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Vapourized Cannabis Extract Administration Impairs Memory and Alters Neural Activity in Laboratory Rats

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VAPOURIZED CANNABIS EXTRACT ADMINISTRATION IMPAIRS MEMORY AND ALTERS NEURAL ACTIVITY IN LABORATORY RATS

by

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List of Abbreviations

2-AG ..... 2-arachidonoyl glycerol
CB1 .....type-1 cannabinid receptor
CB2 .....type- 2 cannabinoid receptor
CBC ..... cannabichromene
CBD .....cannabidiol
CBG .....cannabigerol
CBT .....cannabicitran
CEA .....central nucleus of the amygdala
DMTP ..... delayed- match-to-position
DMTS ..... delayed match to sample task
DNMS .....delayed-non-matching to sample
GPR55..... G protein-coupled receptor 55
IR .....immunoreactivity
JWH-015 ..... 2-methyl-1-propyl-3-(1-naphthoyl)indole
L768242..1-(2, 3-dichlorobenzoyl)-2-methyl-3-(2-[1-morpholino]ethyl)-5-methoxyind ole
MCTs ..... medium chain glycerides
NAS .....nucleus accumbens
PBS ..... phosphate buffered saline
PEGs ..... polyethylene glycols
PVN ..... paraventricular nucleus of the hypothalamus
PVT ..... paraventricular thalamic nucleus
S1 .....primary somatosensory cortex
THC ..... Δ^9-tetrahydrocannabinol
THC-A ..... tetrahydrocannabinolic acid
THC-A .....tetrahydrocannabinolic acid A
THCV ..... tetrahydrocannabivarin
TRP .....transient receptor potential
VEA .....vitamin E acetate
WIN-2 .....WIN55,2122

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Abstract

Although people have smoked cannabis for millennia, administration of cannabis by heating the dried plant material or distilled extracts to a temperature just below the combustion point (i.e., vapourization or "vaping") has rapidly increased over the past few years (Javadi-Payder, Cole, and Taffe, 2018; Manwell, Charchoglyan, Brewer, Matthews, Heipel, and Mallet, 2014). Conversely, most of the cannabinoid research using rodent models has used parenteral routes of administration to examine the effects of purified cannabis extracts such as Δ^9-tetrahydrocannabinol (THC), making it difficult to directly compare laboratory research to human drug use. Building on previous studies showing that THC injection in rats impairs memory via a CB_1 receptor-mediated mechanism (Mallet & Beninger, 1998), the present study used the delayed-match-to-position (DMTP) task to show that acute exposure to a vapourized cannabis extract (three exposure levels and a control vapour condition) dose-dependently impaired memory in rats. Cannabis vapour-induced memory impairments were attenuated--albeit not significantly--by the CB_1 receptor inverse agonist / antagonist rimonabant (3 mg/kg, i.p.). In a separate experiment, brains of rats were harvested 2 hours after exposure to cannabis or control vapour, then drug-induced changes in neural activity were quantified using Fos immunochemistry (IR). Results revealed that cannabis vapour significantly increased neuronal activity in the paraventricular nucleus of the hypothalamus (PVN), paraventricular thalamic nucleus, and the primary somatosensory cortex (S1). This study demonstrates for the first time that exposure to vapourized cannabis impairs memory in rodents, and also serves to provide a novel methodological approach to studying the effects of inhaled cannabis in rodents that better approximates modern human cannabis use. Further studies using this novel drug delivery system

can be used to better inform public health guidelines and policy makers about the behavioural and physiological effects of vapourized cannabis.

Chapter 1. General introduction

Cannabis is currently one of the most widely used drugs in Western societies. According to the World Health Organization, 2.5% of the world's population use cannabis compared to only 0.2% who consume cocaine and 0.2% who consume opiates. Following the recent legalization of recreational cannabis use by the Canadian government, there has been an increase in the frequency of cannabis use, with 27% of Canadians reporting using cannabis in the past 12 months--an increase from 22% in 2018 (Canadian Cannabis Survey, 2019).

The term cannabis refers to the products derived from the plant Cannabis sativa. The cannabis plant contains around 540 chemical substances, also known as cannabinoids, with the key components being Δ-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) (Javadi-Paydar, Nguyen, Kerr, Grant, Vandewater, Cole and Taffe, 2018; ElSohly, Mehmedic, Foster, Gon, Chandra, and Church, 2016). THC is one of the main psychoactive ingredients found in cannabis. THC is a cannabinoid receptor agonist and acts on the brain's endocannabinoid system. Like other cannabinoids, THC binds to the cannabinoid receptors CB1 (found in the central nervous system) and CB2 (found in the peripheral nervous system) (Wu, Jew & Lu, 2012; Morgan, Schafer, Freeman and Curran, 2010; Wright, Vandewater and Taffe, 2013).

1.1 Cannabinoids and the endocannabinoid system

Since the discovery of an endogenous cannabinoid system, research into the mechanism of action, abuse, and possible therapeutic potential of cannabinoids has increased (Grotenhermen, 2005). The two main endogenous cannabinoids are arachidonoyl ethanolamide (anandamide) and 2-arachidonoyl glycerol (2-AG). Endocannabinoids are unique amongst other ligands in that they are retrograde signaling molecules made on demand from the cleavage of

membrane lipid precursors and released into the extracellular space. Additionally, endocannabinoids differ from most drug chemicals that affect the brain through chemical synaptic signaling. Essentially, cannabinoids such as THC act on the brain by interfering with the neuronal signaling system and mimicking the effects of endogenous cannabinoid ligands (Alger, 2013).

The CB1 and CB2 receptors mediate the effects of cannabinoids. However, research suggests that other receptors, such as Transient Receptor Potential (TRP) channels, also mediate some cannabinoid effects (Lu & Mackie, 2017). Furthermore, these cannabinoid receptors inhibit the release of neurotransmitters such as GABA and glutamate from pre-synaptic neurons. Activation of CB1 receptors can increase or decrease neural activity (Howlett, 2002). For example, if the CB1 receptors are located on glutamate terminals, CB1 activation reduces glutamate release. When CB1 activates an excitatory neurotransmitter like glutamate, overall neural activity decreases (Howlett, 2002).

CB1 receptors are abundant in the brain, particularly in the central nervous system, cortex, basal ganglia, hippocampus, and cerebellum (Lu & Mackie, 2017). Conversely, CB2 receptors are not as numerous in the brain as CB1 receptors. THC is a low-efficacy agonist that, when activated, may antagonize CB1 receptor signaling or act as a CB1 receptor agonist (Lu & Mackie, 2017). The psychoactive effects observed following cannabis use may be attributable to their increased efficacy (Lu & Mackie, 2017). Given the complexity of the brain, cannabinoids could affect multiple processes in several ways, including high-order behavioural functions, motor functioning, and other homeostatic processes (Alger, 2013; Zou & Kumar, 2018).

1.2 Behavioural and physiological effects of cannabis in humans

Cannabis induces many behavioural and cognitive effects. Research of the subjective effects of cannabis dates back to the late 1950's, when the most commonly reported positive effects included euphoria and feelings of relaxation (Green et al., 2003). Users also report increased mood (e.g., happiness or increased laughter), altered sensory experience, and increased insight/thinking (Green et al., 2003). One study found that over 90% of users reported a decrease in perception of time and increased appetite (Berke et al., 1974). Berke et al. (1974) found that over 10-15% of users reported elation, brighter surroundings (mostly colors), and an enhanced appreciation of music. Approximately 4-8% of users reported pain relief, improved sleep, and cognitive benefits, following acute intoxication (Berke et al., 1974).

On the contrary, some users report negative mental health effects including paranoia, depression, anxiety, and hallucinations. For example, one study found that approximately 10% of users reported feelings of depression, 14% reported experiencing hallucinations, and 15% reported feeling anxious (Kirk and De Wit, 1999), especially when the cannabis was smoked (Bruijnzeel, Qi, Guzhva, Wall, Deng, Gold, Febo and Setlow, 2016). Another study found that more than half of the study participants reported feelings of drowsiness, decreased social interactions, and decreased memory from smoking cannabis (Block et al., 1998; Hart et al., 2001). Furthermore, increased heart rate has been reported repeatedly in many studies and is considered a reliable physiological sign of intoxication (Heishman et al., 1997). Lastly, suppressed locomotor activity is also a frequently reported behavioural change following acute cannabis intoxication (Manwell et al., 2014).

1.3 Pharmacokinetics/pharmacodynamics

The two most common methods of cannabis consumption by human populations are inhalation—typically by smoking the dried plant materials--and oral consumption, typically by swallowing a food substance containing psychoactive cannabis extracts (Lefever et al., 2017; Morean et al., 2015). The 2020 Canadian Cannabis Survey found that those who report using Cannabis in the past 12 months prefer to use cannabis by inhalation or orally. Historically, cannabis products were mainly consumed using traditional tobacco pipes by smoking crushed dried flowers. However, during the late 1800s and early 1900s, paper-rolled “joints” increased in popularity (Lefever et al., 2017). The revolutionary production of cannabis vapourizers began in the late 1900s and early 2000s. Vapourizers used today typically contain a battery-powered heating chamber that heats the plant or cannabis extract oil to a temperature below the combustion threshold, yielding a chemical-infused vapour (Meehan-Atrash & Rahman, 2021). In recent years, cannabis vapourizers have grown in popularity. Many cannabis vapourizers on the market today use pre-filled cartridges with varying quantities of cannabis extracts (usually sold in 0.5 g or 1 g cartridges) with varying THC and CBD ratios (Meehan-Atrash & Rahman, 2021).

According to the Canadian Cannabis Survey (2013), about 9% of Canadians aged 15 years and older reported using electronic vapourizers (Canadian cannabis Survey, 2013). However, e-vape use has since dramatically increased to 29% in 2021 (Canadian cannabis survey, 2021).

The preparation of cannabis vape cartridges requires several steps. First, the dried cannabis plant is extracted using organic solvents such as ethanol or isopropanol, dry ice, butane, or supercritical CO_2 (Giroud et al., 2015). Subsequently, heating the dried plant (to approximately 110-160° C) converts the inactive tetrahydrocannabinolic acid A (THC-A), into

psychoactive THC. During this process, heat-decarboxylation will convert other cannabinoids into their neutral counterparts (Giroud et al., 2015). Finally, the resulting wax-like residue can be mixed with pure propylene glycol and placed into an airtight cartridge for consumption (Giroud et al., 2015). Currently, there are a variety of vape devices (see Figures 1 and 2) and cannabis cartridges with different THC or CBD concentrations available on the market today.

Commercially available vapourized cannabis products contain a variety of chemical constituents. One study conducted by Guo et al. (2021) found over 100 different terpenes (containing carbon and hydrogen) from 12 commercially available cannabis vape cartridges. Guo et al. (2021) also identified over 19 cannabinoids present amongst the samples and found that the most common cannabinoids were THC, cannabinol (CBN), cannabicitran (CBT), cannabigerol (CBG), tetrahydrocannabivarin (THCV), and cannabichromene (CBC). Delta-9-THC by-products such as Delta-8-THC and Delta-10-THC were also found. It is important to note that the pharmacological and toxic effects of many cannabinoids, such as Delta-8-THC, Delta-10-THC, CBC, and CBG, have not been well studied (Williams, 2020). Many additives, including Vitamin E acetate (VEA), polyethylene glycols (PEGs), and medium chain glycerides (MCTs), were the most commonly identified additives in commercially available cannabis vape cartridges (Guo et al., 2021; Blount et al., 2019; Blount et al., 2020). Consequently, cannabinoid constituents, additives, terpenes identified, and other possible unidentified by-products are not well studied (Guo et al., 2021).

There are many reasons why cannabis users prefer smoking the plant over consuming it orally. Inhaled THC enters the brain quickly, allowing the user to experience drug effects much faster than when taken orally (Lefever et al., 2017; Alger, 2013; Grotenhermen, 2003). The experienced user can become adept at sensing intoxication and can titrate THC levels much

easier than if taken orally (Alger, 2013). One study found that oral THC produced peak, long-lasting serum levels one hour or more following administration (Hlozek et al., 2017; Barrus et al., 2016). However, findings suggest that when cannabis is smoked, it takes approximately 20-30 seconds for THC to cross the blood-brain barrier when coming from the lungs. This inhaled route provides peak effects within minutes of consumption (Barrus et al., 2016).

It is evident that there are qualitative differences in the effects produced by acute cannabis exposure using different routes of administration (Manwell et al., 2014; Lefever et al., 2017). These observed differences are likely due to the pharmacokinetic variations between an injected and inhaled delivery system. Evidence shows that an injected delivery route produces an initial, short-lasting, low amplitude peak, and we see a relatively steady serum levels and concentrations in the brain (Manwell et al., 2014; Hlozek et al., 2017). A recent study by Hlozek and colleagues (2017) found a second peak following injected THC, with a maximum peak level after 8 hours. Interestingly, the THC metabolite 11-OH-THC also showed a second maximum peak at 8 hours following injection, but this same peak was seen after only 4 hours following injection of only CBD (Hlozek et al., 2017). This evidence indicates a possible two-phase model whereby the initial release of the cannabinoid is released into the bloodstream immediately following injection while simultaneously being depot bound within the skin and fat tissues from which are subsequently released from, supporting a second peak (Hlozek et al., 2017; Barrus et al., 2016).

Additional evidence revealed that an inhaled delivery route allows THC to cross the blood-brain barrier very quickly, reaching peak plasma levels within 5-10 minutes following inhalation (Hlozek et al., 2017; Huestis, 2017; Manwell et al., 2014). Serum levels decrease rapidly, but brain levels remain elevated for one hour following administration, indicating that

peak serum levels show a difference in time course compared to peak behavioural and cognitive effects (Hlozke et al., 2017). Recent studies report that inhaled THC acts rapidly given its lipophilic properties; it is initially absorbed by lung tissue and perfused quickly into the brain (Alger, 2013; Barrus., 2016).

Overall, these pharmacokinetic differences may explain the qualitative differences observed within the current rodent and human models of consumption.

In recent years, animal models have been used to better understand the possible adverse effects of human cannabis use. It is difficult to assess the consequences of cannabis alone in humans given that polydrug use is the norm (Newsom & Kelly, 2007), and users vary length and frequency of exposure as well as the route of administration. Moreover, commercial cannabis products vary in dose, ratio of THC/CBD, and source (i.e., home grown or purchased commercially). Although smoking is the most common route of consumption by humans (Barrus et al., 2016), the preclinical research to date has emphasized the use of injected purified forms of THC or other cannabinoids that prove varying, inconsistent results with what has been reported with human inhalation use (Taffe et al., 2019).

Unlike human cannabis users, rodents injected with THC show slightly reduced feeding behaviour 4 hours following exposure (Manwell et al., 2014). We also know that feeding behaviour is not influenced by type of food or varying macronutrient compositions (Verty et al., 2014). To better replicate human cannabis administration, some studies have used rodent models to examine the effects of inhaled cannabinoid administration. These studies reveal that inhaled THC significantly increased food consumption (Manwell et al., 2014; Nguygen et al., 2019) 1 hour after exposure, whereas none of the doses of injected THC significantly increased food

consumption (Manwell et al., 2014). It is evident that the route of administration and time course are both important considerations when describing the behavioural effects of THC.

As previously mentioned, humans report diminished social interaction when intoxicated from cannabis (Green et al., 2003). This is somewhat consistent with injected rodent models of cannabinoid exposure and with inhaled rodent models of delivery (Bruijnzeel et al., 2016) using a social interaction or social preference task. It is important to note that this literature is lacking with only a small number of published findings to support this social interaction phenomenon. This important interaction or preference should be addressed by further studies that aim to examine the acute effects of inhaled cannabis exposure in order to supplement and strengthen the current literature on this topic.

Effects of cannabinoids on anxiety-like behaviours have been well-studied (e.g., Murphy et al., 2017; Ruiz et al., 2020, Bruijnzeel et al., 2016; Manwell et al., 2019). The animal literature is consistent with human findings in that injected forms of THC, at relatively high doses, are generally anxiogenic (Murphey et al., 2017), while lower doses of injected THC are generally anxiolytic (Ruiz et al., 2020). The effects of inhaled forms of THC on anxiety-like behaviour have not been well studied, but it has been suggested that cannabis smoke containing both THC and CBD is anxiolytic (Bruijnzeel et al., 2016).

Several studies have been conducted in order to assess cognitive effects of cannabinoid use. Many of these studies have found a relationship between cannabinoid exposure and cognitive impairments, highlighting deficits in working memory, object recognition, and short-term memory capacity (O'Shea et al., 2005, 2006). Of the reported cognitive effects of cannabinoids, memory impairment is one of the most studied (Mallet and Beninger, 1996). Memory impairments have been demonstrated in animals using several memory tasks, including

the radial maze task, the Morris water maze, and variations of the delayed match to sample task (DMTS). Additionally, Heyser et al. (1993) examined the effects of injected THC on a DMTS task in rats and found that administration of THC significantly reduced performance at longer delays. Similarly, another study examined impairments in working memory following THC, CBD, or THC/CBD exposure (Fadda et al., 2004). Results revealed that deficits in the DMTS task and the open-field water maze were found following only THC exposure and not following CBD or THC/CBD exposure, revealing that potentiation and antagonism of THC-induced spatial memory deficits may be dependent on the ratio of CBD and THC (Fadda et al., 2004).

Moreover, recent investigations have confirmed that the effects of THC and the synthetic cannabinoid WIN 55,212-2 (WIN-2) affect performance on tasks of working memory, indicating that cannabinoids like THC and WIN-2 may disrupt the processing of information (encoding) and recalling information needed for a particular trial (Hampson & Deadwyler, 2000). Finally, Mallet and Beninger (1996; 1998) showed that the administration of either THC or anandamide impaired memory and subsequently demonstrated that rimonabant attenuated these impairments, serving to confirm the involvement of CB_1 receptors in both THC- and anandamide-induced memory impairment.

1.4 Differences in routes of administration used in human and animal studies

The rodent cannabinoid literature focuses on injected methods of consumption, but this model has largely failed to replicate any of the appetitive/rewarding effects of cannabinoids (Manwell et al., 2014). As previously mentioned, the 2020 Canadian Cannabis Survey reports that current cannabis users today prefer to smoke or orally consume cannabis products. The survey, in fact, makes no mention of injected methods.

Additionally, the current rodent models of cannabinoid exposure (either injected or inhaled) have mostly used highly purified isolated THC and other synthetic cannabinoids. These studies therefore do not represent typical human cannabis use. Inhalation of commercially available e-cigarettes containing cannabis extracts do not only contain purified THC. Rather, they contain other chemicals and cannabinoids in addition to THC, such as THC metabolites, CBD, and added terpenes (Agrawal et al., 2009). Current research has indicated that the added terpenes in commercial products have their own behavioural and toxicologic consequences (Alger, 2013), although these remain sparsely studied. Therefore, the effects seen in human consumption may differ from those seen in laboratory animal studies due to the independent effects of the psychoactive chemicals inhaled along with THC (Agrawal & Lynskey, 2009). Further research needs to be conducted in order to properly examine the acute and chronic effects of human grade cannabis products in animals.

Lastly, some studies have examined the effects of vapourized purified THC (e.g., using a Volcano® device (Manwell et al., 2014). However, this approach also does not fully reflect human vapourized cannabis use, which rarely (if ever) involves the vapourization of purified THC.

This brief review of the literature highlights the main limitations of the current cannabinoid literature. Overall, the cannabis research is inconsistent, non-standardized and relatively sparse in some areas, hindering the ability to accurately compare and contrast current findings.

1.5 The present study

The present study aims to fill in some of the gaps in the literature by using a more modern delivery system of vapourized cannabis to assess the behavioural and neuronal effects in

adolescent rats. To this end, two experiments were conducted. The first experiment, described in chapter 2, examined neuronal activation patterns induced by acute cannabis vapour using Fos immunochemistry. More specific aims and hypotheses will be presented in chapter 2. Given that findings from the current literature suggest that working memory in rodents is one of the first behavioural impairments observed following acute injected cannabinoid exposure, the second experiment, described in chapter 3, sought to use the delayed-match-to-position (DMTP) task to examine working memory following acute administration of vapourized cannabis and to confirm the involvement of CB1 receptors in these memory impairing effects. Specific hypotheses of this experiment will be outlined in chapter 3. Of note, the use of rimonabant in attenuating the effects of cannabinoid impairments will be discussed throughout chapter 3.

Chapter 2. Cannabinoid effects on neural activity

2.1 Introduction

2.1.1 Neuroimaging in humans Neuroimaging can be a powerful tool used to assess the effects of cannabinoids on the brain in both humans and animals (Cupo et al., 2021). Some fMRI studies have shown that cannabis exposure decreases activation in the cerebellum, the ventral thalamus and the somatosensory cortex, S2 (Walter et al., 2017; Walter et al., 2016). In addition, Bhattacharyya et al. (2009) found that oral THC exposure increased activation in the right inferior, middle, and superior frontal gyri, and the right thalamus during fMRI testing. Recent functional imaging studies have found global cortical impairments of chronic cannabis users, indicating decreased activity during periods of drug abstinence and increased activity when THC or cannabis is administered. In addition, Mathew et al. (1989) found that chronic cannabis users showed decreased global cerebral blood flow compared to individuals who never used cannabis before.

According to functional imaging studies, the limbic system--the system involved in memory, cognition, behaviour, and motor function--shows differential activity in cannabis users compared to non-cannabis users. For example, Mathew et al. (1997) found increased bilateral anterior cingulate activity following acute THC intoxication compared to controls. In addition, Mathew et al. (2002) found increased cerebral blood flow in the hippocampus following THC administration, noting that in the high dose condition (5 mg), global cerebral blood flow increased in activity in the hippocampus and in the amygdala 30 min after drug administration, but at 60 min, these changes diminished.

2.1.2 Neuronal changes in animal models: c-Fos immunoreactivity

C-fos is an immediate early gene involved in cell proliferation and differentiation following extracellular stimuli. Commonly, Fos—the protein product of *c-fos*--has been used as a marker of neuronal activity and has been associated with several neuronal and behavioural responses to certain acute stimuli expression (Bullitt,1990). The effects of cannabinoid exposure on changes in Fos-imunoreactivity (Fos-IR) in the rat brain have been well studied (Allen et al.2003; Ruiz et al.2021; Manwell et al.2020). Several studies found that acute injected THC administration produces greater Fos-IR in regions associated with reward, memory and emotion including the amygdala, regions of the prefrontal cortex, hippocampus, and the nucleus accumbens (Allen et al.2003; McGregor et al.1998; Arnold et al.2001). McGregor et al (1998) examined expression of Fos-IR following THC and anandamide administration. They found that THC and anandamide produced equally high levels of Fos-IR in the paraventricular nucleus of the hypothalamus, the lateral septum, and both increased expression in the central nucleus of the amygdala. However, they found that only THC increased Fos-IR in the nucleus accumbens and caudate putamen, confirming there may be differential activation of cannabinoid receptor types in different regions of the brain. Similarly, some studies have examined the effects of injected THC on Fos-IR and found that changes in Fos-IR are typically seen in the caudate putamen (CPU) (McGregor et al., 1998), the nucleus accumbens (NAS)(McGregor et al., 1998), the central nucleus of the amygdala (CEA) (McGregor et al., 1998), and the paraventricular nucleus of the hypothalamus (PVN) (McGregor et al., 1998). Similarly, one study found significantly dense Fos-IR in the PVN, the lateral septum, CEA, and the ventrolateral periaqueductal gray following administration of the synthetic cannabinoid CP 55,940 (Arnold et al., 2001).

2.1.3 The present study

Previous Fos imaging studies examining neuronal changes following cannabinoid exposure have focused on an injected route of administration using purified forms of THC. Inconsistent with human consumption, differences in routes of administration are used between human and animal studies. To the best of our knowledge, there is no published literature examining changes in Fos expression following acute vapourized cannabis. This study sought to bridge this gap by exposing animals to either cannabis or vehicle vapour and using Fos imaging to quantify neuronal changes and patterns of activity in the brain.

There is a growing body of evidence showing that the mind-altering effects of cannabinoids are the result of the action of THC on CB1 receptors. Previous Fos imaging studies that have examined neuronal changes following by THC administration have shown expression in regions such the amygdala, hypothalamus, nucleus accumbens, and caudate putamen (Allen et al.,2003 ,Arnold et al., 2001, McGregor et al., 1998). As mentioned previously, cannabis extract used in vape devices or smoking dried cannabis flower, contains many other cannabinoid compounds that may antagonize THC. Consequently, the hypotheses of this experiment were inconclusive. Results from this experiment will examine Fos activation patterns produced by acute cannabis vapour that has not been demonstrated.

2.2 Methods

2.2.1. Subjects

Twelve Male SD IGS rats (Charles River Laboratories, St. Constant, Quebec) were housed in pairs in a temperature-controlled room that was maintained on a 12 h light/dark cycle. Animals had *ad libitum* access to food (Envigo 8640 Rodent chow) and water. Prior to the experiment, animals were handled daily for 3 min per day for 3 consecutive days. After handling, animals were habituated to the vapour apparatus for 30 min daily for 3 consecutive days. All experimental procedures were reviewed and approved by the Laurier Animal Care Committee.

2.2.2. Open Vape Apparatus.

Two custom-built open-source E-cigarette vapour exposure devices for rodents modelled on the OpenVape system described by Frie et al. (2020) were used. The semi-sealed exposure chambers were constructed using modified 259 mm x 234 mm x 209 mm Allentown mouse cages. These were connected to independent 12V DC vacuum pumps (model ROB-10398, SparkFun Electronics, Niwot, CO, USA) via silicone tubing (5 mm ID, 8 mm OD) connected to a custom 3D-printed nozzle located 2 cm above the floor. Vacuum pumps were controlled by a custom program running on an Arduino Uno Rev3 single board computer and motor control driver based on an L293D integrated circuit. The Arduino control software delivered a 5-sec puff every 55 sec. Two vacuum pumps, each with its own control circuit, were used to deliver cannabis vapour and vehicle vapour, which were delivered by placing animals individually into an inhalation chamber for 30 min prior to testing. Cannabis vapour pumps were cleaned by flushing with a 50% ethanol solution after every animal to ensure proper flow (see Appendix A for vapour exposure standard operating procedure).

2.2.3. Drugs.

Animals were randomly assigned to received either cannabis vapour (Foray Maui Wowie Cartridge; THC content = 800 mg/g, CBD content = 0 mg/g, Ontario Cannabis Store; https://www.ocs.ca) or control vapour (flavourless, nicotine free e-juice composed of propylene glycol and vegetable glycerin), which were delivered inside a fume hood. Animals were exposed to either condition for 30 min, and then placed back into their home cages for 90 min until perfusion began.

2.2.4. Perfusion and Sectioning.

Two hours following the onset of vapor exposure each animal was deeply anesthetized with 120 mg/kg (i.p.) sodium pentobarbital. Animals were perfused transcardially with 100-150 ml of 0.1M phosphate-buffered saline (pH 7.2, PBS) and 100-150 ml of 4% paraformaldehyde in phosphate-buffered saline. Brains were post-fixed in the skull for 2 h and then removed. Brains were further fixed in paraformaldehyde for an additional 12 h and transferred into PBS and 15% sucrose for 24 h. Brains were then transferred to a 30% phosphate buffered sucrose solution for at least 72 h, and then were sectioned into 50 µm coronal slices using a cryostat (Leica Biosystems, model CM1850).

2.2.5. c-Fos Immunolabelling.

Free-floating sections underwent three consecutive 30-min washes in 0.1M PBS (pH =7.2), followed by a 30 min incubation in 0.9% hydrogen peroxide to quench endogenous peroxidase activity, and a 30 min blocking wash in 3% normal goat serum in PBS. Sections were then incubated for 72 h at 4°C in the primary anti-c-Fos antibody (MilliporeSigma F7799) diluted 1:5000 in phosphate-buffered goat serum (PBGS: 0.1% BSA, 0.2% Triton x-100, 2%

normal goat serum in 0.1M PBS). After the 72-h incubation period, the sections were washed three times for 10 min in PBS and incubated for 60 min in biotinylated goat anti-rabbit IgG (BA-1000, Vector Labratories) diluted 1:500 in PBGS. Tissue was than washed thrice (10 min each) in PBS, and subsequently incubated for 60 min in ExtrAvidin-peroxidase (E2886, Sigma Chemical Co.-Aldrich) diluted 1:1000 in PBGS. After three 30 min washes in PBS, sections were placed in 0.05% diaminobenzidine (DAB) solution containing 0.015% hydrogen peroxide and PBS to visualize the horseradish peroxidase activity. The reaction was terminated 10 min later by washing the sections with PBS. Sections were then mounted onto gelatin-coated slides, dehydrated using ethanol, cleared using Shandon Xylene substitute (Thermo Scientific, Cheshire, UK) and cover-slipped using Permount (Fisher Scientific).

2.2.6 Quantification of Fos-IR cells

The number of immunoreactive nuclei was quantified using brightfield microscopy by an observer blind to experimental conditions. Fos counts were made within a square reticule (0.5 mm X 0.5 mm) or 0.25 mm^2 and viewed under 20X objective. A neuron was considered Fos-positive when the nucleus was round or oval, completely filled, and dark brown or black in colour, as described by Arnold et al. (2001). In total, five brain structures at the -1.8mm bregma region which were distinct from neighbouring structures based on clearly outlined landmarks were included (Figure 2). These were identified with reference to the rat brain atlas of Paxinos and Watson (2005).

2.3. Results

Brain activation following 30 min exposure to cannabis vapour was assessed by c-Fos immunohistochemistry in five brain structures (see Figure 4). Data for the control and drug

groups were analyzed separately for each brain region using independent groups t-tests. Results revealed significant activation by exposure to cannabis vapour in the paraventricular nucleus of the hypothalamus, the paraventricular thalamic nucleus, and the primary somatosensory cortex (Table 1). An independent groups t-test also revealed no significant patterns of Fos expression by exposure to cannabis vapour in the interanteromedial thalamic nucleus and in the primary motor cortex (M1).

2.4. Discussion

Results revealed that vapourized cannabis exposure increased Fos-IR in the PVN, PVT, and SI. As mentioned above, there are currently no reported findings on Fos expression following inhaled cannabis. These findings are consistent with results of previous experiments that used injected cannabinoid receptor agonists (Allen et al., 2003, Arnold et al., 2001, McGregor et al., 1998, Miyamoto et al., 1996).

The current study found increased Fos activation in the PVN following acute cannabis vapour compared to control. One previous Fos imaging study found that administration of THC and anandamide produced increased *c-fos* expression in the PVN (McGregor et al., 1998). Fos activation in the PVN following injected cannabinoid exposure has been well documented (Weidenfeld et al.,1994, Puder et al., 1982). Cannabinoids activate the hypothalamic-pituitary-adrenal (HPA) axis, increasing ACTH and corticosterone concentrations (McGregor et al., 1998). Threats to animals and humans, endocrine responses following a threat, and enhanced memory formation to avoid the threat in the future, are mainly regulated by the limbic system. In rodents, glucocorticoid signalling mediated by the adrenal cortex as result of HPA activation is responsible for responding to psychological and physiological threats (Hillard et al., 2016).

Moreover, when stress is induced, the HPA axis releases corticotrophin-releasing hormone in neurons mainly in the PVN. Therefore, it is plausible that exposing the animal to cannabis vapour induced stress on the animal, increasing expression in the PVN. Finally, Wittmann et al (2007) found that CB1 receptors are present at a greater density within the PVN compared to other hypothalamic regions which may also explain greater expression in this region in animals exposed to cannabis vapour.

Similarly, Fos expression in the PVT was induced by exposure to cannabis vapour. This finding is consistent with the current literature as animals exposed to injected cannabinoids produced greater Fos expression in the PVT compared to controls (Boucher et al., 2011). Interestingly, the PVT has been found to mediate drug-seeking behaviours and specifically attenuating drug, cue-or context induced reinstatement (Huang et al., 2018). One study found that greater Fos expression in the PVT was correlated with greater cocaine-seeking behaviour (Matzeu et al., 2017). Neurons in the PVT project to the nucleus accumbens, which has been linked to drug seeking behaviour (Huang et al., 2018). Examination of Fos expression in the nucleus accumbens following cannabis vapour should be explored in future studies.

This study revealed greater Fos expression in area S1 in animals exposed to cannabis vapour. While research on the somatosensory cortex in relation to Fos patterns following cannabinoid exposure is lacking, one study found an increase in Fos-positive nuclei in the somatosensory cortex following repeated injected THC exposure. Area S1 is involved in receiving and processing somatosensory information. In rodents, S1 is involved in nociception and pain perception (Apkarian et al., 2005, Bushnell et al., 1999, Ploner et al., 2002) and has been found to interact functionally with motor regions (Favorov et al., 2019). The increase in Fos expression in S1 following cannabis vapour may be related to increased nociception and pain

perception while the animal is intoxicated (Manwell et al., 2017). An important implication to consider is the effect of a novel environment on Fos expression. It is well documented in the literature that animals placed into a novel environment will exhibit increase overall Fos-IR (VanElzakker et al., 2008). It is possible that the novelty of the vapour apparatus induced greater neuronal changes. However, this possibility is unlikely given that animals were habituated to the vapour apparatus for 30 min daily for 3 consecutive days.

Finally, our findings did not reveal increased Fos-IR in the primary motor cortex, consistent with locomotor behavioural findings in animals exposed to injected cannabinoids. Previous studies have shown that injected THC and other cannabinoids produce some level of sedation in the animal. There appears to be a biphasic effect on locomotor activity in that lower doses of injected cannabinoids produce increasing locomotion and higher doses decrease locomotor activity (Wegner and Koch, 2009; McGregor et al., 1998). Moreover, an importation implication to consider is the subjective effects reported by human users on motor performance. Many acute and chronic human users consistently report slower reaction time and an overall "body stone" or an overall feeling of sedation (Prashad and Fibey, 2018). These subjective effects are also consistent with some of the human studies examining motor performance following cannabis intoxication, demonstrating significantly reduced reaction times and increased errors on motor performance tasks (Block et al., 1992, Curran et al., 2002).

Overall, this study used Fos-IR to quantify neural activity in regions that were thought to be affected by vapourized cannabis exposure. Although preliminary hypotheses were unclear, the current study provides novel information regarding changes in neural activity following acute vapourized cannabis exposure and demonstrates clear preliminary data suggesting a successful use of an inhaled delivery model that may replace current injected route of administration. Future

studies should aim to investigate additional brain regions that may be affected by acute cannabis vapour.

Chapter 3. Effects of Cannabis on Memory

3.1.1 Acute effects of cannabinoids on working memory in humans and rodents

The effects of cannabinoid exposure on working memory in rodents has long been studied (Hyser, Hampson & Deadwyler., 1993, Nakamura et al.,1991). Working memory is defined as a cognitive process for the temporary storage and usage of information (Baddeley et al., 1974). Maze-based tasks and instrumental tasks (Cohen & Weinstein., 2018) have been widely used to study working memory in animals. Maze-based tasks such as the radial arm maze and the Morris water maze, are based on navigational behaviours used to assess working memory. Instrumental tasks such as the DMTP or delayed-non-matching to sample (DNMS) task, initially presents a stimulus to the animal and following a delay period presents the animal with a choice between the original stimulus and a novel stimulus. The animal must choose which sample stimulus or novel stimulus matches with the original stimulus (Cohen & Weinstein., 2018). Previous studies have demonstrated that acute administration of THC and other cannabinoids (e.g., WIN-2, CP 55,940) given i.p. impaired working memory in the radial arm maze and DMTP/DNMS tasks (Lawston et al., 2000, Abush et al., 2012, Kirschmann et al., 2017). These findings suggest the involvement of CB1 receptors in the effects of cannabinoids on working memory (Cohen & Weinstein., 2018).

3.1.2 DMTP task and working memory

The delayed-match-to-position task is an operant task used to assess working memory in rodents (Dunnet et al., 1988). This task assesses memory performance using a delayed response to a position associated with food reward. The animal is presented with a sample stimulus and then given a choice between the sample and a novel stimulus, following a delay (Dunnet et al., 1988). This task allows the experimenter to manipulate the delay intervals between the presentation of the stimulus and the animal's choice. Memory impairment is demonstrated when the animal makes an incorrect choice as the delay increases. Moreover, increased response latency may indicate a non-mnemonic function such as a motor or motivational deficit.

3.1.3. Rimonabant Attenuates Cannabinoid-Induced Working Memory Deficits

It is well documented that the CB1 antagonist SR 141716A (rimonabant), attenuates cannabinoid induced memory impairments in rodents (Mallet & Beninger, 1998). Specifically, cannabimimetics are known to impair memory in both humans and in animals by binding to CB1 receptors (Terranova et al., 1996). Rimonabant dose-dependently reverses the chemical and pharmacological effects of cannabinoids, and via this mechanism can attenuate cannabinoid-induced memory deficits. For exmaple, it has been shown that rimonabant antagonizes the inhibition of memory induced by WIN-2, anandamide, THC, and other cannabinoids (Terranova et al., 1996). Using a two-component operant task that included a cued discrimination and a delayed match-to-position, Mallet and Beninger (1998), demonstrated that THC selectively impaired choice accuracy in the delayed match-to-position component. Rimonabant had no effect on its own in either task. However, rimonabant attenuated the memory impairing effects induced by THC or anandamide. Additionally, Terranova et al. (1996) reported that rimonabant antagonised memory impairments elicited by WIN-2 and anandamide.

3.1.4 The Present Study

The current cannabinoid literature focuses on an injected route of administration using purified forms of THC to test the effects of cannabis on memory. However, these studies do not use cannabis which as mentioned previously, contains many other cannabinoid constituents. The purpose of the present study was to investigate the effects of cannabis on memory using a more representative model that better applies to human use.

The present study aimed to: 1) develop methods for inhaled administration of a cannabis extract to rodents, 2) examine working memory following acute vapourized cannabis exposure and 3) to confirm the involvement of CB1 receptors in the mechanism of action behind cannabinoid induced memory impairments. It was hypothesized that acute vapourized cannabis exposure would impair memory on the DMTP task and that these impairments would be attenuated by rimonabant, confirming the involvement of CB1 receptors.

3.2.Vapourized Cannabis Extract Impairs Working Memory (Experiment 3A)

3.2.1. Methods (3A)

Subjects.

Eight naïve male SD IGS rats (Charles River Laboratories, St. Constant, Quebec) weighing approximately 350 g at the start of the experiment were used. In a previous experiment animals were exposed to cannabis or vehicle vapour (once for 30 minutes) and locomotor activity was recorded. One animal was removed from the present experiment due to inability to acquire the task. Animals were single-housed and maintained on a 12hr:12hr dark-light cycle. Rats were habituated to testing chambers and the vape apparatus one day prior to the beginning

of the experiment. The Wilfrid Laurier University Animal Care Committee reviewed and approved this study. All experimental procedures were carried out in accordance with the Canadian Council on Animal Care (CCAC) Guide to the Care and Use of Laboratory Animals.

Food Restriction.

Animals were placed on food restriction, and *ad libitum* access to food was removed 4 days prior to the start of the experiment. Baseline body weights were recorded on day 1. Animals were given free access to food for 1 hour per day on days 1 through 5. On days 3 and 4, animals were provided with a small dish containing 4.5 g of 45 mg grain-based dustless precision food pellets (BioServ #F0165, Frenchtown NJ) in their home cage, which were left in their cage until completely consumed. During the response shaping, training, and testing phases, rats were given measured rations of rat chow (Envigo 8640) in their home cages for 1-2 h following behavioural testing. Body weight were not permitted to drop below 85% of their free-feeding values compared to baseline weights, and adjusted for typical strain-specific growth. If animal weights dropped below 90% of their free-feeding weight, food rations were increased for individual rats as required. Animals had *ad libitum* access to water through the course of the experiment.

Operant Chamber Apparatus.

Four identical operant chambers (model ENV-007CT, Med Associates Inc., St. Albans, VT) were used. These were individually housed within four custom-built sound-attenuating chambers, each fitted with a small fan that provided ventilation and served to mask background noise. Each operant chamber contained a house light and two standard white lens stimulus lights located directly above each lever. In addition, each testing chamber contained a stainless-steel

grid floor, two retractable response levers, and a modular dispenser containing 45 mg food pellets. All testing chambers were controlled by custom programs written in Med-PC.

Lever Press Response Shaping.

On day 1 of response shaping, all animals were placed in the testing chamber for a 30-min habituation period with the house lights on, but without the presence of food pellets or levers (refer to Appendix B for MEDState notation control program). On day 2, three 45 mg pellets were delivered to the food cup before the animal's entry. Once the animal was placed in the testing chamber, individual pellets were delivered into the food cup on a variable time 30-sec (ITI) schedule (see Appendix C for MEDState notation control program). Pellets were only delivered if the animal's head was detected in the food cup after presentation of the previous pellet. The session concluded after 50 pellets were delivered or 60 min elapsed, whichever occurred first. On day 3, food pellet dust and water were mixed to create a food mash. A small amount of mash was placed on both levers to encourage exploration. One randomly selected lever was extended for 20 s. The lever was then retracted, and a pellet was delivered. If the animal pressed the lever, the lever was immediately retracted, and a pellet was delivered. This session concluded after 50 trials or 90 min, whichever occurred first. Day 4 of response shaping was identical to day 3; however, the maximum lever presentation was reduced from 20 to 10 s (refer to Appendix C). On subsequent training days, animals were required to press any randomly selected lever at least 10 times in one session to advance to fixed ratio-1 (FR1) training. Of note, two animals did not reach this criterion. They underwent manual response training whereby the researcher manually delivered a food pellet to the animal every time it made close contact with the lever. Manual training was concluded once the animal could press the retractable lever and retrieve food pellets at least 30 times in one session.

FR1 Training.

During the FR1 training, one randomly selected lever was extended and remained extended until the lever was pressed. Then, the cue light associated with the lever was illuminated, and a pellet was delivered. Following a 10-sec intertrial interval (ITI), the next randomly selected lever was presented. Each session concluded following 50 trials or 90 min, whichever occurred first. The FR1 protocol was repeated daily until the animal completed 50 trials over 2 consecutive days (refer to Appendix D for MEDState notation control program). Once achieved, the animal advanced to DMTP training.

DMTP Training.

Unlike FR1 training, the food pellet was not dispensed upon pressing the lever. Rather, the food pellet reward was only dispensed when the animal pressed the lever that matched the sample lever. If the choice was correct, cue lights were turned off, and a food pellet was delivered. If the animal's choice was incorrect, both cue lights were turned off, and no pellet was delivered. The next trial began after a 5-sec time-out period during which time the houselights were turned off. Each delay set successively increased in delay time(sec) between the presentation of the sample lever and the presentation of the choice lever. Delay set 1 consisted of 0, 1, 2, 3, 4, 5, 6 and 7 sec delays; delay set 2 consisted of 0, 1, 2, 4, 6, 8, and 12 sec delays; delay set 3 consisted of 0, 2, 4, 6, 8, 12, 16, 20, and 24 sec delays; delay set 4 consisted of 0, 2, 4, 8, 2, 18, 24 and 32 sec delays (refer to Appendix E for MEDState notation control program). Each session concluded after 60 min. Training continued once per day 7 days per week. Progression from delay sets one through four was achieved when individual animals reached at least 85% correct choices across all trials for 3 consecutive days. Once the animal achieved 85%

correct choices for 3 consecutive days on delay set four, the animal began drug testing. The mean number of days required for animals to reach each training criterion was 3.5 days.

Open Vape Apparatus.

This experiment used the same Open Vape apparatus as described in section 3.1.7.

Drugs.

Animals were exposed to vapour using commercially available cannabis extract cartridges (Foray Maui Wowie Cartridge; THC content = 800 mg/g, CBD content = 0 mg/g) designed for human use sourced from the Ontario Cannabis Store (https://ocs.ca). The vehicle control vapour was derived from e-cigarette pods containing flavourless, nicotine-free e-juice made from vegetable glycerin and propylene glycol. Four exposure levels (i.e., "doses") were used: low, medium, high dose, and vehicle control. A pilot study examining the effects of cannabis vapour on animal intoxication determined that animals exposed to cannabis vapour for 30 min exhibited noticeable signs of intoxication including eyelid ptosis, increased respiration, increased urination, vocalization, and decreased body temperature. These physical and behavioural changes were not present in animals exposed to vehicle control vapour. The changes displayed by the cannabis-treated animals were consistent with observed changes displayed by animals treated with 2 to 5 mg/kg of injected THC (e.g., Manwell et al., 2014, Mallet and Beninger, 1998).

We determined that during our highest exposure level (i.e., "dose") the animal would receive one 5 sec delivery (i.e., 'puff') of cannabis vapour every minute for 30 minutes. That is, the high dose consisted of 30 5-sec 'puffs' of cannabis vapour delivered once per minute. Other doses were obtained by replacing the cannabis vapour with a control vapour for some of the 5-second 'puffs'. That is, all treatments involved administering a 5-sec stream of some sort of

vapour once per minute for 30 minutes, but the number of 'puffs' containing cannabis and the control vapour were adjusted for each group. For the control condition, animals received only control vapour every minute during the entire 30 min chamber exposure. For the "medium dose" condition, animals received a 5-sec puff of cannabis vapour very 3 min for 30 min. For the low dose condition animals received one 5-sec puff of cannabis vapour every 10 minutes during the 30-min chamber exposure.

DMTP Drug Testing.

Delay set four (0, 2, 4, 8, 2, 18, 24, and 32 s delay) was used for all experimental testing. Experimental treatments were presented using a repeated measures design with treatment orders selected in a quasi-random fashion such that treatments were represented approximately equally across days without any systematic treatment sequence repetitions. Animals were individually placed in the vaping apparatus and exposed to their respective treatment for 30 min. Once vapour exposure was complete, animals were placed in testing chambers immediately and were tested for 60 min. Animals were then returned to their home cages. The following day, animals were given a vapour-free washout day and tested on the DMTP task. Animals were required to achieve at least 85% correct trials for 1 day or 80% correct trials over 3 consecutive days to advance to the subsequent treatment (see Figure 3). The mean time required for all animals to reach the criteria was 1.75 days.

3.2.2. Results (3A)

A two-factor (delay X treatment) repeated measures ANOVA was used to assess working memory. Results revealed that both medium and high doses of cannabis vapour administration

significantly reduced performance accuracy on the task when compared to the lower dose of cannabis vapour or vehicle control from a 4s delay onward (Figure 5; main effect of dose: $F_{3, 18}$= 5.08, p=0.03). These negative effects seen when higher doses (medium and high) of cannabis vapour were administered revealed a more pronounced reduction in performance accuracy as the delay increased from 4 sec to 32 sec (Figure 5; main effect of delay: $F_{7, 42}$ = 25.36, p<.001). Thus, when animals were treated with medium and high doses of cannabis vapour, rats displayed poorer task performance as the delay length between the sample and choice phase increased (Figure 5). Bonferroni (adjusted) pairwise comparisons revealed significantly greater performance when the control treatment was administered compared to the high dose condition (p<.0167). The mean latency to press the choice lever was also examined and revealed that high dose cannabis vapour administration increased response latency (sec) compared to all other treatments, across all eight delays. However, this difference was not significant (p's>.05; see Figure 6). Overall, the total number of trials for each treatment ranged between 85 and 100 trials. No significant differences were found (see Figure 7).

3.3: Reversal of CB1 Receptor Antagonist Rimonabant on the Effects of Working Memory (Experiment 3B)

3.3.1. Methods (3B)

To confirm the involvement of CB1 receptors in the behavioural effects observed in Experiment 3A, the ability of the cannabinoid CB1 receptor antagonist / inverse agonist rimonabant to reverse memory impairments was examined. The same animals and memory testing procedures described above were used. In addition, animals were injected with either 3

mg/kg rimonabant or its vehicle control intraperitoneally, and then placed back in their home cage for 10 min. Next, animals were placed into the vape apparatus and exposed to vapour for 30 min. Vapour drug treatments were conducted in a repeated measures fashion as follows: 3 mg/kg injected rimonabant coupled with cannabis vapour, 3 mg/kg injected rimonabant coupled with vehicle vapour, 3 mg/kg injected vehicle coupled with cannabis vapour, or 3 mg/kg injected vehicle with vehicle vapour. As noted in section 3.1.9, the wash-out procedure was applied to this part of the experiment (see Figure 3).

3.3.2. Results (3B)

A two-factor (delay x treatment) repeated measures ANOVA revealed that performance on the DMTP task increased when animals were exposed to cannabis vapour and pre-treated with rimonabant. When animals were not pre-treated with rimonabant, performance decreased. However, these differences were not significant (main effect of dose: $F_{3,18} = 1.21$, p=0.33). Overall performance across treatment groups decreased as the delay increased from 4s to 32s (main effect of delay: $F_{7, 42} = 33.60$, p<.001; see Figure 8). The mean latency to press choice lever was also assessed and revealed that cannabis vapour and vehicle rimonabant administration slightly increased latency (s) compared to other treatments, across all delays. However, this difference was not significant (*p's*>.05; see Figure 8).

3.4. Discussion (Experiment 3A &3B)

3.4.1. Experiment 3A

The current experiment aimed to examine working memory of rats following acute vapourized cannabis exposure on the DMTP task. To the best of our knowledge, this is the first study demonstrating the impairing effects of vapourized cannabis extract on working memory in

rodents. Results from this experiment were in the predicted direction and provided strong evidence that acute cannabis vapour impairs memory in a dose-dependent manner. Response latencies were short, averaging approximately 1-2s, regardless of drug treatment (Figure 6). In addition, performance was not affected during the first 2s of the task regardless of treatment. This finding confirms that these impairments are likely related to impaired working memory and were not caused by impaired non-mnemonic processes such as motor coordination or an alteration of the animals' motivational state.

The main contribution from this experiment suggests that the various THC constituents and other added cannabinoids within cannabis extracts produce similar memory-impairing effects to THC alone (Mallet and Beninger, 1998; Hyser, Hampson & Deadwyler., 1992, Nakamura et al.,1991). Findings also suggest that cannabis extract impairs working memory when administered in vapourized form in a manner similar to that observed with injected THC.

3.4.2 .Experiment 3B

Findings from Experiment 3B revealed results in the predicted direction that were not significant. The administration of rimonabant did not significantly attenuate the memory-impairing effects of vapourized cannabis from Experiment 3B. Our findings from this experiment were inconsistent with previous studies showing that rimonabant successfully attenuated memory impairment produced by injected THC and other cannabinoids, such as anandamide (Mallet and Beninger., 1998; Terranova et al., 1996). To the best of our knowledge, only one research paper conducted by Blaes et al. (2019) has examined the effects of acute exposure to cannabis smoke on working memory performance in rodents. Blaes et al. (2019) found that acute exposure to acute cannabis smoke (via cigarette) only affected choice accuracy in females but not in males on the DMTP task. In addition, Blaes et al. (2019) found that acute

injections of rimonabant (2 mg/kg) significantly reduced choice accuracy in females and had no effect in males. Choice accuracy was only affected when animals received acute injections of pure THC (3 mg/kg) in male and female rats (Blaes et al., 2019). While it is evident that the current experiment has conflicting findings from Blaes et al. (2019), it is important to note that the present study used cannabis extract products that contain many other cannabinoids constitutes and are not the same as cannabis smoke. Second, Blaes' et al. (2019) findings are inconsistent with the growing body of evidence that rimonabant does not affect memory performance and has been shown throughout the cannabinoid literature to attenuate the memory-impairing effects produced by cannabinoids. Most animal and human research demonstrate cognitive deficits, including working memory, following acute administration of cannabinoids (Mallet and Beninger, 1998; Hyser, Hampson & Deadwyler., 1992, Nakamura et al., 1991). In contrast, Blaes et al. (2019) demonstrated that acute exposure to cannabis smoke enhanced memory performance.

3.4.3. Possible confounds for unpredicted results

Due to the lack of research examining the effects of rimonabant, it is difficult to compare this study's findings to past cannabinoid literature directly. While the current study showed attenuation of impaired memory following rimonabant, this enhancement was not significant. As such, there are some possible explanations for the present study's unpredicted findings.

3.4.3.1. Effects of vapourized cannabis are partially CB1 mediated

Findings from this experiment suggest the possibility that the cannabis-induced impairing effects may not be CB1 mediated. There is growing evidence that cannabinoids such as THC act through CB1 receptors and that the impairing effects produced by cannabinoids are the consequence of

activation of these receptors. By administering a CB1 receptor antagonist, like rimonabant, impairing effects should be inhibited or reversed. It is important to reiterate that this study administered a cannabis extract (containing a mix of cannabinoids) and not THC alone. Some cannabinoids (e.g., CBD) are thought to mitigate some of the impairing effects of THC (Klein et al., 2011; Morgan et al., 2010), suggesting that the mix of cannabinoids within vaped cannabis extract may act upon a variety of different cannabinoid and non-cannabinoid receptors and are not specific to CB1 receptors.

3.4.3.2. Order effects and tolerance

Previous studies examining cannabis tolerance have demonstrated that tolerance to cannabis develops rather quickly, likely after the first dose and lasting around 10-12 dose-free days (Anderson, Jackson & Malor, 1975; Henderson, Crawford, Sepulveda, Hale, Lesperance & Morgan, 2021). One possibility for the unexpected findings may be due to the order in which the treatments were administered. The order in which animals received treatments can lead to a tolerance of cannabis. For example, animals who received a high dose of cannabis as a fourth treatment were exposed to greater amounts of cannabis when the second experiment was conducted compared to an animal that received vehicle as a fourth treatment. Moreover, using a within-subject design can cause tolerance to the memory-impairing effects of cannabis, causing diminished overall impairing effects. Another possibility is that the order in which one animal received vaped cannabis versus when another animal received vehicle, might show less of an effect from one animal (who received vaped cannabis) to the other (who received vehicle). In other words, the size of the effects may differ between animals.

If animals developed a tolerance to cannabis, there would be apparent residual tolerance from Experiment 3A in Experiment 3B. However, results from Experiment 3B revealed that a

high dose alone of vapourized cannabis impaired memory. In addition, the decline in choice accuracy was roughly the same in both experiments. Therefore, these results argue that sufficient time has passed between Experiment 3A and 3B and tolerance was not observed nor can account for the unexpected findings in this experiment.

3.4.3.3. Dose, time course, and sample size

Another possibility may be the dose and time course that rimonabant was administrated. Animals were injected with 3 mg/kg of rimonabant followed by a 10 min delay before entering the vapour chamber, whereby animals received a 30 min exposure to one of the four treatments before being tested. Therefore, animals were exposed to rimonabant approximately 40 mins before the task began. Still, the dose and time course that rimonabant was administered was well within the effective range reported to reverse THC-induced impairments (Mallet and Beninger., 1998; Terranova et al., 1996), and therefore the time course used in the present study seems appropriate.

In the present experiment, it was predicted that rimonabant would attenuate the memory impairments produced by vapourized cannabis exposure. While results were in the predicted direction, rimonabant did not significantly improve performance. Although the reason for the lack of improvement produced by rimonabant is unclear, the most probable explanation may be the relatively small sample size (N=7) used. That is, the sample size in the present experiment was small compared to previous studies that successfully demonstrated rimonabant-induced memory enhancement (Mallet and Beninger., 1998; Terranova et al., 1996). Future studies should aim to replicate the present study using a larger sample size.

To summarize, the present results provide strong evidence that acute vapourized cannabis impairs working memory on the DMTP task. On the contrary, the present results do not yet provide enough evidence that these memory-impairing effects of acute vapourized cannabis are specifically CB1 mediated.

Chapter 4. General Discussion

This study aimed to examine the behavioural and neural effects of acute vapourized cannabis in rodents using a modern delivery system that better replicates human consumption. The current animal cannabinoid literature focuses heavily on an injected route of administration, which does not represent human use and relies on pure forms of cannabinoids. While there have been some efforts to replicate models of injected cannabinoids using an inhaled delivery system, very few studies have directly compared any observed behavioural and neuronal differences. Overall, findings from the present study support the behavioural and pharmacological differences in the routes of administration used between injected and vapourized studies.

In the present study, two experiments were conducted. The first experiment, described in Chapter 2, examined neuronal activation patterns induced by acute cannabis vapour using Fos immunochemistry. Results revealed that vapourized cannabis increased Fos-IR in the PVN, PVT, and SI. While preliminary hypotheses were unclear, the current study provides novel information regarding changes in neural activity following acute vapourized cannabis exposure and demonstrates clear preliminary data suggesting a successful use of an inhaled delivery model that may replace the current injected route of administration. The results from this experiment are consistent with findings from injected THC studies (Allen et al., 2003, Arnold et al., 2001,

McGregor et al., 1998, Miyamoto et al., 1996). Comparable to findings from injected THC studies, this experiment showed activation of Fos expression in key brain regions associated with physiological and psychological impact following exposure to cannabinoids. The similarities found in Fos expression throughout the rodent brain when animals are exposed to injected THC and vapourized cannabis extract sugguest that the Fos-IR activity discovered in this experiment is likely due to THC content than other cannabis constitutes. This confirms the notion that THC is likely the driver of the psychoactive and neural effects of cannabis use.

The second experiment, described in Chapter 3, sought to use the delayed-match-to-position (DMTP) task to examine working memory following acute administration of vapourized cannabis and to confirm the involvement of CB1 receptors in these memory impairing effects. Consistent with previous hypotheses, exposure to acute vapourized cannabis significantly impaired working memory in a dose-dependent manner. The administration of rimonabant did not appear to attenuate performance. However, overall trends seem to be in the predicted direction.

4.1. Limitations and future directions

A few aspects of the present study have not yet been addressed. One limitation of the present study is the failure to assess blood levels of vaped cannabis. A previous study by Manwell et al. (2014) assessed the blood concentration of vapourized THC or IP-administered THC in rodents. Manwell et al. (2014) found roughly equivalent mean blood concentrations following exposure to vapourized and injected THC (vapourized THC: 1, 5, and 10 mg: IP-administered THC: 0.25, 0.50, 1.0, and 1.5 mg/kg). However, it should be noted that the dose

comparison of THC or other cannabinoids (in mg) is unclear when using an electronic delivery system of vapourized cannabis extract.

The present study did not use an injected cannabis extract control group. By not having this control group, we cannot make direct comparisons between routes of administration following cannabis extract exposure. While our lab has previously shown changes in Fos expression, a decline in memory, and rimonabant-enhanced performance on the DMTP task following injected cannabinoid exposure, future studies could include an injected THC treatment group to more directly compare these routes of administration.

4.2. Conclusions

Following legalization and the trend worldwide to legalize cannabis, finding from the present experiments using vapourized cannabis serve to better inform human cannabis users of the effects of vapourized cannabis use. According to the Canadian Tabacco, Alcohol and Drugs Survey (CTADS, 2019), an overall 21% (6.4 million) of Canadians reported cannabis use in the past year, an increase compared to 2017 (15% or 4.4 million), defining cannabis as the most used drug in Canada. According to the Canadian Cannabis survey (2021), 29% of Canadian cannabis users report using cannabis vape pens while 53% report consuming cannabis edible products, 65% report using the dried flower and 26% report using cannabis oil for oral use. There is a rapid increase in vapourized cannabis administration and an overall increase in vaping worldwide. Historically, users would inhale the smoke from burning dried cannabis or by burning resin rich parts of the marijuana plant (i.e. hashish) (Ren et al., 2019). Today, cannabis users are consuming cannabis products using different methods of consumption and not much is known about the harms and potential health risks associated with consuming cannabis in this way.

The three experiments on vaped cannabis reported in this study suggest that the current cannabinoid literature does not completely model human drug consumption and that human user should better consider the short-term and relatively unknown long-term effects of vapourizing cannabis. Most of what we know about cannabis comes from studies examining one cannabinoid drug, namely THC. THC is the primary driver of the cognitive impairments observed following cannabis administration via a CB1 –mediated mechanism. Findings from the current study suggest that the neuronal changes and memory impairing effects of acute vaped cannabis are similar to those observed with injected forms of THC. However, rimonabant (Experiment 3B) was unable to block these memory impairing effects. Findings from Experiment 3B suggest that administering multiple cannabinoids (in cannabis) together may not therefore be mediated by the same receptors as THC alone and that multiple receptors may be involved. It is also possible that the memory impairments produced by acute vapourized cannabis are not related to CB1. Several studies have found a novel cannabinoid G protein-coupled receptor 55 (GPR55), that is highly expressed in large dorsal ganglion neurons and have been shown to be activated by various cannabinoids such as THC and methanandamide (a metabolically stable form of anandamide) (Lauckner et al., 2008; Ryberg et al., 2007). Another possibility is that some cannabinoids in cannabis are acting upon the CB2 receptors. Previous cannabinoid studies have examined multiple cannabimimetic indoles and their affinity for either CB1 or CB2 receptors. One study found that cannabimimetic indole 1-(2, 3-dichlorobenzoyl)-2-methyl-3-(2-[1-morpholino]ethyl)-5-methoxyind ole (L768242) and 2-methyl-1-propyl-3-(1-naphthoyl)indole (JWH-015) showed higher affinity for the CB2 receptor and lower affinity for the CB1 receptor (Huffman, 2000). It is plausible that the memory impairments produced by acute cannabis vapour is not related to CB1 and may be more closely related to GPR55 and the CB2 receptor. In addition, it is also

plausible that the memory impairing effects of vapourized cannabis are due in part to another cannabinoid receptor that has not yet been discovered. Future cannabinoid research should aim to further elucidate the role of novel cannabinoid receptors in mediating these effects.

To conclude, the present study serves to provide proof of concept of a novel method of drug administration for use in laboratory rats that more closely mimics human use. Historically, inhaled and oral cannabinoid self-administration research has been difficult to model in animals, but the methodology continues to mature (Lefever et al., 2014; Barrus et al., 2018), and the work presented here represents further progress in this regard. The present study additionally provides preliminary data on the behavioural and neural effects of cannabis using this novel mode of delivery. With cannabis use rising worldwide, there is increasing need to better understand the cognitive and neuronal changes induced by vapourized cannabis. The lack of information and understanding of the effects of other cannabinoids found within cannabis products remain a cause for concern. The present study shows that it is possible to move away from traditional injected routes of administration and conduct basic cannabinoid pharmacology research with greater ecological validity. Future studies that employ this novel drug delivery method will serve to better inform scientists, policy makers, and the public, about the possible health risks associated with this increasingly popular method of cannabis use.

Table 1. Mean (±SEM) *Number (per mm^2) of Fos-Immunoreactive Neuronal Nuclei 90 Minutes After Exposure to 30 min of Cannabis or Control Vapour.*

	Bregma (mm)	Control (n=6)	Cannabis (n=6)
1. Paraventricular nucleus of the hypothalamus	-1.8	104±16.4	188±30.4*
2. Paraventricular thalamic nucleus	-1.8	240±27.6	436±21.6**
3. Primary somatosensory cortex	-1.8	96±39.2	216±15.2*
4. Interanteromedial thalamic nucleus	-1.8	264±66.4	356±36.4
5. Primary motor cortex	-1.8	136±21.2	68±25.6

* $p<0.05$ and ** $p<0.01$ compared to control.

Figure 1: Evolution of the E-cigarette technology.

Figure 2. Parts of an electronic cigarette diagram.

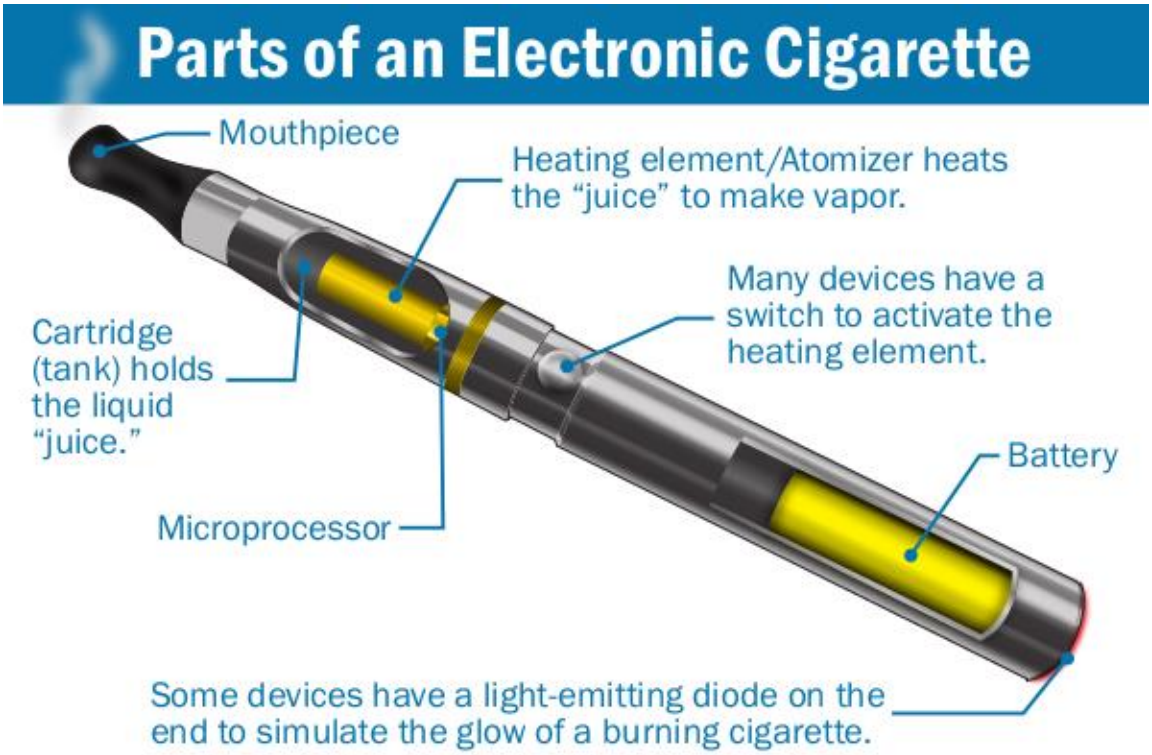
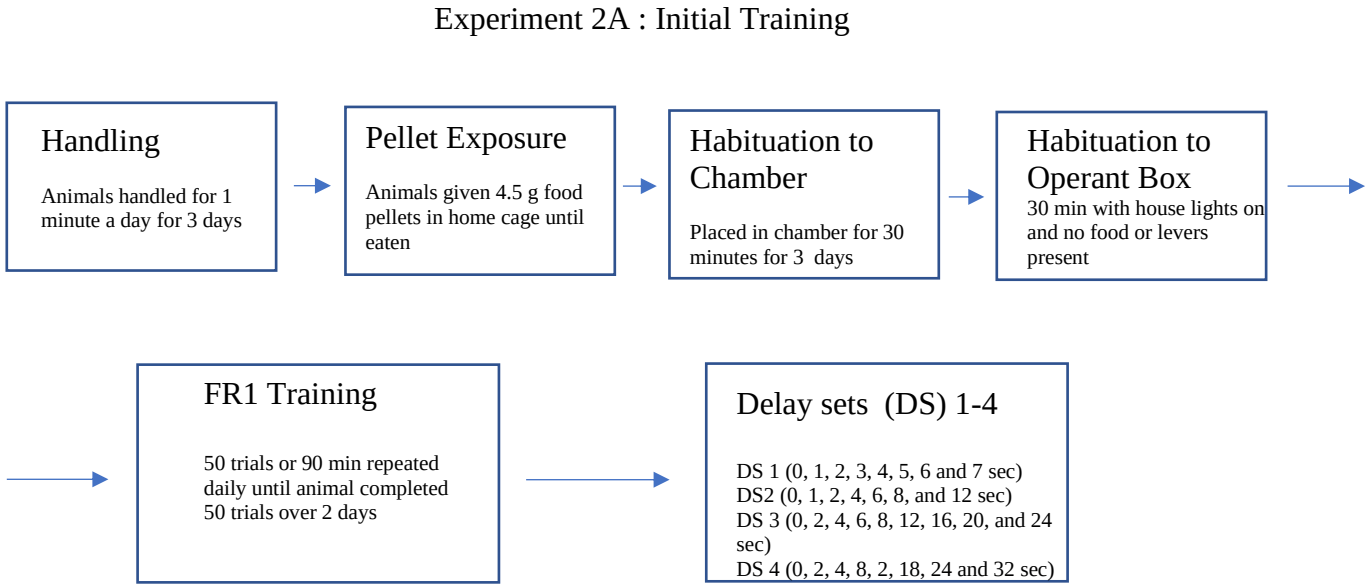


Figure 3. Outline of Experiment 2A initial training and drug testing.



Experiment 2A: Drug Testing

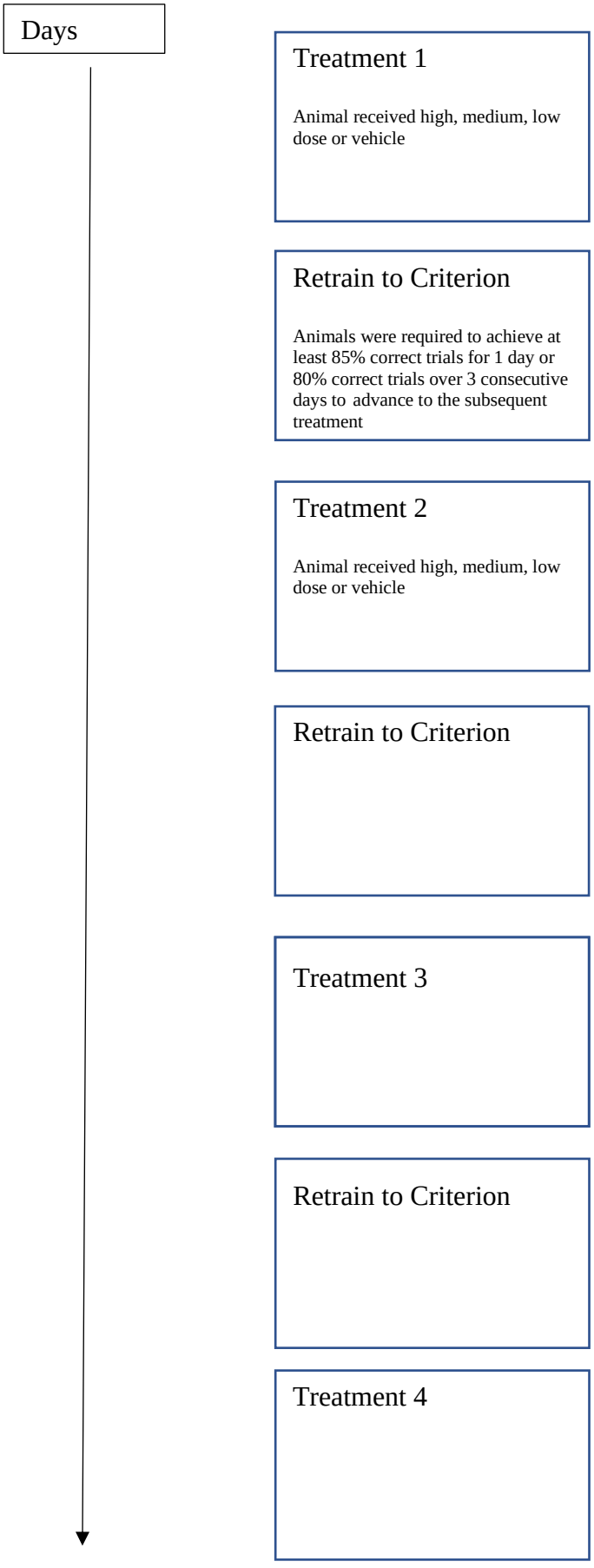


Figure 4. Schematic representation of coronal slices of the rat brain (Paxinos and Watson, 1998). The number of Fos positive cells were counted within the areas shaded in red. The numbers indicated correspond to the brain regions listed in Table 1.

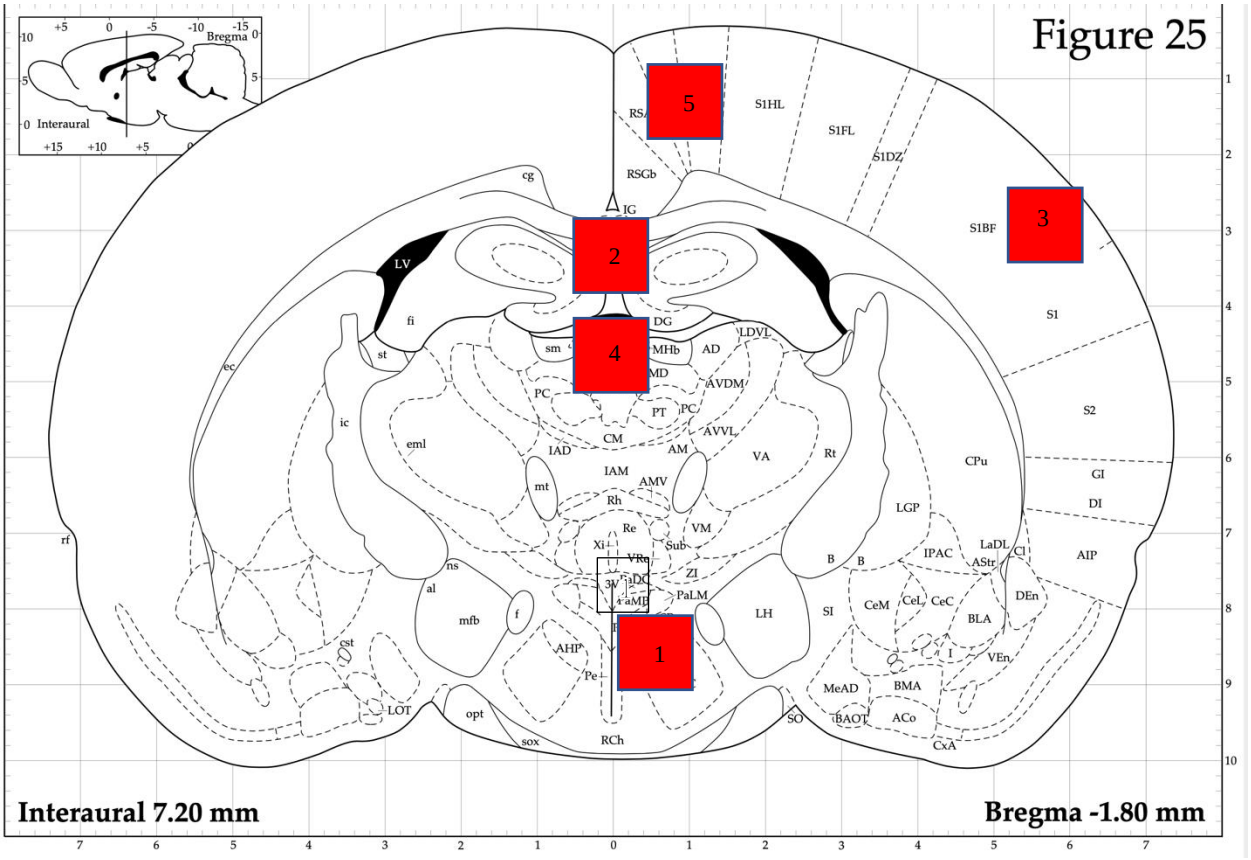


Figure 5. Mean (+/- SEM) percentage of correct responses in the delayed match to position task across eight delay intervals (in seconds) following administration of vehicle, low, medium, or high dose of vapourized cannabis.

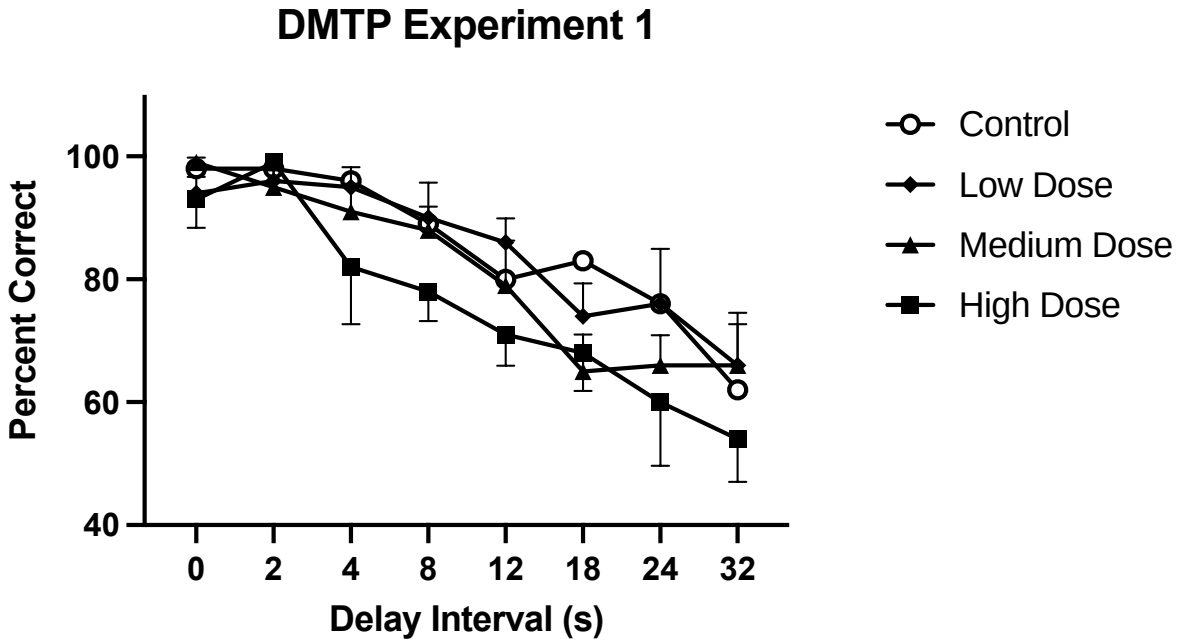


Figure 6. Mean (+/- SEM) latency to press lever (in seconds) in the delayed match to position task across eight delay intervals following administration of vehicle, low, medium or high dose of vapourized cannabis.

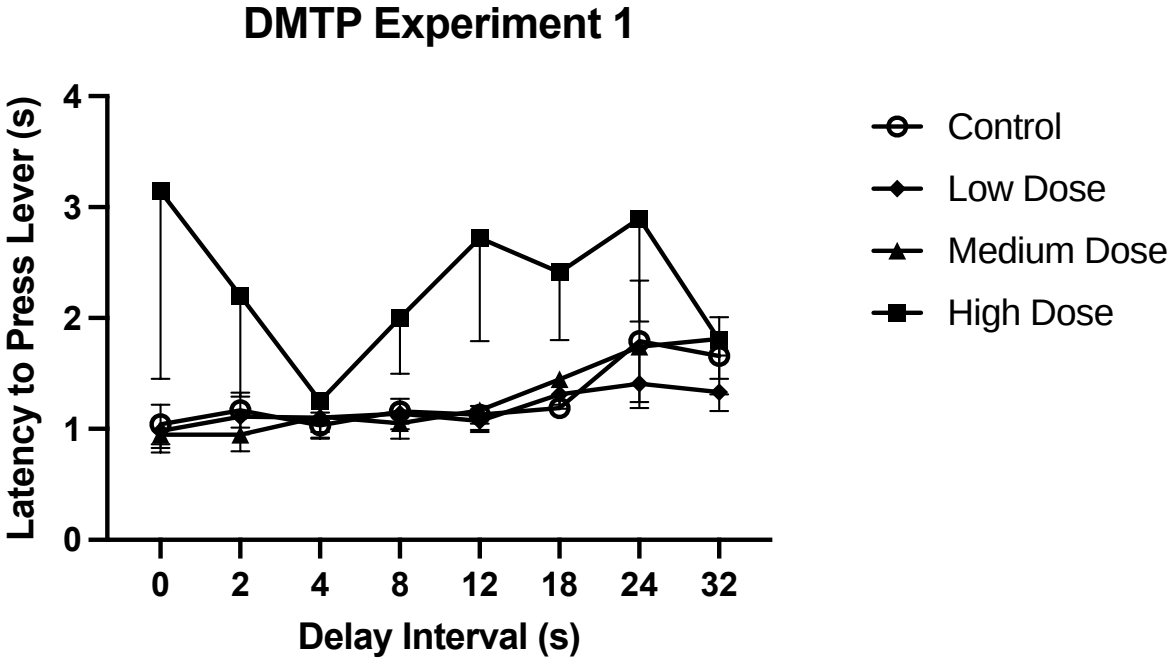


Figure 7. Mean (+/- SEM) number of trials completed in the delayed match to position task following administration of vehicle, low, medium, or high dose of vapourized cannabis.

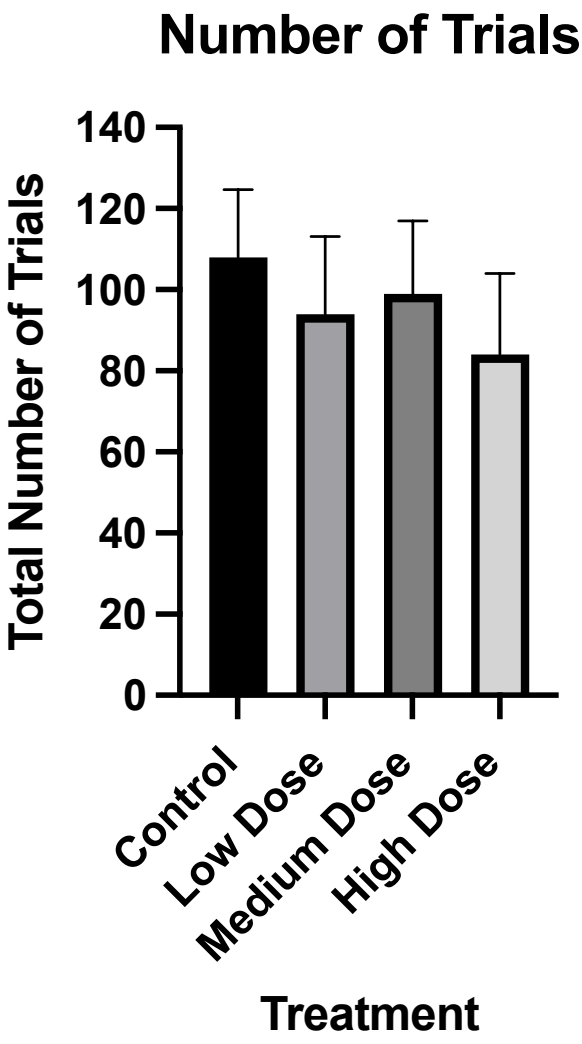
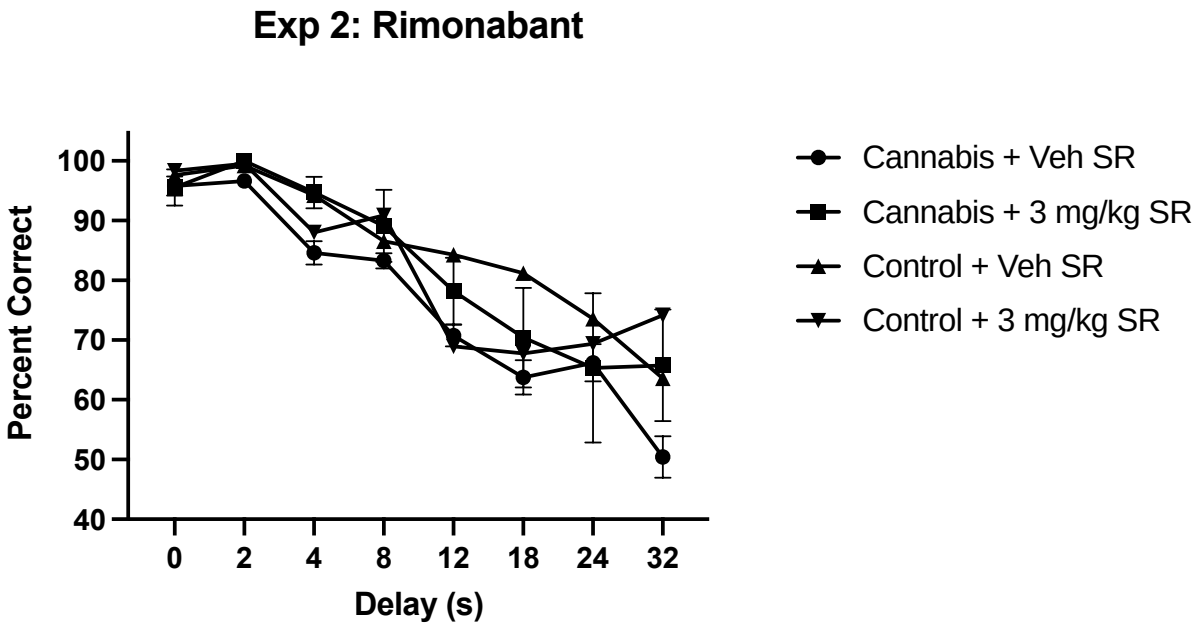


Figure 8. Mean (+/- SEM) percentage of correct responses in the delayed match to position task across eight delay intervals (s) following administration of cannabis vapour + vehicle rimonabant, cannabis vapour + 3 mg/kg rimonabant, control vapour + vehicle rimonabant, and control + 3 mg/kg rimonabant.



Appendix A: Vapourization Cannabis Delivery Protocol

Purpose: This SOP describes the preparation and usage of the vapourization apparatus for rodent inhalation of cannabis extract/vehicle vapour.

Materials:

- Inhalation apparatus with power cord
- Two mouse cages (Allentown)
- Lighter
- ¾" and 1" Diameter shrink tube purchased from amazon (RockDIG 74PCS 3:1 Heat Shrink Tubing Double-wall Adhesive Lined Shrink Tube Assortment Kit 4 Large Size 1 3/4 1/2 3/8 Inch Black Red)
- Six 5 mm ID, 8 mm OD silicone tubes (two cut 8" tubes)
- Two plastic bottles labelled "clean and medium clean" with the clean bottle containing 500ml of 70% ethanol
- Foray 510 thread vape battery and USB charger (https://ocs.ca/products/vaporizer-battery-foray)
- Foray Maui Wowie 0.5g cartridge (https://ocs.ca/products/maui-wowie-510-thread-cartridge)
- Flavourless, nicotine-free ejuice

Procedure: all to be completed under a fume hood

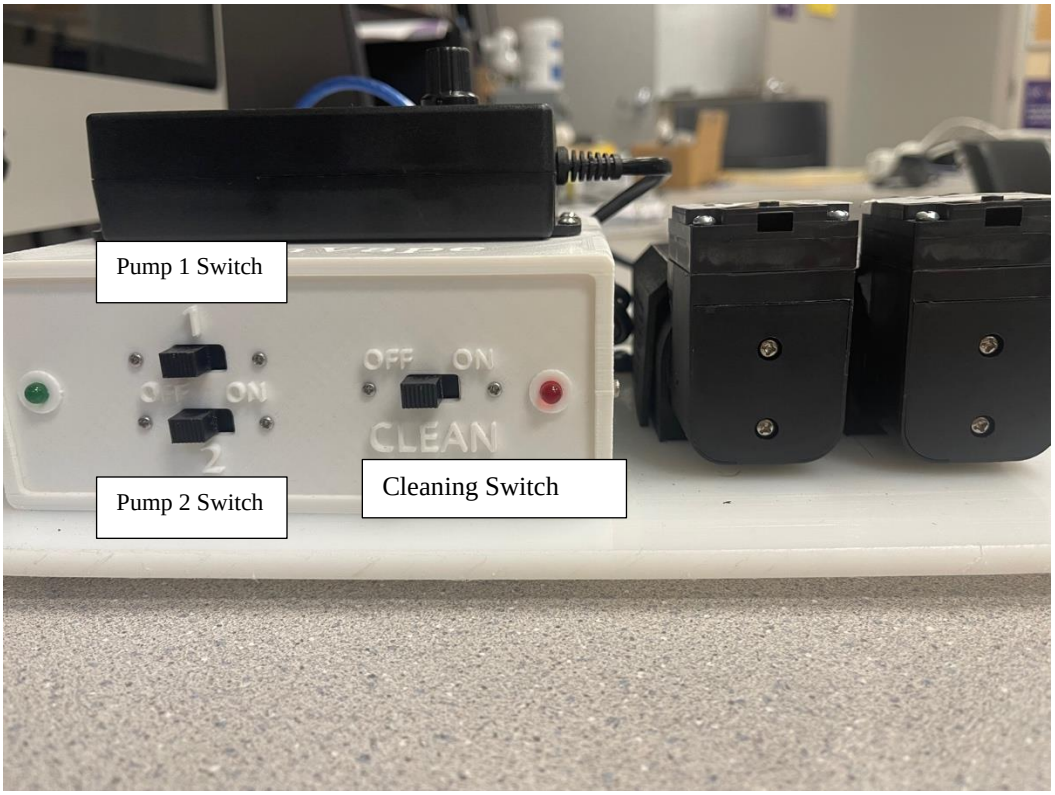
1. Plug in vape apparatus
2. Ensure that the voltage meter located on the power bar is set to 9V. Do not exceed 12V
3. Attach one silicone tube to the input (left) valve of the vacuum pump and one tube to the output (right) valve
4. Fill a small glass beaker with 500ml of 70% ethanol. Pour the 500ml of ethanol into a plastic container. Have another empty plastic container for waste. WARNING: do not use a glass beaker. The cannabis oil will ruin the beaker.
5. Place the input tube into the container filled with 500ml of ethanol and the output tube into the empty container. Switch the cleaning mode switch on.
6. Allow the pump to run in cleaning mode until all 500ml of ethanol is gone. This container should now be labelled "medium dirty."
7. Once all 500ml is filled into the medium dirty container, allow the pump to run for two minutes.
8. Remove both input and output tubing (label as cleaning tubes) and replace them with one output tube for the cannabis pump and one output tube for the vehicle pump.
9. Cut another piece of silicone tubing so that the tubing measures 8" in length. Do this twice.
10. Take one of the 8" tubing and insert it into the 12mm 3D printed nozzle. Using your ¾" shrink tube, place one end of the shrink tube over the nozzle and place the foray vape pen with foray cartridge into the other end of the shrink tube. Using a lighter, carefully heat the shrink wrap to eliminate the gap between the tube and the foray vape pen. Repeat this step with the vehicle control pen. Use the 1" shrink tube for the vehicle pen.
11. Ensure that the cleaning mode is switched off. Before placing animals in the apparatus, ensure the device is working. Switch pump one and pump two on. Pump one control the

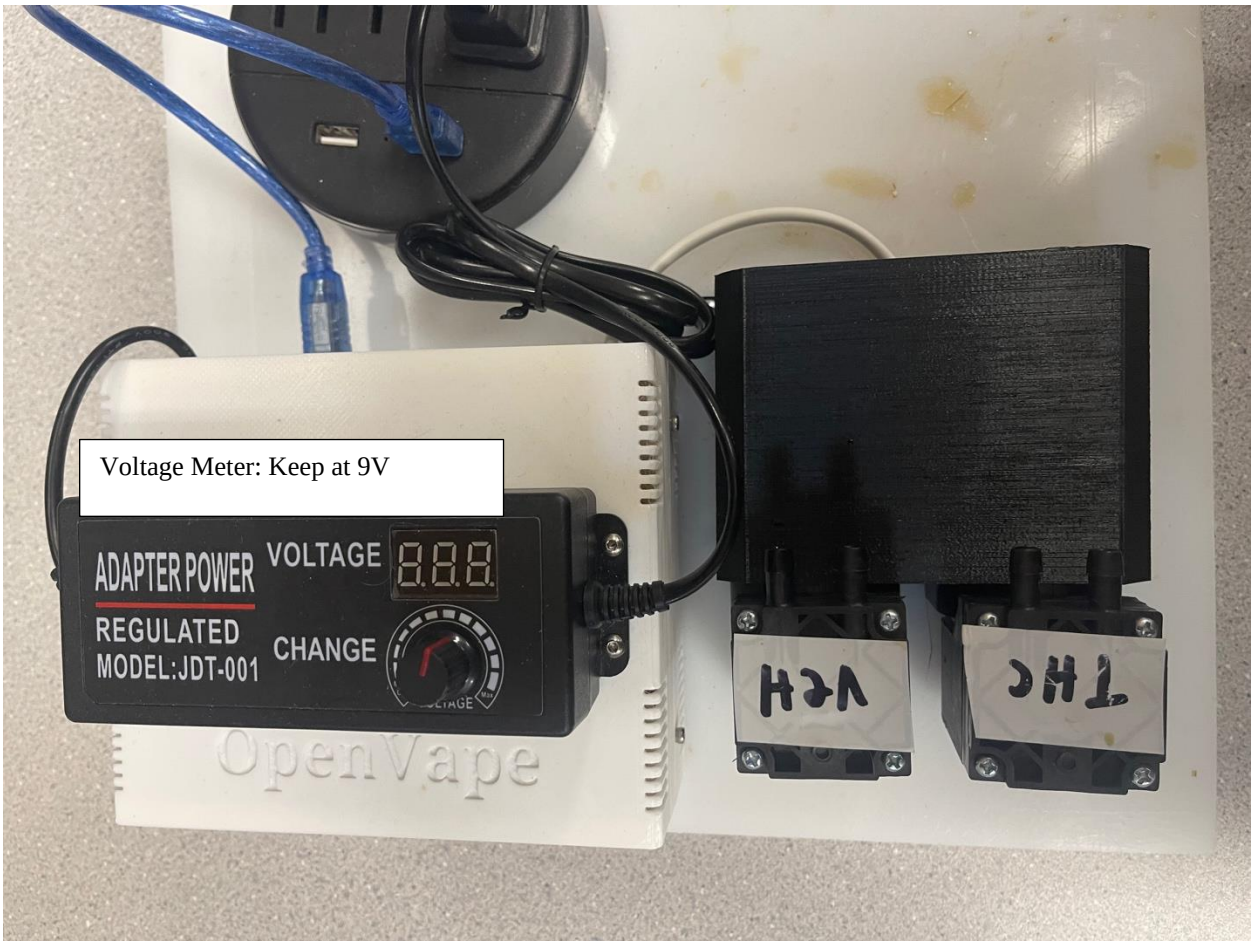
left pump and pump two controls the right pump. The left pump labelled "Cannabis" controls the cannabis pump and the right pump labelled "Vehicle" controls the vehicle pump. If the bottom of the foray pump lights up blue, the cannabis pump is working. Likewise, if the "LUX" symbol on the bottom of the vehicle pen lights up, the vehicle pen is working.

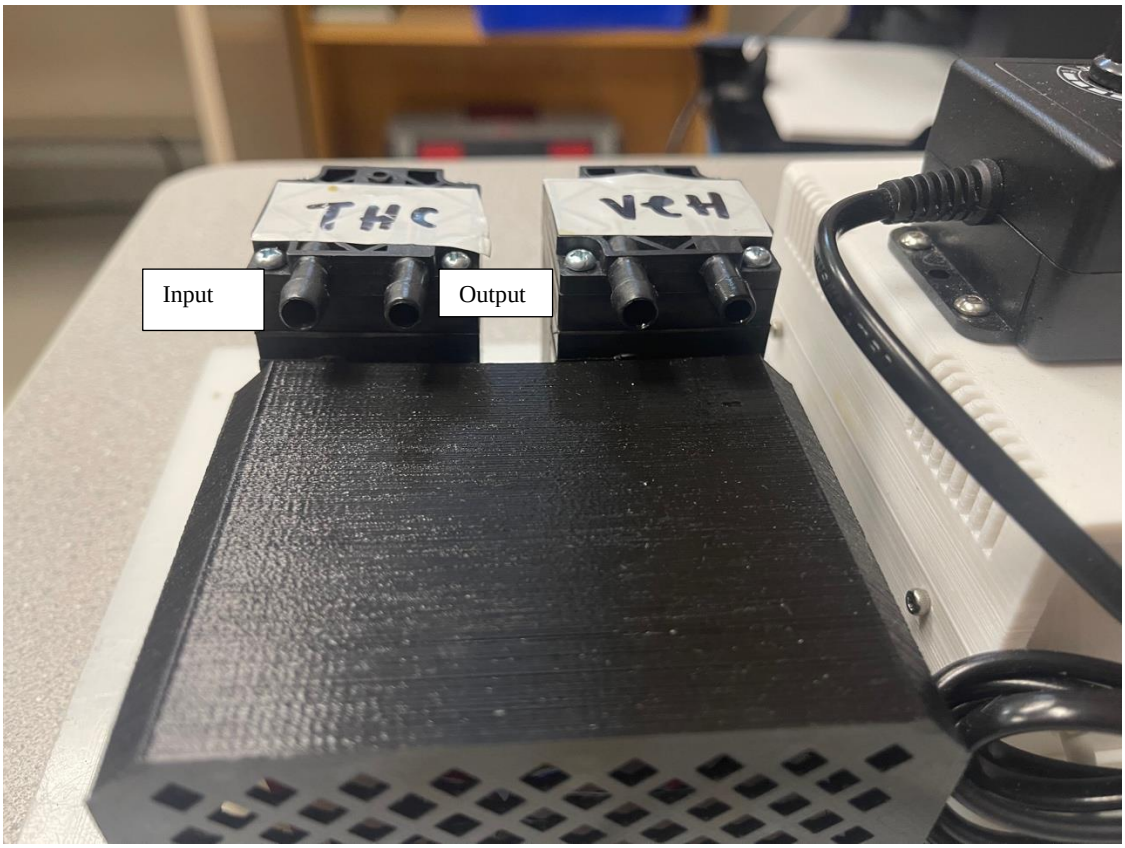
12. To turn off both pumps, switch both pumps off.
13. Attach the output tube's free end to the nozzle on the back of the mouse cage.
14. Place each animal into the mouse cage. Be sure to label the front of the cage with appropriate treatment and rat ID.
15. Depending on the age and size of the animal, add a few heavier items on top of the cage to prevent the animal from lifting the lid.
16. Switch both pumps to on. Ensure that both vape pens are running (See step 11) and begin the timer for thirty minutes. The pump will send a puff of vape smoke to the animal cage for 5 seconds every 55 seconds.
17. If vape pens are not lighting up or you can see that they are not delivering smoke to the cages:
    - The battery may be dead
    - The pump may be clogged
    - The cartridge may be empty
18. Note: the cannabis pump should be cleaned after every animal. The vehicle pump should be cleaned at the end of the day.

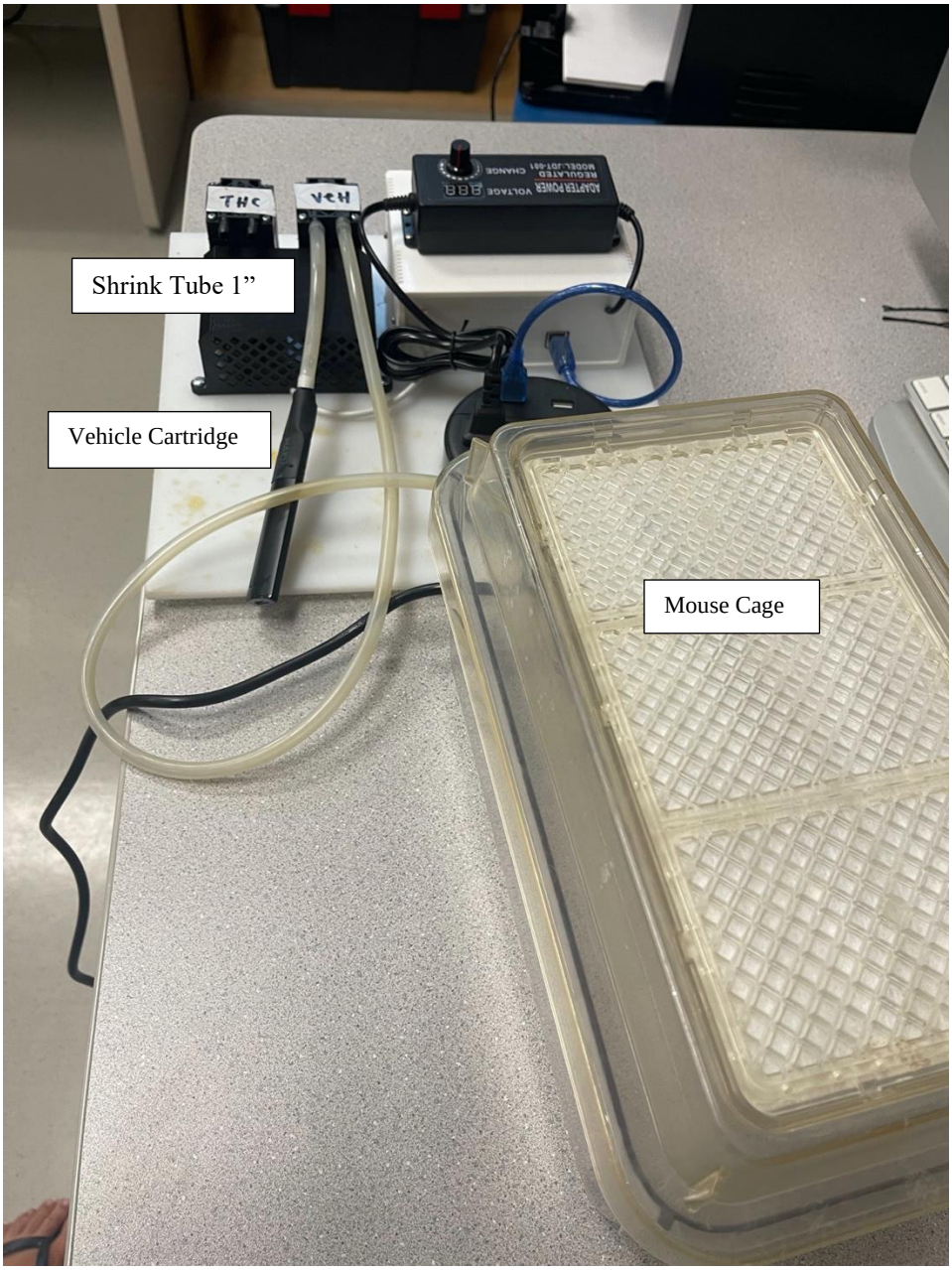
Cleaning protocol: to be completed after initial cleaning (Step 7)

1. You should always have two plastic containers (Clean and medium dirty). Rise the pump with the diluted ethanol (500ml). Once finished, use new, clean 500ml of ethanol and run that through the same pump. Your medium dirty ethanol will become your "dirty" ethanol, and your clean ethanol will become your medium dirty ethanol. You will replenish the clean ethanol with new stock. You should replace clean ethanol every third day.


Pump 1 Switch
1
OFF ON
2
Pump 2 Switch
OFF ON
CLEAN
Cleaning Switch


Voltage Meter: Keep at 9V
ADAPTER POWER
VOLTAGE
REGULATED
MODEL:JDT-001
CHANGE
OpenVape


THC
VCH
Input
Output


THC
VEH
Shrink Tube 1”
Vehicle Cartridge
Mouse Cage

Appendix B: Habituation code written in MEDState notation

```
\This is the habituation program
\Filename, DMTP-DAY01-HAB.mpc
\Date: OCT-9-2015

\Session runs for 30 minutes with houselights on
\Every minute the display is updated to show minutes elapsed

\This section is for Outputs
\1-left lever operate
\2-right lever operate
\3-feeder
\4-left stimulus light
\5-right stimulus light
\6-white noise
\7-houselight
\8-low tone
\9-high tone
\16-pump
^HL=7

\This section is for Inputs
\1-left lever response
\2-left lever position
\3-right lever response
\4-right lever position
\5-hopper head entry
\6-feeder error
^HE=5


\VARIABLE SUMMARY
\M=Elapsed session time (minutes)
\H(30)=Number of head entries (per minute)
\T=Total head entries for entire session

DIM H=30

S.S.1,
S1,
   #START: ON ^HL; SET M = 0; SHOW 1, ELAPSED (MIN), M ---> S2
S2,
   1': IF M=30 [@FOURTRUE, @FOURFALSE]
      @FOURTRUE: SHOW 1, ELAPSED (MIN), M; OFF ^HL --->STOPABORTFLUSH
      @FOURFALSE: SHOW 1, ELAPSED (MIN), M; ADD M --->S2

S.S.2,
S1,
  #START: SET H(M)=0; SET T=0 ---> S2
S2,
  #R^HE: ADD H(M), T; SHOW 2, HEAD ENTRIES, T ---> SX
```

Appendix C: AutoShaping code written in MEDState notation

```
\ Filename ASHAPE.mpc
\ Autoshape lever presses to uncued left & right levers
\ Procedure for Mallet operant chambers
\ Based on A. Santi R2SHAPE Dec 2010
\ P. Mallet - v. 1.0 Mar 16, 2011

\ Inputs for lever responses normally should be Toggle(T) and Normal(+). The Toggle setting requires the rat
\ to press and release the lever before another response is detected. The Normal setting results in an input
\ when the lever is pressed. Normal settings work with normally open contacts.
\ Retractable Lever cables: Response cable is an input indicating a lever press
\                   Operate cable is an output "ON" extends the lever "OFF" retracts the lever
\                   Position cable is an input cable indicating extension (On)or retraction (Off).

\ Nose poke units - The control amplifier operates on 28 V DC and produces a ground output whenever the beam is
broken.
\ Inputs should be set to Level Mode (L) and Inverted (-).

\ Inputs for Head Detection and Pellet Detection must be set to Level Mode (L)
\ and Inverted (-). These settings are made with the jumpers on the module itself.
\ To test the modules for the correct setting use MED Test. When the head or pellet
\ is not in the tray (i.e.,empty), the LED indicator on the interface module should be off.
\ The Level setting results in an input being detected each time the card is polled for
\ as long as the input is maintained. In addition to head entry detection this can be used
\ time how long a lever is held down.

\ Pellet Sentry function for pellet feeders - only provide an alarm signal input if there are
\ five consecutive failures of a pellet being dropped successfully. This function would be rarely used.
\ They are not configured in this basic set up. Note that: A sensitive infrared photo-beam detector
\ is in the delivery tube of the dispenser. If pellet delivery is not confirmed, a built-in microprocessor
\ will repeats the delivery sequence up to five times within 150 milliseconds.


\ ***** Input Definitions

\Front Panel
^LeftLeverResponse  = 1  \Toggle-Normal
^RightLeverResponse = 2  \Toggle-Normal
^LeftLeverPosition  = 3  \Toggle-Normal
^RightLeverPosition = 4  \Toggle-Normal
^FrontHeadEntry     = 5  \Toggle-Normal
^FeederError        = 6  \Not used in this program
^ManualFeederButton = 7

\ ^FrontPelletTroughDetect = Not configured
\ ^FrontPelletDispenserInput = Not configured

\Back Panel = Not configured

\ ***** Output Definitions

\ FrontPanel
^LeftLeverOperate  = 1
```

```
^RightLeverOperate = 2
^FrontPelletOutput = 3
^LeftCueLight      = 4
^RightCueLight     = 5
^HouseLight        = 7

\ Disk variable settings
DISKVARS = K,I
DISKCOLUMNS = 10
DISKFORMAT = 5.2
DISKOPTIONS = FULLHEADERS

\ ***** VARIABLES
\ K = Summary Data Array
\ I = Parameter array for experimental sesssions

DIM K = 7
\ K(0) Elapsed Session RunTime
\ K(1) Number of trials with Left LeverPress
\ K(2) Number of trials with Right LeverPress
\ K(3) Number of trials without Left LeverPress
\ K(4) Number of trials without Right LeverPress
\ K(5) Number of KeyBoardPellets
\ K(6) Number of trials

\ Lever Trial Configurations
LIST E = 1, 2, 1, 2
\ Definitions of each trial configuration.
\ Left  = 1
\ Right = 2

\Experiment Parameters (I)
LIST I = 50, 0, 0, 0, 60, 40, 0, 20, 0, 1.0, 0
Var_Alias Max #Reinforcers = I(0)
VAR_Alias Session Number = I(1)
\VAR_ALIAS RunTime = I(2)
\VAR_ALIAS Elapsed Session Time = I(3)
VAR_ALIAS Max Session Duration (min) = I(4)
Var_Alias ITI Duration (sec) = I(5)    \# resolution ticks stored in I(6) variable.  Calculated at beginning
Var_Alias Lever Extension Duration (sec)= I(7) \# resolution ticks stored in I(8) variable.  Calculated at beginning
Var_Alias Infusion Duration (sec) = I(9) \# resolution ticks stored in I(10) variable.  Calculated at beginning

\Variable Descriptions
\ A = Table format for results
\ B = Trial Counter
\ C = Current lever location presented
\ D = ITI Duration Values = 4,8,16,32
\ E = Lever Location Value List = 1,2,1,2
\ F = First trial identifier (flag, TRUE=FIRST, FALSE=CORRECTION TRIAL)
\ G
\ H =
\ I = Parameter array for Experimental Session
\ J = Current Signal Value (During PSE Testing: Value Obtained from sampling the values in arrays M,N,O,P)
\ K = Summary Data Array
\ L =
\ M =
```

```
\ N =
\ O
\ P
\ Q = Temporary variable for timing the signal duration (Q = (J*1")CurrentSignal*TimeBase 1
\ R = Index for Signal Duration in the  Data Array
\ S
\ T
\ U
\ V = Long Response 1=long 0=short
\ W = Dim 3 W(1)=hopperduration; W(2)=ITI duration; W(3)=Temp ITI variable;
\ X = elapsed time for response
\ Y


S.S.1,    \ Main Control State Set for program

S1,
   0.001": SHOW 2,Max #Reinforcers,I(0); SHOW 3, ITI duration, I(5);
          SHOW 4,Lever Extension Duration,I(6); SHOW 5,Session,I(1) ---> S2
S2,
   #START: SET I(6) = I(5)*1"; SHOW 1,Elapsed(Min), I(3)/60; SHOW 2,Max#Pellets, I(0); SHOW 3,Trials
Completed,K(4);
          SHOW 4,Trials w Press, K(1)+K(2); SHOW 5,Trials w/o Press, K(3)+K(4); OFF ^LeftLeverOperate,
^RightLeverOperate;
          SET I(10) = I(9)*1";
          ON ^HouseLight ---> S3
S3,
   I(6)#T: SET I(8) = I(7)*1"; RANDD C = E;
        IF C = 1 [@True, @False]
           @True: ON ^LeftLeverOperate ---> S4
           @False: ON ^RightLeverOperate ---> S4

S4,
    #R^LeftLeverResponse: OFF ^LeftLeverOperate; Z1; ON ^LeftCueLight, ^RightCueLight; ADD K(1)---> S5
    #R^RightLeverResponse: OFF ^RightLeverOperate; Z1; ON ^LeftCueLight, ^RightCueLight; ADD K(2)---> S5
    I(8)#T: OFF ^RightLeverOperate, ^LeftLeverOperate; Z1; ON ^LeftCueLight, ^RightCueLight;
       IF C = 1 [@True, @False]
          @True: ADD K(3)--->S5
          @False: ADD K(4)--->S5

S5,
  #R^FrontHeadEntry: ADD K(6); Off ^LeftCueLight, ^RightCueLight ---> S6

S6,
  0.01": SHOW 2,Max#Pellets,I(0);SHOW 3,Trials Completed,K(6);SHOW 4,Trials w Press,K(1)+K(2);
         SHOW 5,Trials w/o Press,K(3)+K(4);
        IF K(6) = I(0) [@True, @False]
          @True: ---> S7
          @False: ---> S3
S7,
   .01": OFF ^RightLeverOperate, ^LeftLeverOperate, ^Houselight, ^LeftCueLight, ^RightCueLight; Set I(2) = I(3)/
60 ---> STOPABORTFLUSH



S.S.2, \ Record and Display Elapsed Session RunTime
```

```
 S1,
   #START --->S2
 S2,
   1": Set I(3)= I(3) + 1; SHOW 1,RunTime(Min), I(3)/60 ---> S3
 S3,
   0": if I(3)/60 >= I(4) [@SESSION_END_True, @SESSION_END_False]
    @SESSION_END_True: OFF ^LeftLeverOperate, ^Houselight, ^LeftCueLight, ^RightCueLight; Set I(2) =
I(3)/60; ---> STOPABORTFLUSH
    @SESSION_END_False: ---> S2
```

S.S.3, \Monitor manual feeder button

```
 S1,
   #START --->S2
 S2,
   #R7: Z1; ON ^LeftCueLight, ^RightCueLight; ADD K(5); ADD K(6); SHOW 6,Manual Pellets, K(5) ---> S3
 S3,
   #R^FrontHeadEntry: OFF ^LeftCueLight, ^RightCueLight ---> S2
```

S.S.4, \Deliver pellet

```
S1,
  #Z1 ---> S2
s2,
  0.1": ON ^FrontPelletOutput ---> S3
S3,
  0.1": OFF ^FrontPelletOutput ---> S1
```

Appendix D: FR1 training code written in MEDState notation

```
\ FR.mpc
\ Basic FR Program
\ By Adam Celejewski
\ For DIG-703
\ Based on on stock Med PC code


\ Constants Used in this Program

\ Inputs
^LeftLeverR      = 1
^ManualReward    = 7
^RightLeverR     = 2
^HeadEntry       = 5
^ManualButton    = 8


\ Outputs
^LeftLeverOp      = 1
^RightLeverOp     = 2
^FrontPelletOutput           = 3
^BackPelletOutput            = 16
^LeftCueLight   = 4
^RightCueLight  = 5
^HouseLight = 7


\ Data Variables
\  A = lever response counter

\  C(I) = Latency to each active resposne

\  E = Reward Counter
\  F = Free Reward Counter

\ Working Variables
\  I = Subscript for IRT Array C


\  L = Lever code;       Uses M list to randomly select one of two lever inputs. L Corresponds directly to hardware
lever input
\  M =  Lever List;     List for RANDD selction of for lever

\  R = Runing time           For Counter display
\  T = Used to Increment Counts at 0.1 Second Intervals for IRT's. For active lever
\  V = VAR ALIAS variable array for modifying experimental variables

\  Array sizes and Lists
DIM C = 10000
LIST M = 1,2,1,2     \


\Experimental variables configuration aray
LIST V = 1,10,50,90,1.0,0
```

```
VAR_ALIAS FR                    = V(0)     \Fixed Ratio
VAR_ALIAS ITI                   = V(1)     \ITI Duration
VAR_ALIAS Max Rewards           = V(2)     \Max number of rewards i.e. trials before session terminates
VAR_ALIAS Session Duration (Min) = V(3)    \Session duration in minutes
Var_Alias Infusion Duration (sec) = V(4) \# resolution ticks stored in V(5) variable.  Calculated at beginning

\ Z-Pulses Used in this Program
^Reward              = 1  \Activates Feeder ---> Stops feeder for duration of ITI
^ResponseCounterStop   = 2  \Stops resposne counter for duration of ITI  \Response counter start and stop for
preventing coutning lever preses while lever retracting
^ResponseCounterStart  = 3  \Starts Response Counter

DISKVARS = A, C, E, F, R, V
\DISKFORMAT = 6.2


\***********************************************
\             Main Procedure
\***********************************************
S.S.1,

S1,
  #START: SHOW 1,FR =,V(0);
        SET V(1) =V(1)*1"; SET V(5) = V(4)*1"; SHOW 4, Free Rewards, F;
        ON ^HouseLight --->S2

S2,
   V(1)#T: RANDD L = M;   Z^ResponseCounterStart;
      IF L = 1 [@Left, @ Right]                          \Note: 6 and 7 corespond to hardware config;\ 1 =LeftLever 2 =
RightLever Inputs
          @Left: ON ^LeftLeverOp,^LeftCueLight     --->  S3
          @Right: ON ^RightLeverOp,^RightCueLight   ---> S3
S3,
    V(0)#R^LeftLeverR ! V(0)#R^RightLeverR: ADD E; SHOW 3,Rewards,E;
           Z1;
           OFF ^LeftCueLight, ^RightCueLight;
           OFF ^LeftLeverOp, ^RightLeverOp;
           Z^Reward; Z^ResponseCounterStop ---> S4


S4,
    #R^HeadEntry: OFF ^LeftCueLight, ^RightCueLight ---> S2

\***********************************************
\       Response Counter & Screen Update
\***********************************************
S.S.2,
S1,
  #START: SHOW 2,Response,A, 3,Rewards,E ---> S2
  #Z^ResponseCounterStart: --->S2
S2,
  #R^LeftLeverR ! #R^RightLeverR: ADD A; SHOW 2,Response,A ---> SX
  #Z^ResponseCounterStop: ---> S1

\***********************************************
\       Manual Reward Counter & Screen Update
```

```
\***********************************************
S.S.3,
S1,
  #START: SET G = 0 ---> S2
S2,
  #R^ManualReward: ADD F; Z1 ---> S3
S3,
  0.01": SHOW 4,Free Rewards,F ---> S2



\***********************************************
\  The following State Sets are added to
\  collect IRT and Reinforcement Time Codes
\  that can be read by the SoftCR Cumulative
\  Recorder program.  The IRT's can also be
\  converted to real time values in any spread-
\  sheet by stripping the code value to the
\  right of the decimal point (use an int
\  function) and dividing by 10.
\***********************************************
S.S.5,  \ Increment time "T" with resolution 0.1 seconds
S1,
  #START: ---> S2

S2,
 0.1": ADD T  ---> SX


\************************************************
\ ADD A STEP ELEMENT TO ARRAY C FOR EACH RESPONSE
\************************************************
S.S.6,  \ This code is for Relative or Incremental
       \ values.  If Absolute or Cumulative values are
       \ desired, delete the code ", T = 0" in S2.
S1,
  #START: SET C(I) = -987.987 ---> S2

S2,
  #R^LeftLeverR ! #R^RightLeverR: SET C(I) = T + 0.10, T = 0; ADD I;                \Collects IRTs from active
lever
          IF I >= 10000 [@TrueArrayFull, @FalseContinue]
            @ArrayFull: ---> S1
            @Cont: SET C(I) = -987.987 ---> S2



\************************************************
\   Session Timer
\************************************************
S.S.7,
S1,
   #Start: SHOW 5,SessionTime,R/60 ---> S2

S2,
```

```
   1": ADD R; SHOW 5,SessionTime ,R/60;
      IF (R/60 >= V(3)) OR (E >= V(2)) [@TRUE, @FALSE]
            @True: ---> StopAbortFlush
            @False: ---> SX


S.S.8, \Deliver pellet
S1,
  #Z1 ---> S2
s2,
  0.01": ON ^FrontPelletOutput ---> S3
S3,
  0.5": OFF ^FrontPelletOutput ---> S1



\*************************************************
\   Manual pellet delivery
\*************************************************
S.S.9,
S1,
         #R^ManualButton: ---> S2
S2,
         0.01": Z1 ---> S1
```

Appendix E: DMTP program written in MEDState notation

Note: Delay set 1 shown. Delay (s) increases from set 1 to 4. Delay set 1 (1, 2, 3, 4, 5, 6, 7 sec), delay set 2 (0, 1, 2, 4, 6, 8, 10, 12 sec), delay set 3 (0, 2, 4, 8, 12, 16, 20, 24 sec), delay set 4 (0, 2, 4, 8, 12, 18, 24, 32 sec).

```
\DELAYED MATCHING TO POSITION PROGRAM by Daniel Kueh May 14, 2005
\MODIFIED by Paul Mallet Feb 1, 2022

\COUNTERS SUMMARY
\C(0) = DELAY INTERVAL
\C(1) = NUMBER OF TRIALS
\C(2) = NUMBER OF CORRECT TRIALS (REINFORCEMENT)
\C(3) = TRIALS WITH LEFT SAMPLE
\C(4) = TRIALS WITH RIGHT SAMPLE
\C(5) = LEFT CORRECT RESPONSES
\C(6) = RIGHT CORRECT RESPONSES
\C(7) = LEFT CORRECT RESPONSES (DEL 1)
\C(8) = RIGHT CORRECT RESPONSES (DEL 1)
\C(9) = LEFT CORRECT RESPONSES (DEL 2)
\C(10) = RIGHT CORRECT RESPONSES (DEL 2)
\C(11) = LEFT CORRECT RESPONSES (DEL 3)
\C(12) = RIGHT CORRECT RESPONSES (DEL 3)
\C(13) = LEFT CORRECT RESPONSES (DEL 4)
\C(14) = RIGHT CORRECT RESPONSES (DEL 4)
\C(15) = LEFT CORRECT RESPONSES (DEL 5)
\C(16) = RIGHT CORRECT RESPONSES (DEL 5)
\C(17) = LEFT CORRECT RESPONSES (DEL 6)
\C(18) = RIGHT CORRECT RESPONSES (DEL 6)
\C(19) = LEFT CORRECT RESPONSES (DEL 7)
\C(20) = RIGHT CORRECT RESPONSES (DEL 7)
\C(21) = LEFT CORRECT RESPONSES (DEL 8)
\C(22) = RIGHT CORRECT RESPONSES (DEL 8)
\C(23) = LEFT ERROR RESPONSES
\C(24) = RIGHT ERROR RESPONSES
\C(25) = LEFT ERROR RESPONSES (DEL 1)
\C(26) = RIGHT ERROR RESPONSES (DEL 1)
\C(27) = LEFT ERROR RESPONSES (DEL 2)
\C(28) = RIGHT ERROR RESPONSES (DEL 2)
\C(29) = LEFT ERROR RESPONSES (DEL 3)
\C(30) = RIGHT ERROR RESPONSES (DEL 3)
\C(31) = LEFT ERROR RESPONSES (DEL 4)
\C(32) = RIGHT ERROR RESPONSES (DEL 4)
\C(33) = LEFT ERROR RESPONSES (DEL 5)
\C(34) = RIGHT ERROR RESPONSES (DEL 5)
\C(35) = LEFT ERROR RESPONSES (DEL 6)
\C(36) = RIGHT ERROR RESPONSES (DEL 6)
\C(37) = LEFT ERROR RESPONSES (DEL 7)
\C(38) = RIGHT ERROR RESPONSES (DEL 7)
\C(39) = LEFT CORRECT RESPONSES (DEL 8)
\C(40) = RIGHT CORRECT RESPONSES (DEL 8)
\C(41) = PERCENT CORRECT RESPONSES
\C(42) = NUMBER OF HEAD ENTRIES
```

DISKVARS = A,C,E,F,G,H,I,R,V,W

\VARIABLE SUMMARY
\A = DELAY INTERVAL SELECTION
\E = DATA ARRAY FOR RECORDING LATENCY TO RESPOND TO CHOICE LEVER (DEL 1)
\F = DATA ARRAY FOR RECORDING LATENCY TO RESPOND TO CHOICE LEVER (DEL 2)
\G = DATA ARRAY FOR RECORDING LATENCY TO RESPOND TO CHOICE LEVER (DEL 3)
\H = DATA ARRAY FOR RECORDING LATENCY TO RESPOND TO CHOICE LEVER (DEL 4)
\I = DATA ARRAY FOR RECORDING LATENCY TO RESPOND TO CHOICE LEVER (DEL 5)
\R = DATA ARRAY FOR RECORDING LATENCY TO RESPOND TO CHOICE LEVER (DEL 6)
\V = DATA ARRAY FOR RECORDING LATENCY TO RESPOND TO CHOICE LEVER (DEL 7)
\W = DATA ARRAY FOR RECORDING LATENCY TO RESPOND TO CHOICE LEVER (DEL 8)
\J = INCREMENT OF ARRAY E
\K = TIME CONVERSION FOR DELAY
\L = INCREMENT OF ARRAY F
\M = MINUTES IN CLOCK
\N = SECONDS IN CLOCK
\O = INCREMENT OF ARRAY G
\P = INCREMENT OF ARRAY H
\Q = INCREMENT OF ARRAY I
\U = INCREMENT OF ARRAY W
\S = INCREMENT OF ARRAY R
\T = TIME INCREMENT
\X = ONE OF THE LEVERS OF ARRAY D DETERMINED RANDOMLY EACH CYCLE

\Z PULSES
\Z1 = LEFT CORRECT RESPONSES (DEL 1)
\Z2 = RIGHT CORRECT RESPONSES (DEL 1)
\Z3 = LEFT CORRECT RESPONSES (DEL 2)
\Z4 = RIGHT CORRECT RESPONSES (DEL 2)
\Z5 = LEFT CORRECT RESPONSES (DEL 3)
\Z6 = RIGHT CORRECT RESPONSES (DEL 3)
\Z7 = LEFT CORRECT RESPONSES (DEL 4)
\Z8 = RIGHT CORRECT RESPONSES (DEL 4)
\Z9 = LEFT CORRECT RESPONSES (DEL 5)
\Z10 = RIGHT CORRECT RESPONSES (DEL 5)
\Z11 = LEFT CORRECT RESPONSES (DEL 6)
\Z12 = RIGHT CORRECT RESPONSES (DEL 6)
\Z13 = LEFT CORRECT RESPONSES (DEL 7)
\Z14 = RIGHT CORRECT RESPONSES (DEL 7)
\Z15 = LEFT CORRECT RESPONSES (DEL 8)
\Z16 = RIGHT CORRECT RESPONSES (DEL 8)
\Z17 = TIMER
\Z18 = RECORD LATENCY TO RESPOND TO CHOICE LEVER (DEL 1)
\Z19 = RECORD LATENCY TO RESPOND TO CHOICE LEVER (DEL 2)
\Z20 = RECORD LATENCY TO RESPOND TO CHOICE LEVER (DEL 3)
\Z21 = RECORD LATENCY TO RESPOND TO CHOICE LEVER (DEL 4)
\Z22 = RECORD LATENCY TO RESPOND TO CHOICE LEVER (DEL 5)
\Z23 = RECORD LATENCY TO RESPOND TO CHOICE LEVER (DEL 6)
\Z24 = RECORD LATENCY TO RESPOND TO CHOICE LEVER (DEL 7)
\Z25 = RECORD LATENCY TO RESPOND TO CHOICE LEVER (DEL 8)

\ARRAY D
\TWO DIFFERENT PROGRAMS
\1 = LEFT SAMPLE

```
\2 = RIGHT SAMPLE

\CONSTANTS

\INPUTS
^LEFTR = 1
^RIGHTR = 2
^HE = 5

\OUTPUTS
^LEFT = 1
^RIGHT = 2
^FOOD = 3
^HL = 7
^LLITE = 4
^RLITE = 5

LIST D = 1 , 2
LIST A = 0", 1", 2", 3", 4", 5", 6", 7" \DELAY INTERVALS IN SECONDS
Var_Alias Delay 1 = A(0) \Default is 0 sec
Var_Alias Delay 2 = A(1) \Default is 1 sec
Var_Alias Delay 3 = A(2) \Default is 2 sec
Var_Alias Delay 4 = A(3) \Default is 3 sec
Var_Alias Delay 5 = A(4) \Default is 4 sec
Var_Alias Delay 6 = A(5) \Default is 5 sec
Var_Alias Delay 7 = A(6) \Default is 6 sec
Var_Alias Delay 8 = A(7) \Default is 7 sec


DIM C = 43
DIM E = 999
DIM F = 999
DIM G = 999
DIM H = 999
DIM I = 999
DIM R = 999
DIM V = 999
DIM W = 999

\BOXCHECK
S.S.1,
S1,
 1": ON ^LEFT, ^LLITE --->S2
S2,
   #R^LEFTR: OFF ^LEFT, ^LLITE; ON ^RIGHT, ^RLITE --->S3
S3,
   #R^RIGHTR: OFF ^RIGHT, ^RLITE; ON ^HL --->S4
S4,
   #R^HE: OFF ^HL; ON ^FOOD --->S5
S5,
   0.5": OFF ^FOOD ---> SX

\MAIN CONTROL
S.S.2,
S1, \START SESSION
   #START: ON ^HL; OFF ^LEFT, ^RIGHT, ^LLITE, ^RLITE, ^FOOD;
```

```
   SHOW 2, TRIAL, C(1), 3, HE, C(42), 4, %TOT, C(41), 5, SR+, C(2) ---> S2
S2, \INTERTRIAL INTERVAL
   5": ---> S3
S3, \PRESENT SAMPLE LEVER
   .01": RANDI X = D;
   IF X = 1 [@ONETRUE, @ONEFALSE]
      @ONETRUE: ON^LEFT,^LLITE; ADD C(3) ---> S4
      @ONEFALSE: ON^RIGHT,^RLITE; ADD C(4) ---> S11
S4,
   #R^LEFTR: ---> S5
S5,
   .5": OFF ^LEFT ---> S6
S6, \DELAY INTERVAL FOR LEFT
   .01": RANDD K = A; SHOW 6,DELAY,K/100 ---> S7
S7,
   K#T: ---> S8
S8, \REGISTER PANEL PRESS FOR LEFT
   #R^HE: ADD C(42); SHOW 3, HE, C(42); OFF ^LLITE, ^RLITE ---> S9
S9, \PRESENT CHOICE LEVERS
   .01": ON^LEFT, ^RIGHT; Z1; Z3; Z5; Z7; Z9; Z11; Z13; Z15; Z17; ---> S10
S10, \REGISTER CORRECT LEFT RESPONSE
   #R^LEFTR: ADD C(5) ---> S18
   #R^RIGHTR: ADD C(24) ---> S21
S11,
   #R^RIGHTR: ---> S12
S12,
   .5": OFF ^RIGHT ---> S13
S13, \DELAY INTERVAL FOR RIGHT
   .01": RANDD K = A; SHOW 6,DELAY,K/100 ---> S14
S14,
   K#T: ---> S15
S15, \REGISTER PANEL PRESS FOR RIGHT
   #R^HE: ADD C(42); SHOW 3, HE, C(42); OFF ^LLITE, ^RLITE ---> S16
S16, \PRESENT CHOICE LEVERS
   .01": ON^LEFT, ^RIGHT; Z2; Z4; Z6; Z8; Z10; Z12; Z14; Z16; Z17 ---> S17
S17, \REGISTER CORRECT RIGHT RESPONSE
   #R^RIGHTR: ADD C(6) ---> S18
   #R^LEFTR: ADD C(23) ---> S20
S18, \REINFORCEMENT
   .5": OFF ^LEFT, ^RIGHT; ON ^LLITE, ^RLITE, ^FOOD; ADD C(2); SHOW 5, SR+, C(2) ---> S19
S19, \COLLECT FOOD
   #R^HE: OFF ^FOOD, ^LLITE, ^RLITE ---> S24
S20, \ERROR LEFT
   .5": OFF ^LEFT, ^RIGHT, ^HL ---> S22
S21, \ERROR RIGHT
   .5": OFF ^LEFT, ^RIGHT, ^HL ---> S23
S22, \LEFT TIMEOUT
   5": ---> S25
S23, \RIGHT TIMEOUT
   5": ---> S27
S24, \FINISH
   1": ---> S2
S25, \CORRECTION FOR ERROR LEFT
   5": ON ^HL---> S26
S26, \PRESENT RIGHT LEVER
   .01": SET X = 2;
```

```
   IF X = 2 [@TWOTRUE, @TWOFALSE]
      @TWOTRUE: ON^RIGHT; ADD C(4) ---> S11
      @TWOFALSE: ON^LEFT; ADD C(3) ---> SX
S27, \CORRECTION FOR ERROR RIGHT
   5": ON ^HL ---> S28
S28, \PRESENT LEFT LEVER
   .01": SET X = 1;
   IF X = 1 [@THREETRUE, @THREEFALSE]
      @THREETRUE: ON^LEFT; ADD C(3) ---> S4
      @THREEFALSE: ON^RIGHT; ADD C(4) ---> SX

S.S.3,
S1,
   #START: SHOW 1, ELAPSED (MIN), M---> S2
S2,
   1": ADD N; IF N=60 [@FOURTRUE, @FOURFALSE]
      @FOURTRUE: ADD M; SET N=0; SHOW 1, ELAPSED (MIN), M --->SX
      @FOURFALSE: SHOW 1, ELAPSED (MIN), M --->SX

S.S.4,
S1,
   #START: ---> S2
S2,
   .01": IF C(5)+C(6)>0 [@FIVETRUE,@FIVEFALSE]
      @FIVETRUE:SET C(41)= ((C(5)+C(6))/(C(5)+C(6)+C(23)+C(24))) * 100; SHOW 4, %TOT, C(41) --->SX
      @FIVETRUE:SET C(41)= -999 --->SX

S.S.5,
S1,
   #START: ---> S2
S2,
   60': SET C(41)= ((C(5)+C(6))/(C(5)+C(6)+C(23)+C(24))) * 100 ---> S3
S3,
   .01": ALERTON ---> STOPABORTFLUSH

S.S.6,
S1,
   #START: ---> S2
S2,
   .01": SET C(1)= C(3)+C(4); SHOW 2, TRIAL, C(1) ---> SX

S.S.7, \LEFT CORRECT RESPONSES (DELAY 1)
S1,
   #START: ---> S2
S2,
   #Z1: IF K = A(0) [@SIXTRUE, @SIXFALSE]
      @SIXTRUE: ---> S3
      @SIXFALSE: ---> SX
S3,
   #R^LEFTR: ADD C(7); Z18 ---> S2
   #R^RIGHTR: ADD C(26); Z18 ---> S2

S.S.8, \RIGHT CORRECT RESPONSES (DELAY 1)
S1,
   #START: ---> S2
S2,
```

```
   #Z2: IF K = A(0) [@SEVENTRUE, @SEVENFALSE]
      @SEVENTRUE: ---> S3
      @SEVENFALSE: ---> SX
S3,
   #R^RIGHTR: ADD C(8); Z18 ---> S2
   #R^LEFTR: ADD C(25); Z18 ---> S2

S.S.9, \LEFT CORRECT RESPONSES (DELAY 2)
S1,
   #START: ---> S2
S2,
   #Z3: IF K = A(1) [@EIGHTTRUE, @EIGHTFALSE]
      @EIGHTTRUE: ---> S3
      @EIGHTFALSE: ---> SX
S3,
   #R^LEFTR: ADD C(9); Z19 ---> S2
   #R^RIGHTR: ADD C(28); Z19 ---> S2

S.S.10, \RIGHT CORRECT RESPONSES (DELAY 2)
S1,
   #START: ---> S2
S2,
   #Z4: IF K = A(1) [@NINETRUE, @NINEFALSE]
      @NINETRUE: ---> S3
      @NINEFALSE: ---> SX
S3,
   #R^RIGHTR: ADD C(10); Z19 ---> S2
   #R^LEFTR: ADD C(27); Z19 ---> S2

S.S.11, \LEFT CORRECT RESPONSES (DELAY 3)
S1,
   #START: ---> S2
S2,
   #Z5: IF K = A(2) [@TENTRUE, @TENFALSE]
      @TENTRUE: ---> S3
      @TENFALSE: ---> SX
S3,
   #R^LEFTR: ADD C(11); Z20 ---> S2
   #R^RIGHTR: ADD C(30); Z20 ---> S2

S.S.12, \RIGHT CORRECT RESPONSES (DELAY 3)
S1,
   #START: ---> S2
S2,
   #Z6: IF K = A(2) [@ELEVENTRUE, @ELEVENFALSE]
      @ELEVENTRUE: ---> S3
      @ELEVENFALSE: ---> SX
S3,
   #R^RIGHTR: ADD C(12); Z20 ---> S2
   #R^LEFTR: ADD C(29); Z20 ---> S2

S.S.13, \LEFT CORRECT RESPONSES (DELAY 4)
S1,
   #START: ---> S2
S2,
   #Z7: IF K = A(3) [@TWELVETRUE, @TWELVEFALSE]
```

```
      @TWELVETRUE: ---> S3
      @TWELVEFALSE: ---> SX
S3,
   #R^LEFTR: ADD C(13); Z21 ---> S2
   #R^RIGHTR: ADD C(32); Z21 ---> S2

S.S.14, \RIGHT CORRECT RESPONSES (DELAY 4)
S1,
   #START: ---> S2
S2,
   #Z8: IF K = A(3) [@THIRTEENTRUE, @THIRTEENFALSE]
      @THIRTEENTRUE: ---> S3
      @THIRTEENFALSE: ---> SX
S3,
   #R^RIGHTR: ADD C(14); Z21 ---> S2
   #R^LEFTR: ADD C(31); Z21 ---> S2

S.S.15, \LEFT CORRECT RESPONSES (DELAY 5)
S1,
   #START: ---> S2
S2,
   #Z9: IF K = A(4) [@TWELVETRUE, @TWELVEFALSE]
      @TWELVETRUE: ---> S3
      @TWELVEFALSE: ---> SX
S3,
   #R^LEFTR: ADD C(15); Z22 ---> S2
   #R^RIGHTR: ADD C(34); Z22 ---> S2

S.S.16, \RIGHT CORRECT RESPONSES (DELAY 5)
S1,
   #START: ---> S2
S2,
   #Z10: IF K = A(4) [@THIRTEENTRUE, @THIRTEENFALSE]
      @THIRTEENTRUE: ---> S3
      @THIRTEENFALSE: ---> SX
S3,
   #R^RIGHTR: ADD C(16); Z22 ---> S2
   #R^LEFTR: ADD C(33); Z22 ---> S2

S.S.17, \LEFT CORRECT RESPONSES (DELAY 6)
S1,
   #START: ---> S2
S2,
   #Z11: IF K = A(5) [@FOURTEENTRUE, @FOURTEENFALSE]
      @FOURTEENTRUE: ---> S3
      @FOURTEENFALSE: ---> SX
S3,
   #R^LEFTR: ADD C(17); Z23 ---> S2
   #R^RIGHTR: ADD C(36); Z23 ---> S2

S.S.18, \RIGHT CORRECT RESPONSES (DELAY 6)
S1,
   #START: ---> S2
S2,
   #Z12: IF K = A(5) [@FIFTHTEENTRUE, @FIFTHTEENFALSE]
      @FIFTHTEENTRUE: ---> S3
```

```
      @FIFTHTEENFALSE: ---> SX
S3,
   #R^RIGHTR: ADD C(18); Z23 ---> S2
   #R^LEFTR: ADD C(35); Z23 ---> S2

S.S.19, \LEFT CORRECT RESPONSES (DELAY 7)
S1,
   #START: ---> S2
S2,
   #Z13: IF K = A(6) [@SIXTEENTRUE, @SIXTEENFALSE]
      @SIXTEENTRUE: ---> S3
      @SIXTEENFALSE: ---> SX
S3,
   #R^LEFTR: ADD C(19); Z24 ---> S2
   #R^RIGHTR: ADD C(38); Z24 ---> S2

S.S.20, \RIGHT CORRECT RESPONSES (DELAY 7)
S1,
   #START: ---> S2
S2,
   #Z14: IF K = A(6) [@SEVENTEENTRUE, @SEVENTEENFALSE]
      @SEVENTEENTRUE: ---> S3
      @SEVENTEENFALSE: ---> SX
S3,
   #R^RIGHTR: ADD C(20); Z24 ---> S2
   #R^LEFTR: ADD C(37); Z24 ---> S2

S.S.21, \LEFT CORRECT RESPONSES (DELAY 8)
S1,
   #START: ---> S2
S2,
   #Z15: IF K = A(7) [@EIGHTTEENTRUE, @EIGHTTEENFALSE]
      @EIGHTTEENTRUE: ---> S3
      @EIGHTTEENFALSE: ---> SX
S3,
   #R^LEFTR: ADD C(21); Z25 ---> S2
   #R^RIGHTR: ADD C(40); Z25 ---> S2

S.S.22, \RIGHT CORRECT RESPONSES (DELAY 8)
S1,
   #START: ---> S2
S2,
   #Z16: IF K = A(7) [@NINETEENTRUE, @NINETEENFALSE]
      @NINETEENTRUE: ---> S3
      @NINETEENFALSE: ---> SX
S3,
   #R^RIGHTR: ADD C(22); Z25 ---> S2
   #R^LEFTR: ADD C(39); Z25 ---> S2

S.S.23,
S1,
   #START: ---> S2
S2,
   .01": SET C(0)= 32 ---> SX
   #Z17: SET T = 0 --->SX
```

```
S.S.24,
S1,
   #Z17:SET T = 0 ---> S2
S2,
   .1": SET T = (T + 0.1) ---> SX

S.S.25, \RECORD LATENCY TO RESPOND TO CHOICE LEVER (DELAY 1)
S1,
   #Z18: SET E(J) = T; ADD J; SET E(J) = -987.987 ---> SX

S.S.26, \RECORD LATENCY TO RESPOND TO CHOICE LEVER (DELAY 2)
S1,
   #Z19: SET F(L) = T; ADD L; SET F(L) = -987.987 ---> SX

S.S.27, \RECORD LATENCY TO RESPOND TO CHOICE LEVER (DELAY 3)
S1,
   #Z20: SET G(O) = T; ADD O; SET G(O) = -987.987 ---> SX

S.S.28, \RECORD LATENCY TO RESPOND TO CHOICE LEVER (DELAY 4)
S1,
   #Z21: SET H(P) = T; ADD P; SET H(P) = -987.987 ---> SX

S.S.29, , \RECORD LATENCY TO RESPOND TO CHOICE LEVER (DELAY 5)
S1,
   #Z22: SET I(U) = T; ADD U; SET I(U) = -987.987 ---> SX

S.S.30, \RECORD LATENCY TO RESPOND TO CHOICE LEVER (DELAY 6)
S1,
   #Z23: SET R(Q) = T; ADD Q; SET R(Q) = -987.987 ---> SX

S.S.31, \RECORD LATENCY TO RESPOND TO CHOICE LEVER (DELAY 7)
S1,
   #Z24: SET V(S) = T; ADD S; SET V(S) = -987.987 --->SX

S.S.32, \RECORD LATENCY TO RESPOND TO CHOICE LEVER (DELAY 8)
S1,
   #Z25: SET W(B) = T; ADD B; SET W(B) = -987.987 ---> SX
```

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