silica-based ethyl phase), CN (cyanopropyl phase), C18 high density (end-capped silica-based with high number of octadecyl chains), C18-EC (end-capped silica-based octadecyl phase), SH (strong hydrophobic modified polystyrene divinylbenzene phase), GP (general phase resin polydivinylbenzene phase), C8 (silica-based octyl phase), C8-EC (end-capped silica-based octyl phase) (all purchased from Spark Holland, Inc.)

HPLC columns: Allure Biphenyl (50 mm x 3.2 mm, 5 μm) from Restek Inc.; Gemini C\(_6\)-Phenyl (50 mm x 3 mm, 3 μm) and Synergi Polar RP (75 mm x 3 mm, 4 μm) from Phenomenex Inc.; Xterra MS C\(_18\) (a) (50 mm x 3 mm, 5 μm), Xterra MS C\(_18\) (b) (20 mm x 3 mm, 5 μm), and Xterra MS C\(_18\) (c) (100 mm x 3 mm, 3.5 μm) from Waters Corporation.

* The Xterra MS C\(_18\) (50 mm x 3 mm, 5 μm) was used for all remained of the study because it provided the optimal separation.
† Naef et al. (2004) methods were previously validated in Feng et al. (2000) and Moeller et al. (1992).

Other abbreviations: BSFTA, N\(_2\),O-bis(trimethylsilyl) trifluoroacetamide; DMSO, dimethylsulfoxide; EI, electron ionization; ESI, electrospray ionization; HFIP, hexafluoro-2-propanol; MRM, multiple reaction monitoring; MSTFA, N-methyl-N-trifluoroacetamide; NICI, negative-ion chemical ionization; PIS, product ion scan; SIM, selected ion monitoring (SIM) mode in positive (pos.) or negative (neg.) mode; TBAH, tetrabutylammoniumhydroxide; TFAA, trifluoroacetic acid; tetramethylammonium hydroxide pentahydrate; TMCS, trimethylsilyl chloride.
3.4. Experimental

3.4.1. Materials, standards, and chemicals

$\Delta^9$-THC (Dronabinol, >98% purity) was obtained from THC Pharm GmbH (Frankfurt, Germany). Internal standards for $\Delta^9$-THC (1.0 mg/ml), 11-OH-$\Delta^9$-THC (100 µg/ml), $\Delta^9$-THC-D3 (100 µg/ml), and 11-OH-$\Delta^9$-THC-D3 (100 µg/ml) were obtained from Cerilliant (Round Rock, Texas, USA). Each standard was diluted in methanol to working concentrations (0.5 to 600 ng/ml) and stored at 4°C.

All solvents were analytical or HPLC grade; water, acetonitrile, methanol, ethyl acetate, and hexane were obtained from VWR and Fisher Scientific.

All blood samples were collected in ethylenediaminetetraacetic acid (EDTA) treated glass test tubes (BD Vacutainer tubes). Solid phase extraction (SPE) silica-bonded (Si) C18 (BondElut) flash columns were obtained from Varian Canada Inc. (Mississauga, Ontario, Canada).

For experiments involving i.p. drug administration, $\Delta^9$-THC was first dissolved in a small volume of ethanol and then mixed with TWEEN-80 (polyoxyethylene sorbitan monooleate; ICN Biomedicals). The ethanol was evaporated under a stream of nitrogen gas, and the dispersion was then mixed with physiological saline. The final vehicle contained 15 µl TWEEN-80 per 2 ml saline. $\Delta^9$-THC was prepared in concentrations of 0.5, 1.0, 1.5, and 2.0 mg/ml and injected in doses of 1 ml/kg.

For experiments involving pulmonal (inhaled) drug administration, $\Delta^9$-THC was prepared in ethanol at concentrations of 4, 20, and 40 mg/ml, and 250 µl of each was applied to small steel wool pads (liquid pads, Storz & Bickel, Tuttingen, Germany),
yielding final amounts of 1, 5, or 10 mg/pad. The ethanol was then allowed to evaporate completely before vapourization.

3.4.2. Animals

Male Sprague-Dawley rats (Charles River, Canada), weighing 300–500 g, were used in all experiments. Rats were pair-housed in standard plastic shoebox cages (45 x 25 x 20 cm$^3$) maintained at 21-22°C in a colony room on a 12-h reversed light-dark cycle (lights off at 7 AM) and fed standard rat chow (Harlan 8640) and water ad libitum. Testing was conducted during the dark cycle. Experimental procedures followed Canadian Council on Animal Care guidelines and were approved by the Wilfrid Laurier University Animal Care Committee. Rats were acclimatized to the colony and handling procedures prior to experimentation.

3.4.3. Apparatus

A Volcano® vapourizing device (Storz and Bickel, GmbH and Co., Tuttlingen, Germany) was used as described by Hazekamp et al. (2006). Briefly, $\Delta^9$-THC (1, 5, or 10 mg) was vapourized (at heat setting 9, approximately 226°C) and collected into detachable plastic balloons (approximately 8 L), which were then fitted with a release valve and the vapour immediately expelled (over 10 sec) into enclosed plastic boxes (32 x 16 x 12 cm$^3$) containing two rats that inhaled the vapour for 5 min (refer to Figure 3.1). After 5 min, the lids were opened for ventilation. For determination of blood cannabinoid levels, blood was collected and rats sacrificed by cardiac puncture (while under anesthesia) at regular intervals. This device has been previously reported by Hazekamp et al. (2006) to deliver $>50\%$ of the loaded $\Delta^9$-THC into the balloon in a reproducible manner with a pulmonary uptake comparable to smoking of cannabis.
Figure 3.1. The Volcano ® vaporizer device and inhalation boxes. A. In a ventilated fume hood, rats were placed in enclosed boxes while Δ⁹-THC (1, 5, or 10 mg/pad) was vapourized and collected into detachable plastic balloons. B. Filled balloons were then fitted with a release valve and the vapour immediately expelled into enclosed plastic boxes holding two rats that inhaled the vapour for 5 min. C. After 5 min, the lids were opened for ventilation.
3.4.4. Extraction procedure

Calibration standards were prepared fresh in methanol daily and stored on ice. All blood samples were obtained by cardiac puncture performed on rats anesthetized with isoﬂurane gas; immediately upon collection, 1 ml of blood was added to iced test tubes, already containing standards in methanol, then gently mixed and allowed to stand for approximately 1 h on ice (all steps were conducted on ice unless otherwise indicated). Control and calibration samples were prepared by spiking 1 ml drug-free rat blood with standard solutions: concentrations of Δ⁹-THC and 11-OH-Δ⁹-THC standards ranged from 0.5 to 600 ng/ml, whereas DIs were consistently added in amounts of 45, 75 or 150 ng/ml depending upon the range of standard concentrations (e.g., low, medium, or high range).

After equilibration, blood samples were acidified with 200 μl of 10% acetic acid and gently vortex mixed for 15 s. Proteins were precipitated by adding 2 ml acetonitrile, gently vortex mixing for 30 s, equilibrating 3 min, and centrifugation for 20 min at 10,000 × g at 4°C. All supernatant fractions were removed using glass pipettes and cannabinoids extracted by LLE. At approximately a 2:1 (v/v) amount, 4 ml of 0.1 M acetate buffer (pH 4.5) was added to the supernatant fraction, vortex mixed for 15 s, and followed by the addition of 3 ml of hexane/ethyl acetate (95:5, v/v), vortex mixed for 30 s, and centrifuged for 20 min at 10,000 × g at 4°C. Supernatant fractions were removed, placed into small glass test tubes, and then evaporated at room temperature under nitrogen. Residues were reconstituted in 3 ml hexane and cannabinoids were selectively separated by SPE. Bond Elut columns were preconditioned with three 3 ml volumes of hexane and samples were applied to columns using glass pipettes and then washed with another three 3 ml volumes of hexane. Δ⁹-THC was eluted from columns with three 3 ml
volumes of hexane/ethyl acetate (95:5, v/v) and 11-OH-Δ9-THC was eluted with three 3 ml volumes of hexane/ethyl acetate (75:25, v/v); an intervening fraction of three 3 ml volumes of hexane/ethyl acetate (90:10, v/v) was used to remove contaminants. All fractions were kept separate for the remaining steps, including LC/MS analysis. Eluted samples were evaporated at room temperature under nitrogen and reconstituted in 200 μl methanol.

For the comparison of analyte recovery from whole blood or plasma, whole blood samples were treated as described above, but an additional set of samples of whole blood were also used; this second set was first centrifuged, and the RBC and plasma fractions separated, and then each set treated as the whole blood samples described above.

3.4.5. Liquid chromatography

Chromatography was performed on either a Waters YMC Pro C18 (2.0 × 150 mm, 3 μm) column or a Waters YMC ODS-AQ (2.0 x 150 mm, 3 μm) column using Agilent 1100 system's binary pumps equipped with an autosampler. The gradient started at 70/30 v/v methanol/water mobile flow rate of 0.5 ml/min and a nitrogen flow rate of 5.00 ml/min. After 1 minute it linearly changed to 100% methanol which took 6 min and continued for another 6 min. At min 14 of the run, the solvent ratio linearly returned to the initial conditions over the course of 5 min and equilibrated for another 5 min. The eluent was additionally monitored with a single wavelength detector setup at 205 nm.

3.4.6. Mass spectrometry

The LC/MS system consisted of an Agilent 1100 Series –LC-MSD with a single quadrupole mass analyzer. An Agilent multimode ionization source was used to provide the combined APCI and ESI. The drying gas nitrogen flow was set at 5 L/min at 300°C.
and the nebulizer gas pressure at 40 psi. Capillary voltage was set at 2000V, with the corona current at 1 μA, in order to keep optimal conditions for ESI and APCI. The mass spec was set in negative ion mode to scan from 200 to 400 m/z. A post-run extracted ion chromatogram was reconstructed at m/z 313 [Δ⁹-THC-H]⁻, m/z 329 [11-OH-Δ⁹-THC-H]⁻, m/z 316 [Δ⁹-THC-D₃-H]⁻, m/z 332 [11-OH-Δ⁹-THC-D₃-H]⁻.

3.3.7. Validation

For determination of intra- and inter-day accuracy and precision of the assays, calibration standards were added to 1 ml of whole blood using two ranges of Δ⁹-THC and 11-OH-Δ⁹-THC (4.5, 9, 18, 37.5, 75, 150, 300, 600 ng/ml; and 0.5, 1, 2, 5, 10, 2, 50, 100, 200 ng/ml) and consistent amounts of Δ⁹-THC-D₃ and 11-OH-Δ⁹-THC-D₃ (45, 75, or 150 ng/ml) depending upon the range. Accuracy was expressed as the mean % [(mean measured concentration)/(expected concentration) × 100]. Precision was calculated as intra- and inter-day coefficient of variation [% CV = (S.D./mean) × 100]. The LOQ was determined as defined by Valiveti and Stinchcomb (2004) and was the concentration of Δ⁹-THC and 11-OH-Δ⁹-THC that could be obtained with acceptable precision (%CV < 10).

Matrix effects (co-elution of compounds that could affect analyte ionization) were determined by extracting “blank” whole blood and reconstituting with methanol containing a known amount of the analytes. Reconstituted extracts were analyzed and their peak areas compared to those of the analytes added directly to pure methanol. Initial analyte recoveries were performed in triplicate, for every experimental run, in whole blood spiked with Δ⁹-THC and 11-OH-Δ⁹-THC and DIs then compared with analyte peak areas of Δ⁹-THC and 11-OH-Δ⁹-THC and DIs added directly to methanol.
3.5. Results and Discussion

The mass spectra of Δ⁹-THC (m/z 313.2) and Δ⁹-THC-D3 (m/z 316.0) and the mass spectra of 11-OH-Δ⁹-THC (m/z 329.2) and 11-OH-Δ⁹-THC-D3 (m/z 332.4) were identified and a typical extracted or total ion chromatogram with average retention times is shown in Figure 3.2. The total run time for each sample was approximately 20 min. No interfering peaks were observed in conjunction with the drug peaks.

For analytes spiked directly into pure methanol only, retention times were very similar on both columns: on the Waters YMC ODS-AQ column, retention times for Δ⁹-THC, Δ⁹-THC-D3, 11-OH-Δ⁹-THC, 11-OH-Δ⁹-THC-D3 were 12.580, 12.556, 10.887, and 10.874 min, respectively, and on the Waters Pro YMC column, retention times for Δ⁹-THC, Δ⁹-THC-D3, 11-OH-Δ⁹-THC, 11-OH-Δ⁹-THC-D3 were 12.231, 12.249, 10.686, and 10.573 min, respectively. Similarly, for analytes spiked directly into whole blood, retention times were similar for both columns: retention times for Δ⁹-THC, Δ⁹-THC-D3, 11-OH-Δ⁹-THC, 11-OH-Δ⁹-THC-D3 on the Waters YMC ODS-AQ column were 12.580, 12.556, 10.887, and 10.874 min, respectively, and on the Waters Pro YMC C18 column were 12.355, 12.279, 10.598, and 10.571 min, respectively. Both columns have similar octadecylsilane stationary phases and behave similarly in terms of peak resolution and column bleeding.
Figure 3.2. Typical LC/MS extracted or total ion chromatograms for 600 ng/ml $\Delta^9$-THC ($m/z$ 313.1, RT = 12.653 min) and 11-OH-$\Delta^9$-THC ($m/z$ 329.1, RT = 10.867 min) and 150 ng/ml $\Delta^9$-THC-D3 ($m/z$ 316.1, RT = 12.621 min) and 11-OH-$\Delta^9$-THC-D3 ($m/z$ 332.1, RT = 10.839 min) spiked in whole blood run on the Waters ODS-AQ column.
Calibration graphs were constructed using a linear regression of the peak-areas of a) $\Delta^9$-THC product ions divided by $\Delta^9$-THC-D3 ions and b) 11-OH-$\Delta^9$-THC product ions divided by 11-OH-$\Delta^9$-THC-D3 ions. Standard calibration curves for both $\Delta^9$-THC (Figure 3.3a) and 11-OH-$\Delta^9$-THC (Figure 3.3b) in whole blood were linear over ranges of 5 to 600 ng/ml (means of n=3 each). Mean absolute recoveries from whole blood of $\Delta^9$-THC and 11-OH-$\Delta^9$-THC were 108.96% (%CV=5.94, n=6) and 100.85% (%CV=7.77, n=6), respectively; for both cannabinoids, the limits of quantification (LOQ) and detection (LOD) were 5 ng/ml and 2 ng/ml, respectively.
Figure 3.3. Calibration curves for (a) $\Delta^9$-THC and (b) 11-OH-$\Delta^9$-THC recovered from whole blood.
Comparing $\Delta^9$-THC and 11-OH-$\Delta^9$-THC recoveries from whole blood versus RBC and plasma fractions, in the concentration range of 2 to 200 ng/ml all standard calibration curves were linear (Figures 3.4 and 3.5). However, the mean ratio of $\Delta^9$-THC recovered from whole blood was 1.17-fold and 2.25-fold greater than $\Delta^9$-THC recovered from RBC and plasma fractions, respectively, whereas the mean ratio of 11-OH-$\Delta^9$-THC recovered from whole blood was 2.40-fold and 2.22-fold greater than 11-OH-$\Delta^9$-THC recovered from RBC and plasma fractions.

No significant matrix effects were observed for the analytes in whole blood; extracts from blank whole blood samples showed no interfering peaks during method development (total n > 100) or in batch-to-batch stability analyses (minimum n=3 for each run). The mean CV of peak areas of reconstituted samples was < 10 % for both $\Delta^9$-THC and 11-OH-$\Delta^9$-THC indicating that the analytes’ ionization was not affected by co-eluting compounds. Results of the intra-day and inter-day validation assays for $\Delta^9$-THC and 11-OH-$\Delta^9$-THC are shown in Tables 2 and 3, respectively. No significant degradation of samples occurred for samples left in the autosampler at room temperature for at least 24 hours.
Figure 3.4. Calibration curves for $\Delta^9$-THC recovered from whole blood, RBC fraction, and plasma fraction.
Figure 3.5. Calibration curves for 11-OH-Δ⁹-THC recovered from whole blood, RBC fraction, and plasma fraction.
Table 3.2

Intra-day and Inter-day Quality Control Results for $\Delta^9$-THC

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<th>Intra-day variation</th>
<th>Inter-day variation</th>
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%CV: coefficient of variation

Table 3.3

Intra-day and Inter-day Quality Control Results for 11-OH-$\Delta^9$-THC

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</table>

%CV: coefficient of variation
The method development had two main goals: 1) to develop a sensitive and selective method of simultaneous detection and quantification using a relatively less expensive single quadrupole mass spectrometer (in comparison to a triple quadrupole mass analyzer), and 2) to maximize the extraction of Δ⁹-THC and its psychoactive metabolite 11-OH-Δ⁹-THC from whole blood and plasma. First, compounds of interest that have only one hydroxyl group difference, such as Δ⁹-THC and 11-OH-Δ⁹-THC, create challenges for a universal ionization method of selection for a mixture of both compounds. Unless a sensitive and selective triple quadrupole instrument is used, reliable quantification of both compounds using only APCI or ESI methods is not achievable at low levels (below 250 ng/ml). According to the literature, APCI and ESI ionizations are the most commonly used methods for LC however, cannabinoids are not generally ionized as efficiently as most other drugs by ESI or APCI alone (McMaster, 2005; Foltz, 2007; Nordström et al. 2008). A method's sensitivity depends on ionization efficiency; APCI is believed to perform better for hydrophobic compounds, although it is a harsher ionization method resulting in source fragmentation of analyte ions (McMaster, 2005; Foltz, 2007; Nordström et al. 2008). Thus, the challenge was to empirically determine the optimal ionization parameters for maximal sensitivity for both of compounds; hence, the optimal solvent for ESI and APCI ionization needed to be empirically determined. Anticipating the matrix effects of whole blood/plasma that would interfere with analyte ionization, acetonitrile and methanol were compared first in terms of their mobile phase impact on ionization efficiency. As the mobile phase, acetonitrile was superior to methanol for chromatographic peak resolution; however, methanol actually provided 15-200 times greater ionization efficiency for both Δ⁹-THC and 11-OH-Δ⁹-THC, compared to acetonitrile, in both APCI and ESI mode (data not shown). The analytes were separated using isocratic elution with 20-50% of aqueous phase. The gradient
elution method was developed using Δ⁹-THC and 11-OH-Δ⁹-THC and their deuterated standards first spiked into methanol and then later spiked in rat whole blood and serum. Multimode ionization, although not widely used, provided simultaneous and sensitive detection of both compounds; thus, parallel detection of complex mixtures of compounds is suggested based on this study and other recent reports (Nordstöm et al., 2008).

Second, several extraction protocols were assessed, including comparison of various LLE and SPE methods; the most successful method combined a simple LLE with hexane/ethyl acetate (95:5, v/v) followed by SPE on silica columns, with the same solvent but in varied concentrations for Δ⁹-THC (95:5, v/v) and 11-OH-Δ⁹-THC (75:25, v/v). Silica is a highly polar sorbent and acidic when unbound; when the analytes, in a non-polar solvent (pure hexane) were applied to SPE columns, they were easily separated by gradually increasing the concentration of ethyl acetate which increased solvent polarity allowing for ion-exchange and elution of Δ⁹-THC and 11-OH-Δ⁹-THC sequentially from the columns. In fact, of the several different types of SPE columns and extraction solvents tested, this was the simplest, and least expensive, method providing the highest and most reliable extraction efficiencies. Strata X-C (Phenomenex), DPX (Gerstel), Bond Elut Certify II (Varian) and SAX (Whatman) SPE columns were also tested with 5 different fractions of hexane/ethyl acetate and/or using the manufacturer’s recommendations; all tested protocols were only partially satisfying, showing positive results for only one of the compounds (data not shown).

This method was also tested in several pharmacokinetic studies of Δ⁹-THC in rats, specifically comparing recovery efficiencies after pulmonary and parenteral administration, in both dose-dependent and time-dependent analyses. It should be noted that, although most studies report data in units of ng/ml for biological fluids, it is more accurate to report in terms of
ng/ml/kg since weight can significantly affect metabolic rate, especially in rodents wherein doses required to produce similar behaviours can vary between species on an order of magnitude of ten times or more. Amongst rodents, mice typically require higher dosages than rats because they have a higher rate of drug metabolism in general. For example, a dosage of at least 1 mg/kg Δ⁹-THC was necessary to produce CPP in mice (Ghozland et al., 2002), whereas a dosage of 0.075 mg/kg Δ⁹-THC was sufficient to produce CPP in rats (Braida et al., 2004); however, doses of 5 mg/kg Δ⁹-THC or more usually produce CPA in both mice and rats (Ghozland et al., 2002; Braida et al., 2004; reviewed in Murray and Braida 2010; refer to Table 1.2a/b). Furthermore, the IPI used in CPP/CPA studies with mice is typically immediate, whereas the IPI used with rats is often between 10 and 30 minutes, thus reflecting the need to account for different rates of drug metabolism (reviewed in Murray and Braida 2010; refer to Table 1.2a/b). It is particularly relevant here to account for weight since different routes of administration are being compared that can have potentially different effects on the rates of metabolism (i.e., metabolism after subcutaneous injection directly into fat tissue versus inhalation directly into lung tissue). There is a much greater accumulation and storage of cannabinoids in body fat and less vascularized tissues over time (e.g., concentration ratios of 10⁴:1 for fat:other tissues), including unmetabolized Δ⁹-THC and its metabolites (e.g., fatty acid conjugates of 11-OH-Δ⁹-THC) (reviewed in Grotenherman, 2003).

Representative blood concentrations are shown in Table 3.4 and Figures 3.6 and 3.7; whole blood collected at 5 min time intervals from rats receiving Δ⁹-THC either pulmonary (1, 5, and 10 mg inhaled vapour) or parentally (1, 1.5, and 2.0 mg/kg injected i.p.) showed both dose-dependent and time-dependent concentration changes in Δ⁹-THC and its metabolite 11-OH-Δ⁹-THC. For pulmonary-administered Δ⁹-THC, maximal blood concentrations of Δ⁹-THC peaked at
20 min at 81.45 ng/ml/kg, whereas 11-OH-Δ⁹-THC peaked at 30 min (592.03 ng/ml/kg). However, since time-points were initiated at 20 min, it is likely that 81.45 ng/ml/kg does not represent a true peak for Δ⁹-THC; it is more probable that it is a point on the descending portion of the concentration curve. In comparison, for parenterally-administered Δ⁹-THC, maximal blood concentrations of both Δ⁹-THC and 11-OH-Δ⁹-THC peaked at 20 min at 551.81 and 360.2 ng/ml/kg respectively.

This method proved successful in its relative simplicity and sensitivity for the extraction of Δ⁹-THC and its derivates from biological fluids after pulmonal and parenteral drug administration; due to differences in the chemical nature of Δ⁹-THC and 11-OH-Δ⁹-THC, a simplified LC-MS protocol for detection and quantification, with the maximal yield of both analytes from whole blood after cannabinoid intake/exposure, including the proper use of internal standards, has not been previously reported for humans or animals.
Figure 3.6. Dose- and time-dependent concentrations of Δ⁹-THC (top) and 11-OH-Δ⁹-THC (bottom) recovered from whole blood in rats exposed to vapourized Δ⁹-THC (1, 5, or 10 mg) at 5 min intervals.
Figure 3.7. Dose- and time-dependent concentrations of Δ⁹-THC (top) and 11-OH-Δ⁹-THC (bottom) recovered from whole blood in rats exposed to injected (i.p.) Δ⁹-THC (1.0, 1.5, or 2.0 mg/kg) at 5 min intervals.
Table 3.4

Comparison of Δ⁹-THC and 11-OH-Δ⁹-THC Concentrations in Whole Blood after Δ⁹-THC Administration

<table>
<thead>
<tr>
<th>Pulmonary administration</th>
<th>Parenteral administration</th>
</tr>
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<tbody>
<tr>
<td>(1, 5, and 10 mg vapourized)</td>
<td>(1.0, 1.5, and 2.0 mg/kg injected i.p.)</td>
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<table>
<thead>
<tr>
<th>Dose (mg)</th>
<th>Time (min)</th>
<th>Recovered Concentration (ng/ml/kg)</th>
<th>Recovered Concentration (ng/ml/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Δ⁹-THC</td>
<td>11-OH-Δ⁹-THC</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>13.55</td>
<td>108.88</td>
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<tr>
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<td>13.82</td>
<td>112.77</td>
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<td>17.68</td>
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<td>12.73</td>
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</table>
3.6. Conclusions

The method development evolved in four directions: (i) separation and sensitive detection of cannabinoids, (ii) maximal extraction of cannabinoids from whole blood, (iii) comparison of extraction efficiencies between whole blood and RBC and plasma fractions, and (iv) comparison of recovery efficiencies after pulmonary and parenteral cannabinoid administration. First, a sensitive and selective detection method for cannabinoids was developed, with a single quadrupole mass spectrometer; this method achieved high sensitivity for Δ⁹-THC and 11-OH-Δ⁹-THC using multimode ionization on an Agilent 1100 system equipped with multimode ionization source. Second, the simultaneous extraction and detection of Δ⁹-THC and its metabolites is complicated primarily because of differences in their hydrophobic and hydrophilic natures; Δ⁹-THC is a very hydrophobic compound but its primary metabolite, 11-OH-Δ⁹-THC, is more polar. A method that allows for maximal recovery from whole blood was developed which is relatively uncomplicated and inexpensive using a simple LLE and SPE followed by liquid chromatographic separation in methanol. Third, in order to adequately compare extraction efficiencies between whole blood and RBC and plasma fractions, the addition of DI's directly to whole blood was a primary precondition; mean absolute recovery of cannabinoids was more than two-fold greater in whole blood than when the plasma fraction was separated. This finding is interesting because it is contrary to earlier reports indicating that extraction efficiencies from whole blood are up to 50% lower than plasma putatively due to greater plasma protein binding (> 90%) and poor distribution into RBCs (Schwilke et al., 2009; Giroud et al., 2001; Klausner et al., 1975; Widman et al., 1973, 1974). However, there are only a few published reports that directly compared extraction
ratios for whole blood to plasma (e.g., Schwilke et al., 2009; Skopp et al., 2002; Giroud et al., 2001); none of those experiments incorporated an acidification step prior to protein precipitation, which can improve recovery of cannabinoids from whole blood and other biological samples, possibly by dissociating non-covalently bound $\Delta^9$-THC from blood proteins (Kintz and Cirimele, 1997; Chiarotti and Costamagna, 2000; del Mar Ramirez Fernandez et al., 2008). In addition, extractions from whole blood were immediately (i.e., no intervening storage period where samples could significantly degrade) and directly compared to those from plasma and RBC fractions; again, steps that were not performed in the previous reports. Fourth, recovery efficiencies of cannabinoids after pulmonary and parenteral administration were assessed; both dose- and time-dependent analyses were achieved for inhaled and injected $\Delta^9$-THC and its metabolite 11-OH-$\Delta^9$-THC in whole blood.

The procedure presented here employed a reliable method of analyzing levels of $\Delta^9$-THC and its primary psychoactive metabolite, 11-OH-$\Delta^9$-THC, in small amounts of whole blood from rodents following $\Delta^9$-THC administration via injection or vapour inhalation. This method has high specificity, sensitivity, and selectivity for detecting psychoactive cannabinoids in whole blood and plasma, using a minimized protocol for extraction, detection, and quantification that produces an efficient pharmacokinetic analysis for the purposes of comparing biological levels of psychoactive cannabinoids in studies of animal behaviour.
CHAPTER 4:

4.1. Comparative Effects of Pulmonary and Parenteral Δ⁹-Tetrahydrocannabinol Exposure on Extinction of Opiate-Induced Conditioned Aversion

Chapter 4 is based on research presented at the Society for Neuroscience (Manwell and Mallet, 2009; Ford et al., 2009) and a manuscript prepared by Laurie Manwell in collaboration with Dr. Paul Mallet. All experiments presented here were conducted, analyzed and reported on by Laurie Manwell with training and supervision provided by Drs. Charachoglyan and Brewer at the Mass Spectrometry Facility and under the supervision of Dr. Mallet at Wilfrid Laurier University.

Title: “Comparative Effects of Pulmonary and Parenteral Δ⁹-Tetrahydrocannabinol Exposure on Extinction of Opiate-Induced Conditioned Aversion in Rats”
4.2. Abstract

Evidence suggesting the endogenous cannabinoid (ECB) system can be manipulated to facilitate or impair the extinction of learned behaviours — specifically regarding drug-induced aversive memories — has important consequences for research on opiate withdrawal and abstinence. Manwell et al. (2009) demonstrated that URB597, which blocks deactivation of endogenous CB₁ ligands, significantly facilitated extinction of a naloxone-precipitated morphine withdrawal-induced conditioned floor aversion, whereas the CB₁ antagonist/inverse agonist SR141716 significantly impaired extinction. Here, we evaluated the potential of the exogenous CB₁ ligand, Δ⁹-tetrahydrocannabinol (Δ⁹-THC), to also facilitate extinction of the conditioned aversion. Since recent evidence suggests Δ⁹-THC may produce differential effects on behaviour and memory depending upon the route of administration (e.g., Naef et al., 2003, 2004; Niyuhire et al., 2007; Ford et al., 2009; Manwell and Mallet, 2009), and most studies evaluating the effects of Δ⁹-THC on memory in animal models administer Δ⁹-THC parenterally (e.g., intraperitoneal or intravenous injection) whereas the most common route of administration for human users is pulmonary (e.g., smoking or vapourizing marijuana), we specifically evaluated the effects of both pulmonary and parenteral Δ⁹-THC exposure on rates of extinction learning. Rats trained to associate a naloxone-precipitated morphine withdrawal with a floor cue were administered Δ⁹-THC — pulmonary (1, 5, 10 mg vapour inhalation) or parenteral (0.5, 1.0, 1.5 mg/kg intraperitoneal injection) — prior to each of 20 to 28 extinction/testing trials. Inhaled Δ⁹-THC facilitated extinction of the conditioned aversion in a dose- and time-dependent manner: 5 and 10 mg facilitated extinction compared to vehicle and 1 mg Δ⁹-THC. In addition, the CPA was maintained up to 1 wk after extinction testing for only the rats previously administered 1 mg
inhaled Δ⁹-THC. Injected Δ⁹-THC significantly impaired extinction only for the 1.0 mg/kg dose: it prolonged the CPA 4-fold longer than the vehicle, 0.5 and 1.5 mg/kg doses. These data suggest that the dose and route of Δ⁹-THC administration have important consequences for its pharmacokinetic and behavioural effects, specifically, that pulmonary exposure at higher doses facilitates, whereas pulmonary and parenteral exposure at lower doses impairs, rates of extinction learning for conditioned cue aversion. Pulmonary-administered Δ⁹-THC may prove beneficial for pharmacological potentiation of extinction learning for aversive memories, such as those supporting drug-craving/seeking in opiate withdrawal-syndrome or post-traumatic stress disorders.

4.3. Introduction

Accumulating evidence points to the critical role of the endogenous cannabinoid (ECB) system in the extinction of aversively motivated behaviours and its putatively nonessential role in the extinction of reward-based and appetitively-motivated behaviours (Marsicano et al., 2002; Varvel and Lichtman, 2002; Chhatwal et al., 2005; Hölter et al., 2005; Pamplona et al., 2006; Lutz, 2007; Niyuhire et al., 2007; Varvel et al., 2007; Harloe et al., 2008; Manwell et al., 2009). Marsicano et al. (2002) demonstrated a central role for the CB₁ receptor and two of its endogenous ligands, arachidonoyl ethanolamide (AEA) and 2-arachidonoylglycerol (2-AG), in the extinction of auditory-fear conditioned behaviours in mice. Experiments showed that both CB₁-deficient mice, and wild-type mice administered the CB₁ antagonist/inverse agonist, SR141716, displayed impairments in both short- and long-term extinction of fear-conditioned freezing to a tone, while suffering no deficits in the acquisition and consolidation of the classically conditioned behaviour (Marsicano et al., 2002). These effects were related to elevated
levels of AEA and 2-AG in the basolateral amygdala, putatively acting to selectively inhibit GABA (γ-aminobutyric acid)-mediated inhibitory neural synapses (Marsicano et al., 2002). Varvel, Lichtman and colleagues also found deficits in extinction learning in the aversively motivated Morris water maze tasks. First, compared to untreated wild-type (CB⁺/⁺) mice, CB₁-deficient (CB⁻/⁻) and SR141716-treated mice exhibited normal acquisition rates in learning the location of a fixed platform, but showed significant impairments in learning the reversal task, suggesting that failure to extinguish memory of the first location was interfering with learning a secondary location (Varvel and Lichtman, 2002; Varvel et al., 2005). In addition to direct CB₁ manipulation, the fatty acid amide hydrolase (FAAH), which degrades ECB ligands such as AEA and 2-AG, can be manipulated by genetic deletion or pharmacological inhibition (e.g., with OL-135 or URB597) to increase natural levels of ECBs. Compared to untreated wild-type (FAAH⁺/⁺) mice, both FAAH-deficient (FAAH⁻/⁻) and OL-135 (FAAH inhibitor)-treated mice showed enhanced extinction rates and increased rates of acquisition; in addition, the effects of OL-135 were blocked by co-administration of SR141716 (Varvel et al., 2007). Interestingly, the exogenous CB₁ ligand, Δ⁹-tetrahydrocannabinol (Δ⁹-THC), did not affect extinction rates when administered at doses of 0.1, 0.3, 1 or 10 mg/kg intraperitoneally (i.p.) 30 min prior testing (Varvel et al., 2007).

However, recent evidence suggests Δ⁹-THC may produce differential effects on behaviour and memory depending upon the route of administration (e.g., Naef et al., 2003, 2004; Niyuhire et al., 2007; Ford et al., 2009; Manwell and Mallet, 2009). For example, Niyuhire et al. (2007) reported that, while injected Δ⁹-THC (1, 3, 10 mg/kg) dose-dependently disrupted both acquisition and recall of the platform location in the Morris water maze task, inhaled smoke from
marijuana (50, 100, and 200 mg) only impaired performance at the highest dose (estimated to have 4.2 mg Δ⁹-THC before burning). Although Niyuhire et al. (2007) suggested that previous pharmacokinetic studies (e.g., Wilson et al., 2006) indicated that brain levels of Δ⁹-THC from 1 mg/kg intravenous exposure may be roughly equivalent to smoke from 200 mg marijuana, they did not directly quantify bioavailable levels of Δ⁹-THC. Our own investigations suggest that because there are different concentrations of Δ⁹-THC and its primary psychoactive metabolite 11-OH-Δ⁹-THC in whole blood after pulmonary or parenteral administration of Δ⁹-THC, further research is necessary to evaluate the potential differences that the route of administration may have on extinction learning for opiate-induced CPA. For example, our experiments in Chapter 3 demonstrated different dose-dependent and time-dependent concentration changes in Δ⁹-THC and its metabolite 11-OH-Δ⁹-THC in whole blood after pulmonary and parenteral administration of Δ⁹-THC. Pulmonary-administration of Δ⁹-THC resulted in rapid peak blood concentrations of Δ⁹-THC before 20 min, followed by more gradual peak blood concentrations of 11-OH-Δ⁹-THC at 30 min. However, parenterally-administered Δ⁹-THC appeared to produce maximal blood concentrations of Δ⁹-THC and 11-OH-Δ⁹-THC at 20 min, followed by a more gradual decline in Δ⁹-THC, but a steeper initial decline and potential rebound plateau for 11-OH-Δ⁹-THC, in the subsequent 20 min. Since the most common route of exposure to Δ⁹-THC for humans is pulmonary, yet most animal models use parenteral administration of Δ⁹-THC, further research should evaluate the potential effects on behaviour that may arise from different rates of Δ⁹-THC metabolism after inhalation or injection of Δ⁹-THC. In the following series of studies in Chapter 4, we will compare the effects of pulmonary and parenteral administration of Δ⁹-THC on extinction learning of naloxone-precipitated morphine withdrawal-induced CPA.
Manwell et al. (2009) demonstrated that the FAAH inhibitor URB597 significantly facilitated extinction of conditioned aversion but not conditioned preference learning in rats. Specifically, URB597 promoted extinction of a naloxone-precipitated morphine withdrawal-induced conditioned floor aversion, whereas the CB₁ antagonist/inverse agonist SR141716 significantly impaired extinction. In comparison, extinction rates of morphine-induced conditioned floor preference and operant responding for sucrose reward in rats were not affected by URB597, SR141716, or the CB₁ antagonist/inverse agonist AM251. However, whether inhaled cannabinoids would similarly facilitate opioid-induced withdrawal-induced conditioned aversion is currently unknown. This can have important implications for research on opiate withdrawal and abstinence and for our understanding of the effects of exogenous cannabinoids on cognitive functions. The present experiments extend the results of the conditioned aversion experiment by Manwell et al. (2009, Exp. 3) and evaluate the effects of inhaled and injected Δ⁹-THC on extinction rates of naloxone-precipitated morphine withdrawal-induced conditioned aversion. Although most studies evaluating the effects of Δ⁹-THC on memory in animal models employ various routes of injection, human users typically smoke cannabis. Indeed, the vast majority of animal research on the pharmacological and behavioural effects of Δ⁹-THC and its metabolites administer Δ⁹-THC parenterally (e.g., intraperitoneal or intravenous injection), whereas the most common route of administration for human users is pulmonary (e.g., smoking or vapourizing marijuana). Combustion of cannabis releases many compounds in addition to Δ⁹-THC, including other phytocannabinoids, toxins and carcinogens (Turner et al., 1980a/b; reviewed in Reece, 2009). In comparison, vaporization of pure Δ⁹-THC provides a more accurate assessment of the direct effects of Δ⁹-THC and its primary psychoactive metabolite, 11-OH-Δ⁹-
THC. Here, we directly compared bioavailable levels of Δ⁹-THC and its primary psychoactive metabolite 11-OH-Δ⁹-THC, in blood after injection and inhalation of pure Δ⁹-THC, not marijuana smoke containing unknown quantities of Δ⁹-THC and numerous other cannabinoids and toxicants. First, the method of analyzing levels of Δ⁹-THC and 11-OH-Δ⁹-THC (Chapter 3) was repeated to determine the dosages that would result in the most comparable blood levels of cannabinoids after exposure via injection or vapour inhalation; second, these dosages were used in two behavioural experiments aimed at assessing the effects of inhaled and injected Δ⁹-THC on extinction of a conditioned place aversion in Manwell et al. (2009, Expt. 3).

4.4. Materials and Method

4.4.1. Subjects

Male Sprague-Dawley rats (Charles River, Canada), weighing 300–500 g, were used in all experiments. Rats were pair-housed in standard plastic shoebox cages (45 x 25 x 20 cm³) maintained at 21-22°C in a colony room on a 12-h reversed light-dark cycle (lights off at 0700 h) and fed standard rat chow (Harlan 8640) and water ad libitum. Testing was conducted during the dark cycle. Experimental procedures followed Canadian Council on Animal Care guidelines and were approved by the Wilfrid Laurier University Animal Care Committee. Rats were acclimatized to the colony and handling procedures prior to experimentation.

4.4.2. Drugs and analytical chemicals

Morphine (Medisca, St-Laurent, QC) was prepared in physiological saline at a concentration of 20 mg/ml and administered subcutaneously (s.c.) in a volume of 1 ml/kg at 24 h prior to conditioning. Naloxone (Sigma) was prepared in physiological saline at a concentration of 1 mg/ml and administered (s.c.) in a volume of 1 ml/kg at 10 min prior to conditioning.
Δ⁹-THC (Dronabinol, > 98% purity) was obtained from THC Pharm GmbH (Frankfurt, Germany). For experiments involving pulmonary (inhaled) drug administration, Δ⁹-THC was prepared in ethanol at concentrations of 4, 20, and 40 mg/ml and 250 µl of each was applied to small steel wool pads (liquid pads, Storz & Bickel, Tuttlingen, Germany), yielding final amounts of 1, 5, or 10 mg/pad. The ethanol was then allowed to evaporate before vapourization. For experiments involving i.p. administration, Δ⁹-THC was dissolved in a small volume of ethanol and then mixed with TWEEN-80 (polyoxyethylene sorbitan monooleate; ICN Biomedicals). The ethanol was evaporated under a stream of nitrogen gas, and the suspension was then mixed with physiological saline. The final vehicle contained 15 µl TWEEN-80 per 2 ml saline. Δ⁹-THC was administered in a volume of 1 ml/kg body weight.

Internal standards for Δ⁹-THC (1.0 mg/ml), 11-OH-Δ⁹-THC (100 µg/ml), Δ⁹-THC-D₃ (100 µg/ml), and 11-OH-Δ⁹-THC-D₃ (100 µg/ml) were obtained from Cerilliant (Round Rock, Texas, USA). Each standard was diluted in methanol to working concentrations 5 to 600 ng/ml and stored at 4°C.

All solvents were analytical or HPLC grade; water, acetonitrile, methanol, ethyl acetate, and hexane were obtained from VWR and Fisher Scientific.

All blood samples were collected in ethylenediaminetetraacetic acid (EDTA) treated glass test tubes (BD Vacutainer tubes). Solid phase extraction (SPE) silica-bonded (Si) C18 (BondElut) flash columns were obtained from Varian Canada Inc. (Mississauga, Ontario, Canada).
4.4.3. Apparatus

The conditioning apparatus consisted of a black acrylic rectangular box (60 x 25 x 25 cm$^3$) with a wire-mesh lid. During conditioning trials, tactile cues on both sides of the box were identical. During pretest and choice tests, one side of the chamber had a plastic floor with raised bumps and the other side had a plastic floor with holes (counterbalanced); a small plastic smooth floor, defined as a neutral zone (9 x 25 cm), separated the two cued floors. The amount of time (sec) each rat spent on each of the floors was recorded and subsequently analyzed by the ANY-maze behavioural video-tracking software (Stoelting, Wood Dale, Illinois). Pretests did not indicate a significant difference between time spent on the plastic bumps or holes floors indicating that the apparatus provides an unbiased test of conditioned preference/aversion.

A Volcano® vapourizing device (Storz and Bickel, GmbH and Co., Tuttlingen, Germany) was used as described by Hazekamp et al. (2006). Briefly, $\Delta^9$-THC (1, 5, or 10 mg/pad) was vapourized (at heat setting 9, approximately 226°C) and collected into detachable plastic balloons (approximately 8 L), which were then fitted with a release valve that expelled the vapour over 10 sec into enclosed plastic boxes (45 x 25 x 20 cm$^3$) containing two rats that inhaled the vapour for 5 min (refer to Figure 3.1). Animals were removed 5 min later and either tested in the conditioning apparatus (Experiments 4.2 and 4.3) or sacrificed at regular intervals for determination of blood cannabinoid levels (Experiment 4.1) using the same method described in Experiment 3. This device has been previously reported to deliver $>50\%$ of the loaded $\Delta^9$-THC into the balloon in a reproducible manner with a pulmonary uptake comparable to smoking of cannabis in humans (Hazekamp et al., 2006).
4.5. Procedure

4.5.1. Experiment 4.1: Pharmacokinetic analyses of pulmonary and parenteral administration of Δ⁹-THC

A series of experiments was conducted to determine the expected blood concentrations of Δ⁹-THC and 11-OH-Δ⁹-THC in rats during specific time-points relevant to Experiments 2.3, 4.2 and 4.3. A detailed description of the development and validation of the analytical methods of cannabinoid exposure methods, extraction, identification, and quantification in whole blood used here was previously reported by Manwell and Mallet (2009) and described fully in Chapter 3; the methods used here were identical. Briefly, rats were exposed to either pulmonary-administered Δ⁹-THC (1, 5, 10 mg/pad inhaled vapour) or parenterally-administered Δ⁹-THC (0.25, 0.50, 1.0, and 1.5 mg/kg injected i.p.) and blood concentrations of Δ⁹-THC and 11-OH-Δ⁹-THC determined at 20 and 40 min post-administration. All blood samples were obtained by cardiac puncture performed on rats anesthetized with isoflurane gas; immediately upon collection, 1 ml of blood was added to iced test tubes, already containing standards in methanol, then gently mixed and allowed to stand for approximately 1 h on ice. After equilibration, blood samples were acidified, proteins were precipitated, and cannabinoids extracted by LLE. Supernatant fractions were removed, placed into small glass test tubes, and then evaporated at room temperature under nitrogen. Residues were reconstituted in 3 ml hexane and cannabinoids were selectively separated by SPE. All fractions were kept separate for the remaining steps, including LC/MS analysis.
4.5.2. Experiment 4.2: Effect of pulmonary-administrated Δ⁹-THC on naloxone-precipitated morphine withdrawal-induced conditioned aversion

Forty rats (n=10/group) were assigned to extinction groups (0, 1, 5, and 10 mg Δ⁹-THC) matched on the basis of a 20 min pretest score and pair housing. They were given two conditioning trial cycles (separated by 72 h), each consisting of a 3-day schedule separated by 24 h (previously described in detail by Manwell et al., 2009, Exp. 3). For each conditioning cycle, on Day 1 rats were injected (s.c.) with 1 ml/kg saline 10 min prior to placement in the chamber with a distinctive floor cue for 30 min. On Day 2, rats were injected (s.c.) with 20 mg/kg morphine in their home cages. On Day 3, rats were injected (s.c.) with 1 mg/kg naloxone 10 min prior to placement in the chamber with the opposite distinctive floor cue (as on Day 1) for 30 min. Extinction testing trials began 72 h after the final conditioning cycle. For all extinction tests, rats (n=10/group) were administered vapoured Δ⁹-THC — (0, 1, 5, or 10 mg/pad) in groups of two rats per box (with their pair-housed partner) for 5 min then injected (s.c.) with 1 ml/kg saline 10 min prior to a choice test trial — with both conditioned floors and the neutral floor — which lasted for 20 min. Extinction testing continued every 24 h for 20 consecutive days. One week after the final extinction test, a 20 min drug-free choice test was also conducted.

4.5.3. Experiment 4.3: Effect of parenterally-administrated Δ⁹-THC on naloxone-precipitated morphine withdrawal-induced conditioned aversion

Forty-eight rats (n=12/group) were assigned to four extinction groups (0, 0.5, 1.0, and 1.5 mg/kg Δ⁹-THC) matched on the basis of a 20 min pretest score and pair-housing (2 were removed prior to the end of testing). The procedure was exactly the same as Experiment 4.2 except for the following: a) 72 h after the last conditioning trial, a 20 min drug-free choice test
was administered to probe for the strength of an aversion, and b) for all extinction tests, in groups of two rats per box with their pair-housed partner, rats were administered $\Delta^9$-THC (0, 0.5, 1.0, and 1.5 mg/kg) parenterally (i.p.) 20 min prior to testing, including an injection (s.c.) of 1 ml/kg saline 10 min prior to a choice test trial — with both conditioned floors and the neutral floor — which lasted for 20 min. Extinction testing continued every 24 h for 28 consecutive days. One week after the final extinction test, a 20 min drug-free spontaneous recovery (SR) choice test was also conducted. In addition, one week after the spontaneous recovery test, a reinstatement test (RS) was performed to determine the potential of naloxone-precipitate morphine withdrawal to reinstate the conditioned aversion, and consisted of the following: on Day 1, rats were injected (s.c.) with 1 ml/kg saline 10 min prior to a 20 min choice test; on Day 2, rats were injected (s.c.) with 20 mg/kg morphine in their home cage; and on Day 3, rats were injected (s.c.) with 1 mg/kg naloxone 10 min prior to a choice test to determine the effect of prior extinction treatments on reinstatement of the conditioned aversion.

4.6. Data Analysis

Similar to Manwell et al. (2009, Exp. 3) in Chapter 2, the data for the 20 extinction trials in Experiment 4.1 was analyzed in blocks of 4 trials in a 2 x 5 (Floor x Blocks of Trials) repeated measures ANOVA and a mixed factor ANOVA for the drug-free choice test data 1 week after the final extinction trial. In addition, the time spent on the neutral floor (intersecting zone) and overall locomotor activity was also evaluated. Statistical significance was defined as $p < 0.05$.

The data for the drug-free probe test and the 28 extinction trials in Experiment 4.3 was analyzed as blocks of 4 trials in a 2 x 8 (Floor x Blocks of Trials) repeated measures ANOVA and a mixed factor ANOVA for the drug-free SR choice test data 1 week after the final
extinction trial and the RS test 2 weeks after the final extinction trial. In addition, the time spent on the neutral floor (intersecting zone) was also evaluated. Statistical significance was defined as $p < 0.05$.

4.7. Results

4.7.1. Experiment 4.1: Pharmacokinetic analyses of pulmonary and parenteral administration of Δ$^{9}$-THC

The results of these new pharmacokinetic studies of Δ$^{9}$-THC in rats were similar to preliminary studies reported in Chapter 3 that compared recovery efficiencies after pulmonary and parenteral administration, in both dose-dependent and time-dependent analyses. Representative blood concentrations are shown in Table 4.1 and Figures 4.1 and 4.2; whole blood collected at 20 and 40 min from rats receiving Δ$^{9}$-THC either through pulmonary administration (1, 5, and 10 mg inhaled vapour) or parental administration (0.25, 0.50, 1.0, and 1.5 mg/kg injected i.p.) showed both dose-dependent and time-dependent concentration changes in Δ$^{9}$-THC and its metabolite 11-OH-Δ$^{9}$-THC. For pulmonary-administered Δ$^{9}$-THC, blood concentrations of Δ$^{9}$-THC at 20 and 40 min were determined in the following ranges, respectively: a) between 157.50 – 155.67 ng/ml/kg for 1 mg; b) between 223.06 – 209.38 ng/ml/kg for 5 mg; and c) between 402.31 – 301.06 ng/ml/kg for 10 mg. For parenterally-administered Δ$^{9}$-THC, blood concentrations of Δ$^{9}$-THC at 20 and 40 min were determined in the following ranges, respectively: a) between 151.37 – 173.17 ng/ml/kg for 0.25 mg/kg; b) between 210.54 – 244.47 ng/ml/kg for 0.50 mg/kg; c) between 244.46 – 395.33 ng/ml/kg for 1.0 mg/kg; and d) between 349.33 – 410.89 ng/ml/kg for 1.5 mg/kg.
For pulmonary-administered $\Delta^9$-THC, blood concentrations of $11$-OH-$\Delta^9$-THC at 20 and 40 min were determined in the following ranges, respectively: a) between 86.45 – 120.27 ng/ml/kg for 1 mg; b) between 173.19 – 219.86 ng/ml/kg for 5 mg; and c) between 496.26 – 233.04 ng/ml/kg for 10 mg. For parenterally-administered $\Delta^9$-THC, blood concentrations of $11$-OH-$\Delta^9$-THC at 20 and 40 min were determined in the following ranges, respectively: a) between 353.70 – 244.86 ng/ml/kg for 0.25 mg/kg; b) between 357.57 – 267.34 ng/ml/kg for 0.50 mg/kg; c) between 410.87 – 294.82 ng/ml/kg for 1.0 mg/kg; and d) between 445.95 – 402.96 ng/ml/kg for 1.5 mg/kg.
Figure 4.1. Dose- and time-dependent concentrations of Δ⁹-THC (top) and 11-OH-Δ⁹-THC (bottom) recovered from whole blood in rats exposed to vaporized Δ⁹-THC (1, 5, or 10 mg) at a 20 min interval.
Figure 4.2. Dose- and time-dependent concentrations of Δ⁹-THC (top) and 11-OH-Δ⁹-THC (bottom) recovered from whole blood in rats exposed to injected Δ⁹-THC (0.25, 0.50, 1.00, or 1.50 mg/kg) at a 20 min interval.
4.7.2. Experiment 4.2: Effect of pulmonary-administrated $\Delta^9$-THC on naloxone-precipitated morphine withdrawal-induced conditioned aversion

Pulmonary administration of $\Delta^9$-THC facilitated the rate of extinction of a naloxone-precipitated morphine withdrawal-induced conditioned aversion and in a dose- and time-dependent manner. Compared to vehicle, both 10 and 5 mg/pad of inhaled $\Delta^9$-THC showed facilitated extinction rates of the conditioned aversion, whereas 1 mg/pad did not differ significantly from vehicle. However, the CPA was maintained up to 1 wk after extinction testing only for rats previously administered 1 mg/pad inhaled $\Delta^9$-THC; thus 1 mg/pad inhaled $\Delta^9$-THC appears to have impaired extinction, compared to vehicle, as indicated by aversion maintenance up to one week after extinction testing ended. Figure 4.3 presents the extinction data depicted as five blocks of 4 trials each. There was a main effect of Floor ($F(1, 36) = 21.75, p < 0.001$) and Block of Trials ($F(4, 44) = 5.55, p < 0.001$) but no interaction. Based on the results of Manwell et al. (2009, Exp.3), it was hypothesized that there would be different rates of extinction across trials for the different drug groups, thus, planned pairwise comparisons were conducted indicating the following: during Block 1 (extinction tests 1-4), all groups showed a significant aversion to the withdrawal-paired floor in comparison to the saline-paired floor (all $p$’s < 0.05). During Block 2 (extinction tests 5-8), only the 0 mg $\Delta^9$-THC group showed an aversion ($p < 0.05$). In Block 3 (extinction tests 9-12), the 0, 1, and 5 mg $\Delta^9$-THC groups showed an aversion (all $p$’s < 0.05), but not the 10 mg $\Delta^9$-THC group. In Block 4 (extinction tests 13-16), only the 1 mg $\Delta^9$-THC group showed an aversion ($p < 0.05$). In Block 5 (extinction tests 17-20), only the 0 and 1 mg $\Delta^9$-THC groups maintained an aversion ($p$’s < 0.05), whereas the 5 and 10 mg $\Delta^9$-THC groups maintained extinction of the aversion. In the drug-free choice test 1 week after the final
extinction trial there was a significant Floor by Trial interaction (Block 5 and drug-free test) 
(F(1, 36) = 8.80, p < 0.01) wherein only the 1 mg Δ⁹-THC group maintained the aversion (p < 
0.05).
Figure 4.3. Experiment 4.2. Mean (± sem) seconds spent on the naloxone-precipitated morphine withdrawal-paired floor and on the saline-paired floor during each block of 4 trials for rats exposed for 5 min to vapourized Δ⁹-THC: 0 mg (n = 10), 1 mg (n = 10), 5 mg (n = 10), or 10 mg (n = 10) in 8 L of vapour. Asterisks indicate that rats spent less time on the withdrawal-paired floor than the saline-paired floor during each block of trials (* = p < 0.05).
4.7.3. Experiment 4.3: Effect of parenterally administrated $\Delta^9$-THC on naloxone-precipitated morphine withdrawal-induced conditioned aversion

Parenteral administration of $\Delta^9$-THC impaired the rate of extinction of a naloxone-precipitated morphine withdrawal-induced conditioned aversion for a 1.0 mg/kg $\Delta^9$-THC only. Injected $\Delta^9$-THC significantly impaired extinction only for 1.0 mg/kg: it prolonged the CPA 4-fold longer (16 days) than the vehicle, 0.5 and 1.5 mg/kg groups. There were no differences between groups administered vehicle, 0.5 and 1.5 mg/kg. Figure 4.4 presents the extinction data depicted as 8 blocks of 4 trials each. There was a main effect of Floor ($F(1, 42) = 18.54, p < 0.001$) and a Trial by Floor interaction ($F(7, 294) = 4.20, p < 0.001$). Planned pairwise comparisons indicated the following: during the drug-free probe test, all groups showed a significant aversion to the withdrawal-paired floor in comparison to the saline-paired floor (all p’s < 0.01). During Block 1 (extinction tests 1-4) and Block 2 (extinction tests 5-8), the vehicle and all of the THC groups (0.5, 1.0, and 1.5 mg/kg) showed an aversion (all p’s < 0.05). However, for Block 3 (extinction tests 9-12), Block 4 (extinction tests 13-16), Block 5 (extinction tests 17-20) and Block 6 (extinction tests 21-24), only the 1.0 mg/kg THC group continued to show an aversion (all p’s < 0.05); the vehicle and 0.5 and 1.5 mg/kg THC groups all maintained extinction of the aversion (all p’s n.s.). In Block 7 (extinction tests 25-28), none of the groups showed an aversion (all p’s n.s.).

Effects of $\Delta^9$-THC on locomotor activity were assessed using the total time mobile (in sec ± sem) during pretest, a drug-free probe test, and each block of 4 trials of extinction tests. There was a main effect of Trial ($F(8, 344) = 63.57, p < 0.001$) indicating that locomotor activity decreased equally across all groups from the first to last trial (Figure 4.5).
For the drug-free SR choice test 1 week after the final extinction trial, a 2 x 2 repeated measures ANOVA comparing the last extinction block (tests 25-28) to the SR test showed maintenance of the extinction (all p’s n.s.). Approximately 1 week later, the RS test was conducted. On Day 1, a 2 x 2 repeated measures ANOVA comparing Day 1 of the RS test (the saline prime) to Day 3 of the RS test (the naloxone prime) showed a main effect of Trial (F(1, 43) = 66.46, p < 0.001), a main effect of Floor (F(1, 43) = 8.10, p < 0.01), and a Trial by Floor interaction (F(3, 43) = 4.12, p < 0.05). All groups spent less time on the naloxone-paired floor on both trials (Days 1 and 2); however, all groups also spent more time on the saline-paired floor in the presence of the naloxone prime (on Day 3).
Figure 4.4. Experiment 4.3. Mean (± sem) seconds spent on the naloxone-precipitated morphine withdrawal-paired floor and on the saline-paired floor during each block of 4 trials for rats pretreated with injected Δ⁹-THC: 0 mg/kg (n = 11), 0.5 mg/kg (n = 12), 1.0 mg/kg (n = 11), or 1.5 mg/kg (n = 11). Asterisks indicate that rats spent less time on the withdrawal-paired floor than the saline-paired floor during each block of trials (* < 0.05).
Figure 4.5. Experiment 4.3. Total time mobile (in seconds ± sem) during pretest, a drug-free probe test, and each block of 4 trials of extinction tests for rats pretreated with injected Δ⁹-THC: 0 mg/kg (n = 11), 0.5 mg/kg (n = 12), 1.0 mg/kg (n = 11), or 1.5 mg/kg (n = 11): locomotor activity decreased equally across all groups from the first to last trial.
4.8. Discussion

This study had three primary objectives: 1) to determine and compare the blood levels of Δ⁹-THC and its primary psychoactive metabolite, 11-OH-Δ⁹-THC, after pulmonary and parenteral administration, 2) to demonstrate the effects that the route of administration of Δ⁹-THC has on extinction learning, and 3) to further elucidate the involvement of the ECB system in extinction.

Regarding the first objective, the purpose of Experiment 4.1 was to determine the initial dose(s) of parenterally-administered Δ⁹-THC that would produce blood levels of both Δ⁹-THC and 11-OH-Δ⁹-THC equivalent to those produced by pulmonary-administered Δ⁹-THC. Experiment 4.1 determined that there were roughly equivalent mean blood concentrations after exposure to vapourized and injected Δ⁹-THC; rats receiving either pulmonary-administered Δ⁹-THC (1, 5, and 10 mg inhaled vapour) or parenterally-administered Δ⁹-THC (0.25, 0.50, 1.0, and 1.5 mg/kg injected i.p.) showed both dose-dependent and time-dependent concentration changes in Δ⁹-THC and its metabolite 11-OH-Δ⁹-THC. These results are consistent with those reported in Experiment 3, although there are some differences that may be attributable to the rats in Experiment 3 being much older, heavier (e.g., greater body fat ratio), and having received 1-3 prior injections of naloxone than the rats in Experiments 4.1. All rats used in Experiment 4.1 were completely drug naive and the same age and weight as the rats in the subsequent Experiments 4.2 and 4.3. The results of Experiment 4.1 are also consistent with the sparse and narrow studies reported in the literature evaluating pulmonary and/or parenteral administration of Δ⁹-THC in rodents; of these studies, only Wilson et al. (2002) directly compared pulmonary and parenteral routes of administration.
In Experiment 4.1, similar levels of Δ⁹-THC and 11-OH-Δ⁹-THC were found in whole blood in male Sprague-Dawley rats when there was an approximate 6-fold increase in the amount of Δ⁹-THC that was delivered through pulmonary administration compared to parenteral administration. Wilson et al. (2002) also demonstrated that an approximate 6-fold increase produced similar results in male mice: at 20 min post-exposure, mean Δ⁹-THC whole blood levels were 409±86 and 1132±240 ng/ml for 20 and 60 mg of pulmonary-administered Δ⁹-THC and 365±39 and 1324±38 ng/ml for 3 and 10 mg/kg parenterally-administered Δ⁹-THC, respectively. However, Wilson et al. (2002) also reported a dissociation between Δ⁹-THC levels found in blood and brain that was dependent upon the route of administration; although Δ⁹-THC blood and brain levels were roughly equivalent following pulmonary administration, Δ⁹-THC levels were 2–3-fold greater in brain than in blood after parenteral administration. In their conclusions, Wilson et al. (2002, p. 264) acknowledged that, in earlier studies also in mice, employing parenteral routes of administration either increased or decreased Δ⁹-THC in brain compared to blood; they cautioned that the difference in results across studies, even in the same species, could be attributed in part to “different extraction procedures, drug absorption times, the use of radiolabeled Δ⁹-THC rather than actual drug, data analyses and other methodological considerations” making direct comparisons difficult.

The results of Experiment 4.1 are also consistent with the results of those reported by Valiveti and Stinchcomb (2004); the methods of LC/MS quantification of Δ⁹-THC in Experiments 3 and 4.1 were modeled primarily upon the methods reported in their paper. Valiveti and Stinchomb (2004) evaluated levels of Δ⁹-THC and 11-OH-Δ⁹-THC in rat and guinea pig plasma (and not whole blood) after parenteral administration of Δ⁹-THC;
they did not assess the effects of pulmonary administration of Δ⁹-THC. Valiveti and Stinchcomb (2004) reported maximal levels of Δ⁹-THC (194 ng/ml) and 11-OH-Δ⁹-THC (10 ng/ml) in rat plasma immediately after parenteral administration (e.g., an intravenous bolus dose) of 1 mg/kg Δ⁹-THC which plateaued after approximately 1 h and maintained a steady state for approximately 6 h. Using the same methodology, they reported maximal levels of Δ⁹-THC (197.5 ng/ml) in guinea pig plasma which also plateaued after 1 h, but reported no metabolites for up to 6 h, noting that guinea pigs metabolize Δ⁹-THC differently than other mammals (Valiveti and Stinchcomb, 2004). Valiveti and Stinchcomb (2004) did not report on the strain or sex of the rodents used in their experiments.

The results for male Sprague-Dawley rats in Experiment 4.1 are also consistent with those for the male Sprague-Dawley rats reported by Tseng et al. (2004) using parenterally-administered radiolabeled Δ⁹-THC. Tseng et al. (2004), who tested both male and female Sprague-Dawley rats, administered 5 μCi/10 mg/ml [³H]-Δ⁹-THC parenterally (i.p.) and reported blood serum levels of total radiolabeled cannabinoids (Δ⁹-THC + metabolites) that were equivalent for males and females of 2000 and 1000 DPM/ml serum at 15 and 30 min post-administration respectively. In addition, brain levels of Δ⁹-THC and metabolites in males peaked within 15 min and maintained a steady state similar to blood levels (Tseng et al., 2004). However, there was a significant dissociation between blood and brain levels for females only: a) brain levels of Δ⁹-THC and metabolites were significantly higher in females than males, and b) brain levels in females peaked at 120 min and then steadily decreased (Tseng et al., 2004). When evaluated separately, levels of Δ⁹-THC were not significantly different in brain tissue for
males and females; the difference was attributable to the metabolite 11-OH-Δ⁹-THC which peaked once for males (at 30 min) and twice for females (at 20 and 120 min) (Tseng et al., 2004).

In studies in humans, there is also significant variability across routes of administration. For example, Naef et al. (2004) reported that in males and females, pulmonary administration of a Δ⁹-THC liquid aerosol (0.053 mg/kg) produced peak plasma levels of Δ⁹-THC in the range of 18.7 ± 7.4 ng/ml for approximately 10 to 20 minutes after inhalation, and then rapidly decreased; however, plasma levels after parenteral (i.v.) administration of Δ⁹-THC (0.053 mg/kg) were more variable, ranging from 81.6 to 640.6 ng/ml (mean of 271.5 ± 61.1 ng/ml) within 5 min after injection (Naef et al., 2004). In addition, Naef et al. (2004) reported significantly lower levels of 11-OH-Δ⁹-THC after pulmonary administration, putatively due to lack of hepatic first pass metabolism. Naef et al. (2004) did not report any significant sex differences.

These findings demonstrate that the results of Experiments 3 and 4.1 are consistent with the few studies reported in the literature. They also highlight some of the problems arising from using different administration and extraction methods and important interspecies metabolic differences that make it difficult to relate the pharmacological and toxicological effects of Δ⁹-THC across species of rodents and even extrapolate to humans (reviewed in Grotenhermen, 2003). Interspecies differences in metabolism may be attributable to factors such as size, respiratory rate, body fat ratio, site of Δ⁹-THC metabolism and/or storage, and differences in isoenzymes of the cytochrome P450 (CYP) complex, which is largely responsible for hydroxylation and oxidation of Δ⁹-THC in the liver (reviewed in Grotenhermen, 2003, and Murray and Bevins, 2010). There
are also important sex differences in the pharmacokinetic and behavioural effects of Δ⁹-THC between males and females (reviewed in Fattore and Fratta, 2010). Since the studies discussed here reported results for male animals that are consistent with the current experiments using male rats, the issue of sex differences will be discussed in greater depth in Chapter 5. Thus, for Experiment 4.3, the three highest doses of injected Δ⁹-THC were chosen as to closely approximate doses administered by inhalation in Experiment 4.2. Unlike previous studies, Experiments 4.2 and 4.3 evaluated the effects of two very different routes of administration of Δ⁹-THC on extinction rates of the same naloxone-precipitated morphine withdrawal-induced conditioned aversion in rodents.

Regarding the second objective, the data from Experiment 4.2 clearly support the results of Manwell et al. (2009) which demonstrated that increasing endogenous levels of CB₁ agonists (e.g., increasing AEA availability by inhibiting the enzyme that deactivates it) can facilitate the extinction rate of a conditioned aversion. In Experiment 4.2, exposure to pulmonary-administered Δ⁹-THC also facilitated the rate of extinction learning of a naloxone-precipitated morphine withdrawal conditioned aversion. All rats showed an aversion to the withdrawal-paired floor cue during the first block of extinction trials indicating that Δ⁹-THC administration did not interfere with recall memory. However, pulmonary-administered Δ⁹-THC did facilitate extinction learning in a dose-dependent manner: rats receiving the highest dose of inhaled Δ⁹-THC vapour displayed rapid extinction of the aversion by the second block of trials, followed by rats receiving a moderate dose of inhaled Δ⁹-THC that showed extinction of the aversion by the fourth block of trials. In comparison, rats receiving either the vehicle or the lowest dose of inhaled Δ⁹-THC failed to extinguish the aversion during the course of the experiment. In
addition, a drug-free test one week later revealed that only the lowest dose of Δ⁹-THC maintained the withdrawal-conditioned aversion. This finding suggests that, had extinction testing continued beyond 20 days, rats receiving vehicle only may have been more likely to show extinction of the aversion within the next block of trials than rats receiving the lowest dose of Δ⁹-THC.

The data from Experiment 4.3 indicate that, in contrast to Experiment 4.2, wherein exposure to pulmonary-administered Δ⁹-THC facilitated the rate of extinction learning of the CPA, parenterally-administered Δ⁹-THC impaired extinction rates. All rats showed an aversion to the withdrawal-paired floor cue during both the drug-free probe test and the first and second block of extinction trials; however, only 1.0 mg/kg i.p. Δ⁹-THC significantly prolonged the aversion up to 4-fold longer than vehicle and both the 0.5 and 1.5 mg/kg doses. All groups maintained the extinction during the drug-free probe test one week later. During the reinstatement testing days, all groups spent less time on the naloxone-paired floor on both trials (Days 1 and 2); however, all groups also spent more time on the saline-paired floor in the presence of the naloxone prime (on Day 3).

In Experiments 4.2 and 4.3, during the first block of extinction trials, all groups, regardless of extinction drug dose, demonstrated a strong aversion to the withdrawal-paired conditioned cue; this was consistent for both routes of administration of Δ⁹-THC. However, as early as the second block of extinction trials, significant differences began to emerge for both dose and route of administration. The results suggested that, over repeated exposures, low doses, whether inhaled or injected, had no effect or an impairing effect on extinction learning, whereas high doses had a facilitatory effect on extinction but
only when inhaled. In general, it appears that the doses and routes of administration of Δ⁹-THC were simply insufficient to affect retrieval memory of the CPA in the first block of extinction tests; this is consistent with Experiment 2.3 wherein URB597 did not affect extinction within the first block of trials. It was only after repeated exposure, and thus likely through repeated activation of CB₁, that Δ⁹-THC began to facilitate or impair extinction memory; again, similar to the effects of URB597 and SR141716 in Experiment 2.3. It may be that parenterally-administered Δ⁹-THC takes longer to cross the blood-brain barrier and in doing so, has no effect or an antagonising effect through repeated exposure every 24 h; it may also produce more variable brain levels of Δ⁹-THC and 11-OH-Δ⁹-THC. Since pulmonary-administered Δ⁹-THC immediately crosses the blood-brain barrier, it may readily activate CB₁ receptors in greater density and distribution throughout the brain after repeated exposure every 24 h. In addition, there is some limited evidence that although parenteral and pulmonary-administered Δ⁹-THC produce similar blood levels of Δ⁹-THC and 11-OH-Δ⁹-THC, brain levels are significantly higher after pulmonary-administration (Wilson et al., 2002; Tseng et al., 2004). Although further experimentation is necessary to determine the specific biological mechanisms mediating the dose- and route-dependent effects of Δ⁹-THC on extinction rates of CPA demonstrated here, a few related experiments may help explain the results of Experiments 4.2 and 4.3.

First, Murray and Bevins (2010, Table 1.2b) reviewed the significant differences in the acquisition of behavioural conditioning that can be produced by altering a very narrow range of parenterally-administered (i.p.) Δ⁹-THC doses in rats. For example, Mallet and Beninger (1998) reported that, using an IPI of 30 min, 1.0 to 1.5 mg/kg Δ⁹-
THC produced CPA, whereas a minimal increase or decrease of only 0.5 mg/kg outside of that range did not affect the acquisition of behavioural conditioning (n.s.). In fact, increasing the dosage incrementally up to 8-fold (or decreasing it by 10-fold) did not affect the acquisition of behavioural conditioning (Mallet and Beninger, 1998). Similarly, in Experiment 4.3 on the extinction of behavioural conditioning, only 1.0 mg/kg (i.p.) Δ⁹-THC prolonged the CPA. In addition, since altering the IPI can alter the aversive effects of Δ⁹-THC (Murray and Bevins, 2010), it is possible that the shorter 20 min IPI used in Experiment 4.3 resulted in only aversive effects for 1.0 mg/kg and null effects on the extinction of behavioural conditioning for 1.5 mg/kg in contrast to the results in Mallet and Beninger (1998) on the acquisition of CPA.

Second, temporal conditions of the extinction sessions, which were conducted for 30 min every 24 h, may have contributed to these differences. In place conditioning studies using parenteral routes of administration, increasing the time between training sessions generally results in lower dosages producing CPP and higher dosages producing CPA; these effects are typically a result of biphasic effects of Δ⁹-THC metabolism (Murray and Bevins, 2010). For example, when training session intervals were 24 h, both 2 and 4 mg/kg Δ⁹-THC produced CPP but 1 mg/kg had no effect; in comparison, 48 h training session intervals shifted the dose effect curve such that 1 mg/kg Δ⁹-THC now produced a CPP and 2 and 4 mg/kg produced CPA (Lepore et al., 1995). For Experiment 4.3, it is possible that, for parenterally-administered Δ⁹-THC, a 24 wash-out period was sufficient to impair extinction learning for 1 mg/kg only. It could be that 1 mg/kg i.p. Δ⁹-THC resulted in partial and/or unevenly distributed activation of CB₁ receptors that resulted in an antagonistic and aversive effect when repeated every 24 h. In comparison,
1.5 mg/kg i.p. $\Delta^9$-THC may have produced a greater level of CB$_1$ activation that was not antagonistic, but also not sufficient to facilitate extinction learning. For Experiment 4.2, it is possible that, for pulmonary-administered $\Delta^9$-THC, a 24 wash-out period was insufficient to produce any antagonistic rebound effects for high doses of $\Delta^9$-THC and instead repeated exposure produced a facilitory effect. This may also account for the sustained CPA up to one week after extinction testing for 1 mg/pad $\Delta^9$-THC (compared to vehicle); it is possible that the dosage was not sufficient to facilitate extinction learning, but had an antagonistic effect resulting from chronic subthreshold activation of CB$_1$ in the brain.

Third, chronic exposure to cannabinoids leads to the rapid development of both tolerance and withdrawal (reviewed in Howlett et al., 2004) which could have played a role in Experiments 4.2 and 4.3 during repeated exposure to $\Delta^9$-THC every 24 h for up to 28 d. For example, male Sprague-Dawley rats administered 10 mg/kg (i.p.) $\Delta^9$-THC every 24 h for 3, 7, 14 or 21 days showed rapid desensitization and down-regulation of CB receptors that were time- and region-dependent; these effects were demonstrated from 3 to 7 days (and remaining until day 21) and the effects were more pronounced in the hippocampus (Breivogel et al., 1999). In male Wistar rats, 10 mg/kg (i.p.) $\Delta^9$-THC administered every 24 h for 1 to 5 days also produced a similar pattern of down-regulation of CB$_1$ receptors (Romero et al., 1997). These results contrast with the authors’ earlier findings using much lower doses of $\Delta^9$-THC (3 mg/kg i.p.) and also AEA (3 mg/kg i.p.); when administered acutely (1 day) or chronically (5 days), Wistar rat brains showed significant increases in cannabinoid receptor binding in the hippocampus (Romero et al., 1995). Thus, repeated exposure every 24 h to 5 and 10 mg/pad inhaled
Δ⁹-THC may have resulted in a gradual increase in CB₁ receptor binding during the first block of trials (four days) that then led to facilitation of extinction learning over the following blocks of trials; this pattern is also consistent with Experiment 2.3 wherein URB597 facilitated extinction learning putatively by increasing AEA levels in the brain and thus CB₁ agonism. This is also consistent with the failure to find any effects of URB597 or AM251 on extinction of morphine-induced CPP (Chapter 2) wherein morphine-induced CPP rapidly extinguished within 2 to 3 trials, whereas morphine-withdrawal induced CPA required a minimum of 5 to 8 trials to extinguish. These findings would suggest that repeated activation of CB₁ receptors may have led to increases in subsequent CB₁ binding activity that facilitated extinction learning in rats administered URB597 and inhaled Δ⁹-THC; the above findings also suggest that even the highest dose of injected Δ⁹-THC used in Experiment 4.3 may not have been sufficient to affect cannabinoid receptor binding across repeated exposures.

Fourth, the procedures for all CPA extinction experiments were identical except for the one added procedure required for inhalation of Δ⁹-THC. Thus, it is possible that the immediate effects of vapourized Δ⁹-THC on the brain could have mediated effects of subsequent activation of the hypothalamic pituitary adrenal (HPA) axis and stress response during the injections required for the remaining procedures. For example, Schramm-Sapyta et al. (2007) reported that 5 mg/kg i.p. Δ⁹-THC produced a prolonged increase in the stress hormones adrenocorticotropic hormone (ACTH) and corticosterone in blood serum in male rats for both adolescents (age 28 d) and adults (65 d). Adult rats also demonstrated higher ACTH levels in response to Δ⁹-THC than adolescent rats between 30 and 90 min post-injection (Schramm-Sapyta et al., 2007). However, in both
age groups, injections of vehicle and Δ⁹-THC resulted in increased corticosterone at early time points (15 and 30 min) and remained elevated for more than 90 min only with Δ⁹-THC exposure (Schramm-Sapyta et al., 2007), suggesting that i.p. injection initially increases specific stress hormones and Δ⁹-THC prolongs their circulation in the blood. However, it is unlikely that the effects of Δ⁹-THC on HPA axis function alone account for the difference in results between pulmonary and parenterally administered Δ⁹-THC. For example, in Experiments 4.2 and 4.3, all rats received i.p. injections 20 min prior to all CPA acquisition and extinction trials; thus the effects of elevated corticosterone due to injection would be equivalent for all groups across experiments. The highest dose of Δ⁹-THC administered i.p. in Experiment 4.3 was 3-fold less than that reported by Schramm-Sapyta et al. (2007) and thus may not have been sufficient to increase ACTH levels or prolong corticosterone levels. In addition, none of the doses of Δ⁹-THC in Experiments 4.2 and 4.3 affected expression of the conditioned aversion in the first block of trials and the lowest dose of Δ⁹-THC that was pulmonary administered did not affect extinction rates at all. Furthermore, in Experiment 2.3, parenterally administered URB597 had the same effects on extinction rates with the same injection procedures.

Regarding the third objective, it is possible that there was an interaction between the ECB and opioid systems that mediated the effects reported here, which is consistent with research showing significant structural and functional overlap between the ECB and opioid systems (Navarro et al., 1998, 2001; Mas Nieto et al., 2001; Lichtman et al., 2001; Yamaguchi et al., 2001; Ghozland et al., 2002; Corchero et al., 2004). For example, administration of both endogenous CB₁ receptor ligands, anandamide (5 mg/kg) and 2-arachidonoylglycerol (2-AG) (10 μg/mouse, i.c.v.) and exogenous and synthetic ligands,
Δ⁹-THC and HU-210, have been shown to significantly decrease the unconditioned somatic signs of naloxone-precipitated morphine withdrawal (Vela et al., 1995; Yamaguchi et al., 2001), suggesting that CB₁ agonism in Experiments 2.3, 4.2, and 4.3 might have also reduced the strength of the CPA produced by naloxone-precipitated morphine withdrawal. In both Experiments 4.2 and 4.3, Δ⁹-THC pre-exposure did not reduce the strength of the initial aversion during the first block of trials; similarly, in Experiment 2.3, pretreatment with URB597 did not affect the strength of the aversion in the first block of extinction trials. However, although CB₁ agonism did not initially reduce the strength of the morphine-withdrawal CPA, it may have done so over repeated exposure to amounts of Δ⁹-THC and URB597 that then chronically activated CB₁ receptors; this explanation would be consistent with the results of Romero et al. (1995) demonstrating significant increases in cannabinoid receptor binding after chronic exposure to specific dosages of Δ⁹-THC and AEA (Romero et al., 1995).

Owing to the significant interactions between the ECB system and the opioid system (e.g., Navarro et al., 1998), selectively targeting CB₁ receptors may be important in interventions aimed at extinguishing both the unconditioned (e.g., somatic signs) and conditioned (e.g., negative affect) effects of morphine withdrawal syndrome, which have been proposed to contribute to drug-craving/seeking behaviour in opioid addiction and relapse (Koob, 1996; Bechara et al., 1995, 1998). Previous research has suggested the use of cannabinoids in treating opioid addiction in place of opioid agonists (Mas-Nieto et al., 2001) currently used, such as methadone and buprenorphine (Kreek, 1997; Fischer et al., 1999), which have the potential to also induce dependence (Dyer et al., 1999, 2001; Kuhlman et al., 1998). If the ECB system can be manipulated to facilitate extinction of
previously learned responses, then it may serve as a pharmacotherapeutic target for facilitating the extinction of maladaptive associations between environmental context/cues and the aversive effects of drugs of abuse. In animal models, stress induces significantly greater self-administration of drugs and, in clinical studies, human patients consistently report links between stress and drug abuse (reviewed in Gordon, 2002). According to this 2002 report by the U.S. National Institute on Drug Abuse (NIDA), a significant population of people who abuse drugs were also found to meet the diagnostic criteria for post-traumatic disorder (PTSD): “the incidence of PTSD is very low in the general population, while drug abusers were over three times more likely to report a traumatic event and symptoms that meet clinically diagnostic criteria […] those experiencing both physical and sexual abuse were at least twice as likely to be abusing drugs than those who experienced either abuse alone” (Gordon, 2002, p. 117).

In summary, these experiments support and expand those of Manwell et al. (2009) in which it was demonstrated that URB597, which elevated endogenous levels of CB₁ ligands, facilitated extinction rates whereas the CB₁ antagonist SR141716A impaired extinction rates. These data suggest that the route of administration of Δ⁹-THC has important consequences for its resulting pharmacokinetic and behavioural effects, specifically, that pulmonary exposure facilitates, whereas parenteral exposure impairs, rates of extinction learning for conditioned cue aversion. Thus, pulmonary administered Δ⁹-THC may prove more beneficial for pharmacological potentiation of extinction learning for aversive memories, such as those supporting drug-craving/seeking in opiate withdrawal-syndrome or post-traumatic stress disorders.
CHAPTER 5:
GENERAL DISCUSSION

5.1. The ECB System Plays a Primary Role in Extinction Learning for Behaviours Motivated by Aversion and Not Reward

Recent evidence suggesting that the ECB system can be selectively manipulated to facilitate or impair the extinction of learned behaviours — specifically regarding drug-induced aversive memories — has important consequences for research on opiate withdrawal and abstinence. Data presented here support and expand previous findings suggesting that the ECB system functions primarily in the extinction of aversively motivated behaviors and is mediated by an increase in available endogenous CB₁ receptor agonists, primarily anandamide (AEA), and the exogenous CB₁ receptor agonist Δ⁹-THC, in a manner that is dependent upon both the dose and route of administration. These experiments clearly support a growing consensus in the literature that manipulations of the ECB system selectively modify extinction of aversive, but not appetitive, learning (e.g. Lutz, 2007; Harloe et al., 2008, Hölter et al., 2005; Niyuhire et al., 2007).

In the experiments discussed in detail in Chapter 2, Manwell et al. (2009) demonstrated that the fatty acid amide (FAAH) inhibitor, URB597, which blocks deactivation of endogenous CB₁ ligands, such as AEA, significantly facilitated extinction of a naloxone-precipitated morphine withdrawal-induced conditioned floor aversion, whereas the CB₁ receptor antagonist/inverse agonist SR141716 significantly impaired extinction. Several experiments demonstrated that neither the CB₁ antagonist AM251 nor the FAAH inhibitor URB597 had any effect on extinction learning for morphine-induced conditioned place preference. Similarly, URB597 did not affect extinction of operant
responding for sucrose reward, whereas the CB₁ antagonist/inverse agonist SR141716 did suppress operant responding during extinction, suggesting either a motivational or locomotor suppressant effect. In contrast, URB597 did significantly facilitate extinction learning of a naloxone-precipitated morphine withdrawal-induced conditioned aversion, whereas SR141716 impaired extinction rates. More importantly, the difference between the effects of URB597 and SR141716 emerged over repeated testing indicating that memory retrieval was unaffected and the effects were specific to extinction learning. These effects may have been mediated by elevated AEA in the BLA, an area of the brain that has been implicated in the expression of conditioned place aversion learning (e.g., Zanoveli et al., 2007; Lucas et al., 2008); this would be consistent with similar research on fear conditioning demonstrating that impaired extinction — but not acquisition or consolidation — of fear memory by the ECB system was mediated by elevated AEA, specifically in the BLA (Marsicano et al., 2002). These results demonstrate a selective effect of manipulation of the ECB system on learning motivated by an aversive stimulus, but not on learning motivated by a rewarding stimulus, and are consistent with other recent studies (Harloe et al., 2008; Niyuhire et al., 2007).

Experiments discussed in detail in Chapter 3 focused on method development for the experiments in Chapter 4 which were designed to evaluate the potential of the exogenous CB₁ ligand, Δ⁹-THC, to also facilitate extinction of an opiate-induced withdrawal-induced conditioned aversion. Since preliminary data suggest that Δ⁹-THC may produce differential effects on behaviour depending upon route of administration — and most studies in animals employ parenteral routes of cannabinoid administration, even though human cannabis users self-administer via pulmonary routes — a method was
developed for analysing cannabinoid levels in blood by liquid chromatography/mass spectrometry (LC/MS) to compare bioavailable levels of Δ⁹-THC and its primary psychoactive metabolite, 11-OH-Δ⁹-THC, after pulmonary (1, 5, 10 mg/pad vapour inhalation) and parenteral (0.5, 1.0, 1.5 mg/kg i.p. injection) administration. Thus, the experiments in Chapter 3 evolved in four directions: (i) separation and sensitive detection of cannabinoids, (ii) maximal extraction of cannabinoids from whole blood, (iii) comparison of extraction efficiencies between whole blood and RBC and plasma fractions, and (iv) comparison of recovery efficiencies after pulmonary and parenteral cannabinoid administration. The recovery efficiencies of cannabinoids after pulmonary and parenteral administration were assessed as accurate and reliable and demonstrated both dose- and time-dependent levels of Δ⁹-THC and its metabolite 11-OH-Δ⁹-THC in whole blood in rats.

Experiments in Chapter 4 were designed to meet three primary objectives: 1) to determine and compare the blood levels of Δ⁹-THC and its primary psychoactive metabolite, 11-OH-Δ⁹-THC, after pulmonary and parenteral administration, 2) to demonstrate the effects that the route of administration of Δ⁹-THC has on extinction learning, and 3) to further elucidate the involvement of the ECB system in extinction. Experiments determined that there were roughly equivalent mean blood concentrations after exposure to inhaled and injected Δ⁹-THC, showing both dose- and time-dependent concentration changes in Δ⁹-THC and its metabolite 11-OH-Δ⁹-THC. These results are consistent with the limited studies reported in the literature evaluating pulmonary and/or parenteral administration of Δ⁹-THC in rodents and related studies in humans. Regarding the second objective, the data from Experiment 4.2 found that exposure to pulmonary-
administered Δ⁹-THC also facilitated the rate of extinction learning of a naloxone-precipitated morphine withdrawal conditioned aversion, results which clearly support similar findings by Manwell et al. (2009) on the facilitory effects of URB597 on extinction learning. In contrast, the data from Experiment 4.3 found that parenterally-administered Δ⁹-THC either had no effect or impaired extinction rates; only 1.0 mg/kg i.p. Δ⁹-THC had any effect on the rate of extinction which was to significantly prolong the aversion up to 4-fold longer than the other groups. One explanation may be that parenterally-administered Δ⁹-THC takes longer to cross the blood-brain barrier and in doing so, has no effect or an aversive effect through repeated exposure every 24 h; first-pass metabolism of Δ⁹-THC in the liver may also produce more variable brain levels of Δ⁹-THC and 11-OH-Δ⁹-THC compared to pulmonary-administered Δ⁹-THC. Since pulmonary-administered Δ⁹-THC immediately crosses the blood-brain barrier, it may readily activate CB₁ receptors in greater density and distribution throughout the brain after repeated exposure every 24 h. Regarding the third objective, it is possible that interactions between the EOS and ECB systems mediated the effects reported here; although CB₁ agonism did not initially reduce the strength of the morphine-withdrawal CPA, it may have done so over repeated exposure to amounts of Δ⁹-THC and URB597 that then chronically activated CB₁ receptors.

These findings suggest that repeated CB₁ agonism, through increased AEA levels after FAAH inhibition by URB597 or exposure to inhaled Δ⁹-THC, facilitates extinction learning of the new CS-no US association rather than merely interfering with initial recall of the original CS-US association; the effect of URB597 and Δ⁹-THC on extinction of CPA required numerous re-exposures and emerged only after the first block of trials. In
comparison, repeated CB₁ antagonism/inverse agonism by SR141716 impaired extinction learning; a similar result occurred for injected Δ⁹-THC, although the specific mechanism of action for this result is unclear.

Several studies have distinguished between two processes initiated in memory retrieval — memory reconsolidation (i.e., stabilizing expression of the original association) and extinction learning (i.e., weakening expression of original association) — and identified their distinct temporal and biochemical markers based on strength and age of the memory at the time of retrieval (Santini et al., 2004; Suzuki et al., 2004). For example, Suzuki et al. (2004) demonstrated that protein synthesis and functional CB₁ and NMDARs are necessary for extinction, but not for consolidation or reconsolidation. Recently, Myers and Carelzon (2010b), demonstrated that D-cycloserine (DCS), which is an NMDAR partial agonist, also facilitated extinction learning of a naloxone-precipitated CPA in male Sprague-Dawley rats, similar to the results reported in Experiments 2.3 and 4.2 for URB597 and inhaled Δ⁹-THC respectively. Myers and Carelzon (2010b) suggested that the mechanism of action of DCS involved a neural pathway in common with fear conditioning because DCS also facilitates extinction of conditioned fear in rats and is mediated by the BLA (e.g., Walker et al., 2002; Ledgerwood et al., 2003). Thus, it is possible that CB₁ and NMDARs are key components of neural pathways involved in the extinction of aversively conditioned cues and that their repeated activation is necessary to induce molecular signaling cascades that trigger the protein synthesis required for consolidation of the CS-no US association in extinction learning. Indeed, molecular studies of long-term memory have established that both gene expression and protein synthesis are necessary for consolidation of memory (reviewed in Kandel, 2001),
including classical fear conditioning (Ressler et al., 2002; see Lamprecht and LeDoux, 2004, for review), and gene expression in the BLA has been proposed to trigger secondary transcriptional cascades that stabilize synaptic changes, including transcription factors, cytoskeletal proteins, adhesion molecules and receptor stabilization molecules (Rosen et al., 1998; Ressler et al., 2002). Thus, it is possible that repeated activation of CB\textsubscript{1} receptors results in downstream signaling cascades that produce enduring changes in synaptic connections; this also makes it possible that a partial and/or more variable activation of CB\textsubscript{1} via parenterally-administered Δ\textsuperscript{9}-THC could have been a mediating factor in the failure of injected Δ\textsuperscript{9}-THC to facilitate extinction learning.

5.2. Possible Mechanisms of ECB Action on Extinction Learning

Considered collectively, these experimental findings raise a series of related questions regarding the role of ECBs in extinction learning for memories associated with drugs of abuse. First and foremost, what are the biological mechanisms of action of the ECB system that are involved in extinction learning? Is the action primarily at the level of learning or motivation? Is the manipulation of the ECB system during extinction affecting the original opioid–induced memory (CS–US) directly (e.g., impairing retrieval of the original memory), the process of new learning (CS–no US) (e.g., facilitating consolidation of the new memory), or some other motivational aspect of behaviour? Is the effect involving regulation of other neurotransmitter systems (e.g., dopamine, norepinephrine, corticotropin-releasing factor (CRF), etc…)? Finally and most importantly, how can these mechanisms of ECB action be manipulated to normalize
dysregulated MDS responses in order to help individuals in treatment to re-exert control over drugs of abuse?

Indeed, studies across many different behavioural conditioning paradigms indicate that, similar to conditioning, extinction memory is not localized to a single anatomical structure (reviewed in Quirk and Mueller, 2007), but rather is disseminated throughout the brain, as summarized by Myers and Davis (2002, p. 569): “the “excitatory” and “inhibitory” responses to a CS may be orchestrated by different brain structures (e.g., amygdala versus prefrontal cortex), different populations of cells within a structure (e.g., glutaminergic versus GABAergic neurons), or different types of molecules within individual cells (e.g., kinases versus phosphatases; activators versus repressors of transcription).” Thus, there are several possible mechanisms of action of ECBs during extinction that can help answer these pivotal questions, which may act independently or in concert, and can be summarized as follows:

a) ECB action is at the level of associative memory formation (incentive learning) (i.e., biological transient neuroadaptations and positive and negative drug reinforcement) and distinctions can be made between learning-based and memory-based interactions (see Myers and Carlezon, 2010a);

b) ECB action is at the level of motivation (emotional salience) (i.e., transient changes in affective state induced by the drug alters the strength of the response to environmental cues) (see Myers and Carlezon, 2010a);

c) ECB action is dependent upon pharmacokinetics and pharmacodynamics which determine the allostatic state of the mesolimbic dopaminergic reward system (MDS) (e.g., the primary “allostatic driver” of the shift from use to abuse
to dependence is physiological withdrawal (could also be conditioned withdrawal) (see McEwen, 2005; Erickson, 2007); the route of administration, metabolic rate, and dose interact to produce differential processing of context-dependent cues associated with conditioned reward or conditioned withdrawal) (see Murray and Bevins, 2010).

5.2.1. ECB Action at the Level of Associative Memory Formation and Motivation

ECB action plays an important role in both associative memory formation (incentive learning) and motivation (emotional salience) and relevant distinctions of its role can be made between learning-based and memory-based interactions (see Myers and Carlezon, 2010a). If the ECB system’s primary level of action was merely one that generally affected molecular memory, for example, through regulation of neurotransmitter release, phosphorylation, signaling cascades, and/or formation/retention of dendritic spines, then similar results would be expected for the extinction of CPP and CPA, which is not consistent with experimental findings thus far. Evidence supports the conclusion that the function of the ECB system in extinction learning is different depending on whether the original memory is associated with a rewarding (e.g., morphine) or aversive (e.g., morphine withdrawal) stimulus and thus plays a unique and specific role in the extinction of aversively-motivated behaviours. This may be a consequence of the different neural circuits involved in the acquisition and expression of CPP and CPA that are then reactivated during extinction learning; this may be especially important for opiate-induced conditioning because of interactions between the EOS and ECB systems.
The role of the ECB system in extinction learning may also be particularly relevant to CPA, compared to CPP, because of the multiple related associations that are learned. For example, the procedures involved in the acquisition of CPA typically require some prior exposure to a rewarding stimulus (e.g., morphine, US₁) that may become associated with a specific context (CS₁), which is then followed by exposure to an aversive stimulus (e.g., morphine withdrawal, US₂) either in the same context (CS₁) or more likely a different context (CS₂). In fact, there are key neural circuits in the incentive-motivational mesolimbic dopaminergic system (MDS) that are activated during opiate-induced CPP and CPA (i.e., cues paired with drug administration are known to activate limbic and cortical regions and can induce transient and enduring gene-regulatory processes), but these areas do not overlap completely and can be dissociated, including cues paired with both acute (precipitated) and conditioned withdrawal (reviewed in Myers and Carlezon, 2010a). For example, in opiate-induced CPP, cues paired with morphine-exposure induce conditioned gene expression of Fos protein in the NAcc shell, cingulate gyrus, PFC, and preoptic areas of rats, which may “reflect the cognitive, sensory, or ‘anticipatory’ aspects of conditioning” (Schroeder et al., 2000, p. 151; Schroeder and Kelley, 2002); however, no significant Fos expression is found in the BLA, sensorimotor cortex, or NAcc core. However, in opiate-induced CPA, the BLA and extended amygdala (the central amygdala (CeA), bed nucleus of the stria terminalis (BNST), and NAcc) all show c-fos expression in addition to the VTA, locus ceruleus (LC), hippocampus, and hypothalamus (Frenois et al., 2005). Furthermore, c-fos expression differs primarily at the level of the amygdala when comparing acute withdrawal (retrieval memory) and conditioned withdrawal (memory formation); there is
a double dissociation between the BLA and CeA such that *c-fos* expression is increased in the CeA and decreased in the BLA in acute withdrawal rats, but decreased in the CeA and increased in the BLA for conditioned withdrawal rats (Frenois et al., 2005). In comparison, similar patterns of strongly increased *c-fos* expression in a subpopulation of VTA dopamine neurons were found after naloxone-precipitated morphine (acute) withdrawal and re-exposure to the withdrawal-paired environment (conditioned withdrawal).

These findings demonstrate that similar, but not identical, neural circuits specifically involving the amygdala and VTA underlie both the initial formation of an association — between stimuli and an aversive withdrawal state — and subsequent retrieval of that learned association; this may be the neural mechanism mediating the *incentive-motivational processes* involved in the development of drug seeking behaviour and avoidance of acute and conditioned withdrawal (Frenois et al., 2005). Thus, if activation of subpopulations of neurons in the amgydala are critical during acquisition and retrieval of opiate-induced conditioned withdrawal, their reactivation during extinction may also be necessary in facilitating extinction learning. This would be congruent with Reijmers et al.’s (2007) elegant demonstration that, in the extinction of conditioned fear, the strength of the CS-US association (as indicated by freezing behaviour to the CS) is positively correlated with the number of reactivated neurons in the amygdala; it is so specific that subpopulations of reactivated neurons can be determined for memory of the context (neurons in the BLA) versus memory of the tone (neurons in the LA only) (Reijmers et al., 2007). If neurons in the amygdala are activated by acute and conditioned opiate withdrawal (Frenois et al., 2005), but not opiate-induced
CPP (Schroeder et al., 2000; Schroeder and Kelley, 2002), then it may be the activity of these neurons the ECB system modifies during extinction learning.

Evidence further suggests that reexposure to aversively-conditioned cues results in increased production of AEA which facilitates memory extinction by long-term depression (LTD) of GABA-mediated inhibitory currents in the BLAC; thus, extinction of aversively-conditioned cues may be mediated by a CB₁-induced decrease of activity of inhibitory networks in the BLA leading to disinhibition of principal excitatory neurons (Marsicano et al., 2002). Elevating AEA levels via FAAH-inhibition has also been proposed to be a more functional mechanism of facilitating extinction rather than mere exposure to even a direct acting CB₁ receptor agonist (Varvel et al., 2007). AEA appears therefore to be involved in extinction of conditioned fear and other learned behaviours that are associated with aversive stimuli. Thus, for extinction learning of aversive memories conditioned with opiates, the ECB system may exert its effects through neural connections in the BLA and CeA.

Considering the role of the amygdala in the formation and retrieval of opiate-withdrawal memories, the findings reported in Chapters 2 and 4 — that elevated levels of both endogenous and exogenous cannabinoids altered extinction rates of acute naloxone-precipitated morphine withdrawal conditioned aversions in rats — suggest that cannabinoids act to modify the intensity of the withdrawal state, and thus are involved in regulating the motivational aspects of the memory, rather than just retrieval of the memory. It is also likely that altering the intensity of the retrieved memory (e.g., initial CS-US association) affects learning of the new memory (e.g., CS-no US association). The unique action of the ECB system is very interesting for this reason; it is necessary for
extinction learning of the new CS-no US association, which also requires acquisition, consolidation and retrieval of that new association, that then interferes with expression of the original CS-US association (reviewed in Lutz, 2007; Quirk and Mueller, 2008; Manwell et al., 2009). In laboratory studies with animals, both acquisition and expression of associative memory can be strengthened or weakened; however, “acquisition studies are complicated by the possibility that an experimental manipulation could modulate the intensity of acute drug effects or acute withdrawal as opposed to modulating learning about the relationship between cues and the drug or withdrawal state” (Myers and Carelzon, 2010a, p. 290). This supposition holds true for extinction learning, which also has an acquisition component; increasing or decreasing the salience of the extinction drug during the acquisition phase of extinction learning may also modulate the effect of that drug on the rate of extinction. For example, Experiment 4.2 included a component that may have increased the salience of the extinction drug — pulmonary-administration of Δ⁹-THC required an additional step in a unique context — and thus affected the rates of extinction learning in a manner that was different than parenterally-administered Δ⁹-THC. The result may have been that the introduction of an additional context paired with inhaled Δ⁹-THC initiated acquisition of a different CS-US association that shifted the memory retrieval process more towards extinction (CS-no US) as opposed to reconsolidation (CS-US) of the CPA. However, it is unlikely that this accounts entirely for the difference between inhaled and injected Δ⁹-THC because both injected URB597 and inhaled Δ⁹-THC facilitated extinction in similar manner after the first block of trials.

The fact that extinction training in the presence of URB597 and Δ⁹-THC also did not affect naloxone-primed reinstatement demonstrates that the original CS-US
association may be stronger than the CS-no US association. Similarly, McCallum et al. (2010) demonstrated that preconditioning with URB597 did not have an effect on naloxone-primed reinstatement of CPA, but URB597 was also not used during the extinction trials. It would be predicted that if it were used during both extinction training and reinstatement it would have an effect. For example, if either URB597 or Δ⁹-THC were present during the naloxone-reinstatement both may have had an effect by either blocking the retrieval of the original CS-US association or facilitating retrieval of the new CS-no US association – the latter explanation would be more consistent with the process of extinction learning demonstrated for both URB597 and Δ⁹-THC.

5.2.2. ECB Action Dependent Upon Pharmacokinetics and Pharmacodynamics

Action of the ECB system at the level of associative memory formation (incentive learning) and at the level of motivation (emotional salience) may be dependent upon pharmacokinetics and pharmacodynamics which determine the allostatic state of the MDS (see McEwen, 2005; Erickson, 2007). The pharmacokinetics of cannabinoids, particularly Δ⁹-THC, are dependent upon the route of administration, absorption by the body, distribution to tissues, metabolism by enzymes (mainly in the liver) and elimination from the body; the pharmacodynamics of cannabinoids are the mechanism of action on the body, for example, toxicity, tolerance, withdrawal, dependency, polydrug interactions, and therapeutic potential, and are mostly mediated through agonistic or antagonistic activation of their endogenous receptors (reviewed in Grotenhermen, 2003). Both the pharmacokinetics and pharmacodynamics of cannabinoids can have an effect on the allostatic state of the individual. According to McEwen (2005, p. 315), allostatic is a
more precise term that clarifies important differences between the protective and damaging effects of the body’s response to stressors: “Allostasis refers to the adaptive processes that maintain homeostasis through the production of mediators such as adrenalin, cortisol and other chemical messengers.” Thus, allostatic states can be adaptive during acute stress as they reestablish homeostasis, but can become maladaptive during chronic stress, also known as “allostatic overload,” and can ultimately lead to exhaustion of the system and pathophysiology (McEwen, 2005, p. 315). In terms of the development of substance use disorders (SUDs), Erickson (2007) describes physiological withdrawal as the primary “allostatic driver” of the shift from substance use to abuse to dependence. Taken together, the allostatic state may, in the short term, help the system adapt to repeated exposure to a drug, for example, through a conditioned compensatory response (CCR) (Siegel, 1975; Seigel et al., 1982; Siegel et al., 2000), defined as “a conditioned homeostatic mechanism triggered by drug-paired cues that serves to maintain the system at or close to its normal set point in the presence of drugs that otherwise would perturb this balance” (reviewed in Myers and Carlezon, 2010, p. 286). However, in the long term, the allostatic state loses its functional adaptability with increasing “overload” predisposing the individual to disease, for example, as the drug is increasingly required to merely maintain normal functioning which is severely compromised in the withdrawal state (reviewed in Myers and Carelzon, 2010).

In the development of drug dependence, the route of administration, metabolic rate, and drug dose can all interact to produce differential processing of context-dependent cues associated with conditioned reward or conditioned withdrawal, that are observed in drug-seeking and drug-taking behaviours. The route of exposure can affect
the potency of Δ⁹-THC; for example, intravenous administration of Δ⁹-THC resulted in 45-fold greater antinociceptive activity than subcutaneous administration in mice (Martin, 1985). The route of exposure can also affect the rate of absorption and metabolism of Δ⁹-THC, and how Δ⁹-THC interacts temporally and spatially with receptors in the body (i.e., how long it takes for ligand-receptor binding and the extent of distribution within the circulatory and nervous systems) (reviewed in Grotenhermen, 2003, Koob and LeMoal, 2006, and Murray and Bevins, 2010). There are also important sex differences in the pharmacokinetic and behavioural effects of Δ⁹-THC between males and females (reviewed in Fattore and Fratta, 2010) and age differences between adolescent and adult rodents (Shramm-Sapyta et al., 2007). Specifically, parenteral administration of Δ⁹-THC in rats produces similar serum and brain levels of Δ⁹-THC in males and females, but greater levels of Δ⁹-THC metabolites in females; these differences corresponded to greater Δ⁹-THC precipitated behavioural effects and may be attributed to sex differences in CYP450 enzymes that metabolize Δ⁹-THC and sequestration of cannabinoids in fat tissues (Tseng et al., 2004). Parenterally-administered Δ⁹-THC also induces greater levels of ACTH, more anxiogenic behaviours, less locomotion, and is more aversive at higher doses in male adult rats compared to male adolescent rats (Schramm-Sapyta et al., 2007).

There are only a few studies that have directly compared the behavioural effects of parenteral and pulmonary routes of administration of Δ⁹-THC in rodents (e.g., Wilson et al., 2002, 2006; Niyuhire et al., 2007). First, Wilson et al. (2002) found that in male mice, intravenous and inhaled Δ⁹-THC produced similar levels of Δ⁹-THC in blood and brain at the antinociceptive ED₅₀ dose (median effective dose of 30 mg) and that the behavioural effects of inhaled Δ⁹-THC (antinociception, hypomotility, catalepsy, and
hypothermia) were all significantly antagonized by co-administration of SR141716. Second, Wilson et al. (2006) subsequently reported that although SR141716 induces withdrawal signs (e.g., paw tremors) in mice chronically exposed to either inhaled marijuana smoke or intravenous Δ⁹-THC, only co-administration of intravenous Δ⁹-THC reversed these effects; inhaled marijuana smoke did not prevent precipitated withdrawal by SR141716 (Wilson et al., 2006). To account for this unexpected finding, Wilson et al. (2006) suggested that levels of Δ⁹-THC from the marijuana smoke in the brain after inhalation were insufficient to reverse the effects of SR141716; thus, the mechanism of action was still likely CB₁-mediated. This conclusion was supported by additional findings of a dissociation between Δ⁹-THC levels found in blood and brain that were dependent upon the route of administration; although blood and brain levels were roughly equivalent following inhalation, brain levels were 200-300% greater in brain than in blood after intravenous administration (Wilson et al., 2006). Third, Niyuhire et al. (2007), also using male mice, found that both inhaled marijuana smoke and injected Δ⁹-THC impaired both acquisition and retrieval memory in the Morris water-maze task and that co-administration with SR141716 reversed these effects. This suggests that the mechanism of action was also CB₁ mediated (Niyuhire et al., 2007). Thus, the results of Experiments 4.2 and 4.3 support and expand the limited evidence in the literature that parenteral and pulmonary routes of administration of Δ⁹-THC have potentially different and important pharmacologic effects on behavior.

5.2.3. Can ECB Action Modify Dysregulated MDS Responses in Treatment?

As the transition from drug use to abuse and dependence proceeds, there is a corresponding change in neural circuits, particularly those in the MDS, that mediate the
incentive properties of the drug and stimuli associated with it (reviewed in Self, 2004, p. 249): “Many enduring changes could directly influence the motivational response to drugs and environmental stimuli that trigger drug seeking, whereas other short-lived changes could facilitate incentive learning during chronic drug use and, thus, strengthen drug- and alcohol-related memories.” Self (2004) describes two possible mechanisms of action — involving learning-based versus memory-based systems — that may underlie enduring changes in neuroadaptation and behaviour following transient and chronic drug use and neuroreadaptation after cessation of drug use (see Figure 5.1); both have relevance to the high rate of relapse in users abstinent for months and years following cessation.

The first mechanism involves the transient neuroadapations in response to repeated drug exposure and that alter the strength of the reinforcing properties of the drug; for example, the positive reinforcing effects of the drug (e.g., euphoria) shift to negative reinforcing effects in the absence of the drug (e.g., anxiety and depression). Consequently, there is an increase in the strength of incentive learning such that even after somatic symptoms of withdrawal are absent, the conditioned effects of withdrawal trigger persistent drug-seeking behaviour that is resistant to extinction (see Figure 5.1 A).

The second mechanism, rather than reflecting incentive learning, involves neuroadaptive changes in the strength of the memory itself and occurs during the withdrawal period; for example, during cessation of drug use, there is a time-dependent facilitation of memories that result in increased drug-seeking behaviour (see Figure 5.1 B).
Figure 5.1. Interaction between short- and long-term neuroadaptations to chronic drug use and mechanisms of learning and memory.

(A) Short-term neuroadaptations can sensitize positive reinforcement mechanisms, but also facilitate negative reinforcement mechanisms by causing dysphoria, anxiety and depression in early withdrawal that are temporarily alleviated by drug use. Increases in both positive and negative reinforcement mechanisms would facilitate incentive learning (shown in black), leading to persistent increases in the motivational strength (salience) of drug-related memories even after short-term neuroadaptations normalize in long-term withdrawal (shown in gray); (B) Neuroadaptations that arise during withdrawal from drug use could directly facilitate the motivational strength of drug-related memories (shown in black), thereby increasing the propensity for relapse in prolonged abstinence.

*Reprinted from Self (2004, Figure 4, p. 250), Regulation of drug-taking and –seeking behaviors by neuroadaptations in the mesolimbic dopamine system. Neuropharmacology, 47, (Supplement 1), 242-255, with permission from Elsevier and author.
Conditioned incentive learning is thought to play a pivotal role in craving and drug-seeking behavior, thus clinical treatments that enhance extinction of conditioned incentive learning may be useful in controlling cravings in recovery and relapse prevention. Preclinical studies of the neural circuitry regulating substance use have reported involvement of CB$_1$ receptors in the consumption of alcohol (Mechoulam and Parker, 2003; Wang et al., 2003; Hungund and Basavarajappa, 2004; Colombo et al., 2004; Gessa et al., 2005), cocaine (Arnold, 2005), 3,4-methylenedioxy-methamphetamine (Braida et al., 2005; Sala and Braida, 2005), nicotine (Castañé et al., 2005; Cohen et al., 2002, 2004), and opioids (Fattore et al., 2004, 2005, Viganò et al., 2005). Thus, further evaluation of the CB$_1$-mediated mechanism of facilitated extinction is required.

Negative affective states associated with opiate withdrawal, either somatic or conditioned, are believed to be involved in relapse to drug-craving/seeking behaviour, even after long periods of opiate abstinence. The endogenous cannabinoid and opioid systems share overlapping neural chemistry and circuitry that underlie the reward-related brain mechanisms of addictive behaviours; thus the ECB system may play an important role in modifying the dysregulated MDS responses in individuals undergoing substance dependence treatment, particularly for those addicted to opiates. The incentive-motivational properties of various drugs of abuse can be attenuated by the ECB system (reviewed in Piomelli, 2003; Le Foll and Goldberg, 2005; Maldonado et al., 2006; Justinova et al., 2009) and thus FAAH inhibitors, which increase natural levels of endogenous cannabinoids, are being studied widely, in preclinical research, as potential pharmacological treatments for drug addiction and related cognitive dysfunction (reviewed in Panlilio et al., 2013).
Several lines of experimentation demonstrate that the ECB system selectively affects aversively-motivated memories and the extinction of withdrawal-conditioned behaviours. Since the ECB system is naturally activated “on-demand” (Kano et al., 2009), the release of ECBs in specific brain regions may also attenuate activation of neural circuits in a selective manner. For example, in the VTA, dopamine neurons play a central role in reward-related behaviours, motivation and cognition (Shultz, 2002). Depolarized VTA neurons produce and release ECBs “on demand” which then transiently suppress excitatory inputs by retrograde presynaptic activation of CB<sub>1</sub> receptors; a functional ECB system is necessary for the retrograde signaling between PFC and VTA synapses (Melis et al., 2004). The “on demand” function of the ECB system (Kano et al., 2009) plays a key role in coordinating synaptic signaling that tightly controls VTA DA neuron firing patterns. For example, in vivo, CB<sub>1</sub> activation decreases the probability of VTA DA neuron “spiking and bursting” induced by PFC stimulation, suggesting that the ECB system ultimately modulates “distinct physiological and behavioral functions depending upon the target area” (Melis et al., 2004, p. 10712).

In addition, preclinical research indicates that FAAH inhibitors may be important in treating anxiety and stress related disorders, such as PTSD and depression, which have a high comorbidity with substance abuse (reviewed in Panlilio et al., 2013). Studies also indicate that, in comparison to Δ<sup>9</sup>-THC, FAAH inhibitors such as URB597 do not produce dependence and their effects are more localized to specific populations of neurons expressing CB<sub>1</sub> receptors, rather than producing systemic effects on all CB<sub>1</sub> expressing neurons (reviewed in Panlilio et al., 2013). Thus, pharmacological potentiation of extinction learning, in conjunction with behavioural exposure therapy,
may prove beneficial for treatment of a variety of psychopathologies hallmarked by an inability to extinguish maladaptive behaviours.

There exists a substantial body of preclinical research demonstrating the role of various neurotransmitters and transporters in the processes of neuroadaptation and neuroreadaptation to drugs of abuse, but there is still sparse and insufficient clinical research on the development of these potential targets for substance use disorders (SUD) medications (Volkow and Skolnick, 2012). For example, clinical research on the role of endogenous cannabinoids in regulating stress responses is currently underway. Most recently, Dlugos et al. (2012) demonstrated that psychosocial stress produced significantly elevated levels of serum ECBs, specifically anandamide, and that basal levels of anandamide were negatively correlated with anxiety ratings, suggesting that ECBs play a neuroprotective role in anxiety. The Centre for Addiction and Mental Health in Toronto has recently employed the first clinical use of an analog of URB597 as a positron emission tomography (PET) radiotracer, \([^{11}\text{C}-\text{carbonyl}]\)-URB694 \((^{11}\text{C}\text{CURB})\), to image FAAH activity in humans (Rusjan et al., 2013). Since the ECB system is implicated in several psychiatric disorders, Rusjan et al. (2013) successfully attempted to be the first to map FAAH activity, via imaging and quantification, demonstrating that binding of the URB597 analog is well identified, reversible, and found within the cerebral cortex gray matter, basal ganglia, thalamus, and cerebellum. This study leads the way for evaluating differences in FAAH activity and ECB levels in the brains of patients with psychiatric and substance use disorders.
5.3. Limitations and Future Directions for Research

The findings reported in Ch. 2 and 4 clearly demonstrate the role of the ECB system in the extinction of learned behaviours motivated by opiate-induced conditioned withdrawal; elevating endogenous levels of anandamide by FAAH inhibition and exposure to vapourized $\Delta^9$-THC significantly facilitated rates of extinction learning of CPA, whereas CB1 antagonism and injected $\Delta^9$-THC impaired extinction rates. The dose- and route-dependent effects of $\Delta^9$-THC on extinction rates of CPA demonstrated here suggest that further experimentation would be useful to determine the mechanisms underlying the differential effects.

First, levels of $\Delta^9$-THC and 11-OH-$\Delta^9$-THC should be measured in the brain after pulmonary and parenteral administration of $\Delta^9$-THC to determine if there is a dissociation between blood and brain levels as suggested by some studies (Wilson et al., 2006); the effects for both male and female rats should be compared directly (Tseng et al., 2004).

Second, activation of the HPA system could be assessed by evaluating levels of corticosterone and ACTH that may be produced and/or elevated after $\Delta^9$-THC exposure and if the route of administration has an effect on blood and brain levels of stress hormones; this should be assessed in both adolescent and adult rats (Schramm-Sapyta et al., 2007). Future experiments could evaluate levels of corticosterone in rats exposed to various doses of pulmonary versus parenterally administered $\Delta^9$-THC and/or extinction rates for CPA in the added presence of exogenous corticosterone.

Third, endogenous levels of anandamide were not directly measured and thus it would be useful to evaluate those specifically in the amygdala which is involved in the expression of CPA learning (e.g., Zavoveli et al., 2007; Lucas et al., 2008). It would also
be important to evaluate how these levels change over the weeks of repeated exposure to FAAH inhibition and chronic exposure to $\Delta^9$-THC; such experiments would provide important information on drug-induced neuroadaptations followed by neuroredaptation after successful extinction learning. For example, although both URB597 and inhaled $\Delta^9$-THC significantly facilitated extinction learning and maintained extinction for up to a week after exposure ceased, neither were able to prevent reinstatement of the CPA; of course it is possible that they could have prevented reinstatement if administered during reinstatement testing, which they were not. Thus, both have *neuroadaptive* effects on the brain in their presence and in their absence lasting up to at least a week, but no effects that are representative of longer-lasting *neuroreadaptation* in the brain in their absence.

Finally, another important follow-up study should evaluate the effect of future drug-seeking behaviour after successful extinction. For example, it would be important to determine if prior exposure to chronically elevated endogenous and/or exogenous brain levels of cannabinoids potentially impairs — or possibly even facilitates — drug-induced CPP for various types of drugs, including opiates and marijuana. Thus, it is critical to elucidate the mechanisms underlying how these drugs may contribute to future expression of drug-seeking behaviours.
APPENDIX A

Towards a Global Humanitarian Perspective on Drug Addiction

A review by Statistics Canada estimated that 13% of the population (3.1 million people) over the age of 15 had tried or regularly used illicit drugs, and of these people, nearly 200,000 were considered dependent on illicit drugs; cannabis was the most commonly cited drug of choice at 10% (Tjepkema, 2004). Another report by Statistics Canada on drug offences, which cited over 100,000 police-reported drug offences in Canada, found that this represented a 30 year peak in drug-related crime; cannabis offences constituted the majority of these offences (Dauvergne, 2009). Indeed, the physical, emotional, and socio-economic costs of drug abuse for people are well known. However, it is perhaps underappreciated, by society in general, how exposure to drugs that interact with the endogenous cannabinoid and opioid systems can lead to dysregulation of those systems, resulting in increased drug use, abuse and eventually dependence. What needs to be better understood are the neurophysiologic mechanisms that are involved in the development of addiction. More importantly, the mechanisms by which the behaviours associated with drug addiction – such as drug-craving and drug-seeking – can be successfully extinguished and be prevented from recurring, particularly after long periods of abstinence.

Drug addiction is a multi-factorial humanitarian problem requiring global interdisciplinary research and collaboration; it also requires deep compassion for each and every one of the human beings it affects. Accordingly, the United Nations Office on Drugs and Crime (UNODC) has increasingly shifted its mandate towards research “based on a public health and human rights approach,” including “drug prevention, treatment and
rehabilitation efforts focused on decreasing vulnerability among at-risk groups, including women, youth, prisoners, people who have been trafficked and people living with HIV/AIDS” (UNODC, 2010, p. 43). Indeed, within global criminal markets, estimated annual profits in the past decade from the opiate trade alone are more than double those from trafficking in persons and firearms combined (UNODC, 2010); ironically, enormous profits from the drug trade substantially support both arms and human trafficking, further perpetuating the toxic effects of the illegal drug trade on societies across the globe (Naím, 2005; Malarek, 2006; Batsone, 2007).

Even the UNODC (2009, p. 19) admits that, although more needs to be done to enforce drug laws — the purpose of which is to “deter, incapacitate, and rehabilitate drug offenders” — for problematic drug users, who consume the greatest amount of illicit drugs, further resources must be aimed at intensive intervention and treatment, otherwise profitability will continue to drive drug traffickers into “displacement” markets, essentially replacing lost buyers and substances with new ones, thus perpetuating the spread of crime and corruption. Ironically, the largest population of opioid drug users in the USA takes prescription pain relievers; in 2007, an estimated 5.2 million people misused pharmaceutical opiates (e.g., oxycodone) for non-medical purposes, leading to an overall increase in opiate-dependent treatment admissions and deaths due to poisoning (UNODC, 2009). Included in the initiatives of the Joint UNODC-WHO (World Health Organization) Programme on Drug Dependence Treatment and Care are the mandates to “recognize drug dependence as a preventable and treatable multi-factorial health disorder” and to “recognize the social advantages of investing in treatments” (UNODC, 2009, p. 86). Indeed, one of the world’s greatest
challenges is to create international control systems supporting the production of drugs, such as opium and cannabis, for medical purposes, with provisions to prevent digression to illicit markets.

Accordingly, the joint UNODC-WHO’s recent mandates to “recognize drug dependence as a preventable and treatable multi-factorial health disorder” and to “recognize the social advantages of investing in treatments” (UNODC, 2009, p. 86) are finally congruent with the vast body of research on the development and persistence of substance use disorders reported by the scientific community. Yet the stigma of illegality that is typically associated with substance use disorders is still pervasive and preventing progressive research and treatment for the most vulnerable individuals worldwide. Even the UNODC admits that one of its most significant challenges today is to create international control systems supporting the production of drugs, such as opium and cannabis, for medical purposes, while implementing concurrent measures that help prevent digression of these drugs into illicit markets (UNODC, 2009). Just as importantly, geopolitics also plays a huge role in the persistence of drug use in society, from the failed War on Drugs to the War on Terror (reviewed in Maté, 2008; Scott, 2007, 2010; Greenwald, 2011). For example, the U.S.-led War on Drugs has had significant negative effects on creating solutions for treating drug-dependent individuals worldwide (reviewed in Maté, 2008). In March 2010 in Vienna, the United Nations’ meeting on Drugs and Crime ended in gridlock between the U.S. and several other countries on how to combat illicit drugs with its persistent opposition to “harm reduction” strategies, which include methadone clinics and medically observed needle exchange and use sites for the most at risk of disease and abuse, and the use of medical marijuana to treat many diseases
and ailments (UNODC, 2010). Shockingly, in spite of the fact that the U.S.-led War on Terror has resulted in record levels of Afghan opium production and distribution (Scott, 2010), it continues to oppose decriminalization and treatment-based solutions in favor of prosecution and incarceration (UNODC, 2010). Not surprisingly, the U.S. also has the highest prison population per capita in the world, including the greatest number of people in prison for nonviolent drug-related offences, such as marijuana possession (Maté, 2008; Greenwald, 2011). A brief report by The National Institute on Drug Abuse (NIDA) suggests that one of the challenges to clinical research on substance use disorder (SUD) medications is the stigma associated with treating disorders involving largely illegal substances (e.g., heroin and marijuana); for example, this type of research is impeded by a) the U.S. Federal Drug Administration’s (FDA) stance that abstinence, rather than harm reduction, is the only approach with demonstrated efficacy, b) similar marginalization of patients with chronic SUDs by the healthcare community, and c) lack of economic interest on the part of major pharmaceutical companies (Volkow and Skolnick, 2012). It is critically important to remove these barriers because SUDs are often comorbid with other psychiatric disorders, specifically anxiety and mood disorders such as PTSD, major depressive and bipolar disorders, and other mental illnesses such as schizophrenia, and need to be treated in tandem (Felitti et al., 1998; Dube et al., 2003; Gordon, 2002; Whitfield et al., 2005; Schilling et al., 2007; Isohookana et al., 2013). Thus, one major research question that needs to be answered — in order to create viable solutions — is whether or not drug use is primarily a medical, social or criminal problem.

Growing evidence from neuroscience indicates that drug abuse and dependence arises from a complex interplay of biological, psychological and social factors, and that emphasis should be placed on understanding how early exposure to drugs of abuse and stress produces
maladaptive functional changes during neurodevelopment that can create lifelong vulnerabilities to drug abuse and dependence. It is evident from many studies that drug abuse and dependence is co-morbid with post-traumatic stress disorder and a history of abuse (Dube et al., 2003). The impact of these experiences on the brain are increasingly pointing to addiction as being a medical problem; for example, dysregulation of neurotransmission and brain alterations putatively predispose people to chemical dependence on drugs that mimic certain endogenous ligands to neuroreceptors in the brain (reviewed in Riddle and Pollock, 2003). For example, changes in the nervous system arise in response to changes in the internal or external environment though a process known as neural adaptive plasticity, or neuroplasticity; the greatest potential for neural adaptive plasticity occurs during infancy and early childhood when the brain is the most malleable. Many studies suggest that there are enduring structural and functional changes in the mammalian brain due to prenatal stress and early drug exposure (Andersen, 2003). Importantly, drugs of abuse and stress exposure also both trigger a common synaptic alteration in dopamine neurons that can perpetuate drug-seeking and drug-taking behaviours (Saal et al., 2003; Stewart, 2008). Research should also extend beyond preclinical work, with an emphasis on optimizing treatments for substance use disorders and comorbid psychiatric disorders. Pharmacologic interventions would target the specific neural circuits known to be involved in maladaptive behaviours underlying drug dependence and behavioural treatments would focus on ameliorating or reversing deficits by using experience-dependent learning strategies known to moderate brain regions involved in relapse. Ideally, treatments supporting abstinence from drugs of abuse must reduce the potential for both drug exposure and various stressors to trigger relapse to drug-seeking; blocking these effects at neural levels
may significantly reduce rates of relapse (Andersen, 2003; Saal et al, 2003; Singh et al., 2006; Stewart, 2008; Galve-Roperh et al., 2009).

One future research program should focus on greater understanding of how early exposure to drugs of abuse and stress produces maladaptive functional changes during neurodevelopment and designing treatments for neurological disorders arising from drug-taking and adverse experiences. Indeed, two of the primary predictors of drug use and abuse are early exposure to drugs and stress, particularly at critical points in neurodevelopment (Andersen, 2003). Exposure to drugs and stress during critical periods of brain development can have lasting effects resulting in learning and memory deficits, and a lifetime of vulnerability to psychological disorders and drug abuse. Adverse Childhood Experiences (ACEs) are defined as disturbing or stressful childhood exposures which have serious and enduring effects on the child’s development (Dube et al., 2003). ACEs are categorized as exposure to abuse (physical, emotional, or sexual), neglect (physical or emotional), and growing up in a dysfunctional household with parental discord (battered spouse, separation, or divorce), mental illness, substance abuse, and/or an incarcerated family member (Dube et al., 2003). These disturbing and stressful childhood exposures are reported to contribute significantly to later mental and physical health problems, including drug abuse, alcoholism, depression, non-suicidal self-injury, suicide attempts, antisocial behaviour, hallucinations, chronic heart and lung disease, cancer, liver disease, and skeletal fractures (Felitti et al., 1998; Whitfield et al., 2005; Schilling et al., 2007; Isohookana et al., 2013). ACEs also greatly increase the risk of early initiation of substance use and abuse; the risk of early initiation of illicit drug use for an individual increases 2- to 4-fold for each ACE, and up to 10-fold if he or she has experienced more than five ACEs (Dube et al., 2003). The research also indicates that nearly two-thirds of
injection drug use (e.g., heroin) in the general population may be attributable to traumatic and abusive childhood events (Dube et al., 2003). A review by the National Institute on Drug Abuse stated that, “populations of substance abusers are found to meet the [diagnostic] criteria for post-traumatic stress disorder…those experiencing both physical and sexual abuse were at least *twice* as likely to be using drugs than those who experienced either abuse alone” (Gordon, 2002, p. 117). Even after prolonged abstinence from drug use, stress remains a significant factor in relapse to drug abuse (Stewart, 2008).

However, more research is required to elucidate the specific developmental mechanisms by which early stress and drug exposure produce these subtle changes in the developing nervous system, and that can lead to more pronounced effects on learning and memory which become increasingly salient with age. For example, if deficits are subtle, such as modifications in neural signaling, they may not be noticeable until exacerbated by later-life experiences, including exposure to illicit drugs or anxiety-provoking situations (a neurophysiological process known as *cross-sensitization*). What needs to be better understood are the neurophysiological mechanisms that are involved in the development of addiction, particularly when children are exposed to drugs and stressful experiences at critical periods of neurodevelopment. For the prevention of substance use disorders, future research goals should be aimed at studying the role of ACEs in the development of mental illnesses (e.g., anxiety, depression, bipolar disorder, schizophrenia), and the social stressors (e.g., poverty, access to education, lack of predictable employment) that *precede* the initiation of drug use and relapse in substance dependence disorders throughout an individual’s lifespan. We need to better understand factors that determine the directionality of the relationship between mental illness and addiction and how the directionality *may change* over an individual’s lifespan. We also need to work on educating the public, in particular
those who work with families and children (e.g., social workers, physicians, educators, social policy makers), on the effects of ACEs on maladaptive brain development and the lifelong vulnerability to mental illness and substance use and abuse it creates. We need to focus on transformational education emphasizing a) the need for greater social and cultural support for parents and children during critical periods of brain development, and b) how neuroplasticity throughout the lifespan provides the potential for ameliorating/reversing these effects with psychotherapy, pharmacology, and social supports. Based on evidence, the answer to solving many of the problems of mental illness and addiction in our society must begin and end with family; this means understanding how to better support individuals struggling with substance use disorders and comorbid mental health issues and their families and fostering stronger professional collaboration dedicated to helping those individuals.

“The effects of adverse childhood experiences transcend secular changes such as increased availability of drugs, social attitudes toward drugs, and recent massive expenditures and public information campaigns to prevent drug use. Because ACEs seem to account for one half to two thirds of serious problems with drug use, progress in meeting the national goals for reducing drug use will necessitate serious attention to these types of common, stressful, and disturbing childhood experiences by pediatric practice.”

- Dube et al. (2003, p. 564)

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