THE EFFECTS OF RAT STRAIN, SEX AND THC ON BRAIN AND BEHAVIOUR: FACTORS THAT ALTER THE SENSITIVITY TO DRUGS OF ABUSE

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DEDICATION

In loving memory to GMBK, without whom this thesis wouldn't exist and I wouldn't be here.

ABSTRACT

The laboratory rat has been used for over a century, and through directed and accidental mechanisms, different rat strains were developed. This study examined the effect of strain and sex on metrics of brain size and function and in response to amphetamine. In addition to strain and sex, the adolescent period is sensitive to drugs of abuse, such as marijuana. This study also examined the effects of post-pubertal exposure of the psychoactive component of marijuana, Δ^9-tetrahydrocannabinol (THC), to males of females of two rat strains. Rats were assessed for developmental and adult differences in brain and behaviour, including alterations in sensitivity to amphetamine. This study also implemented parametric and nonparametric statistical tests. Strain and sex contributed to behavioural and neuroanatomical differences between groups exposed to THC during adolescence, and drug experience in adolescence produced strain- and sex-specific effects. This study highlights the background-dependent effects of THC on brain and behaviour.

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Finally, to my family and friends, who encouraged me always, without completely understanding what I was doing and why I was doing it. With specific mention to my father, J.R. Keeley, without whom, at least one of these chapters wouldn't exist as well as the countless discussions of the similarities and differences between the sciences. Finally, mention to my partner, I.D. Blum, who provided more discussions than absolutely necessary and supported me with everything he had along the way, without whom this thesis would be less than what it is.

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AMPH – d-amphetamine
AMYG – amygdala
CA1 – Cornu Ammonis 1
CA3 – Cornu Ammonis 3
CORT – corticosterone
CPP – conditioned place preference
DAB – 3,3' diaminobenzidine
DFCTC – discriminative fear-conditioning to context
DG – dentate gyrus
EPM – elevated plus maze
HP – hippocampus
HPA – hypothalamic-pituitary-adrenal
HR – high responders
ICC – intra-class correlation
i.p. – intraperitoneal
LER – Long-Evans rat
LR – low responders
mPFC – medial prefrontal cortex
MWT – Morris water task
OFC – orbital frontal cortex
PFA – paraformaldehyde
PBS – phosphate buffered saline
PCA – principal component analysis
PND – postnatal day
ROI – region of interest
SRT – skilled reaching task
THC - Δ^9-tetrahydrocannabinol
WR – Wistar rat

CHAPTER 1 - GENERAL INTRODUCTION

Behavioural neuroscience aims to decode the relationship between brain function and behaviour. This field of enquiry is broad and complex, using multiple techniques, ranging from neuroimaging and electrophysiology to genetic manipulation, in order to determine the exact and precise neural correlates of specific behaviours. Only through the use of multiple levels of analyses, from micro- to macroscopic tiers, can we fully understand the function of the brain and its relationship to complex behaviour. Indeed, part of the impetus to understand the brain is the distant hope to prevent and ideally cure diseases related to neurological function, which comprise a large subset of human diseases. Although there are benefits to studying these diseases purely in patients and their families, it is of interest to establish animal models in order to help understand the underlying etiology, pathology and progression of these diseases, not only for the eventual treatment of these diseases but also to learn about the basic processes of the brain.

Multiple animal models have been used throughout the development and progression of neuroscience research, and rodents have proven to be an incredibly popular animal model for the human brain in both health and disease. *Mus musculus*, or the laboratory mouse, is widely used, most recently due to the advent of genetic techniques and their genetic accessibility. However, the laboratory mouse does not perform as well as other models in a multitude of behavioural laboratory tasks. Therefore *Rattus norvegicus*, or the laboratory rat, has remained popular despite its lack of genetic accessibility, although that fact has changed in recent years. The laboratory rat has proven useful in the understanding of rat brain and behaviour as an intermediary for informing the understanding of human brain and behaviour.

Here, I will first discuss the history of the use of laboratory rodents, specifically in reference to the establishment of multiple rat strains. Rat strains have provided the opportunity to study the specific contributions of individual differences to behaviour, as they represent members of the same species from genetically distinct populations. In addition to strain, differences between males and females are observed among laboratory rats, a phenomenon that is also observed among humans. Both of these variables can contribute to differences in multiple behaviours as well as associated brain areas. Of particular interest for the purposes of this thesis, is the contribution of strain and sex to differences in one of the endogenous neurotransmitter systems in the brain, the endocannabinoid system. This system will be discussed briefly, primarily in relation to drug use. Specifically, drug use during adolescence, which seems to be particularly harmful, will be discussed. Therefore, the relationship between adolescence, strain, sex and drug use will be discussed. The goal of this research lay in examining how strain and sex differences might influence the effects of exposure to a drug of abuse during a specific epoch of development, puberty. Identifying intrinsic differences between strain and sex groups as well as differences in response to a drug of abuse may help to identify specific populations and/or groups that are particularly sensitive or resilient to the effects of drugs during the adolescent period.

The development and use of the laboratory rat

R. norvegicus was the first bred animal used in experimental research. The first experiment implementing the Brown Norway rat was a fasting study published in 1828 (as discussed in Krinke, 2000; McCay, 1973), and the first published psychological and behaviour-based experiment to use rats was published in 1898 (Stewart, 1898). Scientists

using rats in these initial experiments received their subjects from rat fanciers, who bred rats to be used in rat baiting. Rat fanciers would breed for appearance and some behavioural characteristics, and from these stocks, the modern laboratory rat was based. Rats are thought to be a good model species for behavioural neuroscience research given their intelligence, sociality and quick breeding cycle. They have been used extensively throughout the years in psychological and neuroscience research to model human brain and body functions and multiple human health problems.

The Wistar Institute, in Philadelphia, through the pioneering genetic and breeding work of Dr. Helen Dean King in the early 1900s (King, 1918a, 1918b, 1918c, 1919), sought to establish a gold standard version of the rat (Clause, 1993, 1998), one that would be used across research laboratories, allowing for the complete understanding of rat physiology. The end goal for the development of this gold standard laboratory rat was to allow experimental results to be comparable across laboratories and research facilities to aid in the understanding of human physiology and disease states. Through multiple years of breeding as well as using multiple selection criteria, including decreased aggression towards experimenters and large litter sizes, the Wistar strain was developed. The Wistar strain, which is physically characterized by its white fur and red eye, is an albino hooded strain, such that, without the expression of albinism, they would have the standard piebald, or hooded, appearance that is characteristic of other common rat strains used today. The Wistar strain, through stocks originating from multiple locations, continues to be used to the present day. Indeed, the Wistar rat (WR) is the parental strain of multiple outbred and inbred rat strains, including the very commonly used Long-Evans hooded rat (LER). The LER strain originates through multiple breedings with the Brown Norway and the Wistar rat (Baker, Lindsey, & Weisbroth, 1979).

Factors that affect behavioural differences

Both WR and LER are used to study the connections between brain and behaviour among other research topics. Behaviour of the laboratory rodent can originate from many sources, including genetic background. The same can also be said of humans, such that some behavioural and physiological traits can be traced solely to the genetic background of the individual. Rat strain can be used as a proxy to understanding differences between genetically distinct populations within the same species. The size and structure of brain areas, which can be altered genetically, through temporary inactivation, permanent damage and other experimental manipulations, can affect behavioural control, depending on the kind of behaviour. Through the examination of changes in behaviour following experimental manipulations, behavioural neuroscience seeks to determine the function of behaviourally associated brain areas.

Individual differences in behaviour between rats can be partially explained by strain differences. Indeed, differences in behaviours related to learning and memory (Andrews, Jansen, Linders, Princen, & Broekkamp, 1995; Cain, Ko, Chalmers, & Ralph, 2004; Thorpe, Deibel, Reddigan, & Fontaine, 2012; Tinius, Beckwith, & Oltmanns, 1989; van der Staay, Schuurman, van Reenen, & Korte, 2009), fear-conditioning (Pare, 1996; Pryce, Lehmann, & Feldon, 1999), sexual receptivity (Sachs, 1996), impulsivity (Hamilton, Potenza, & Grunberg, 2014; Richards et al., 2013) and visual discrimination (Dyer & Swartzwelder, 1978; Mohn & Russell, 1983) have been observed between multiple strains, including LER and WR. These behavioural differences are thought to partially originate from differences in the size and structure of specific brain areas. For example, differences in the size and shape of the optic nerve in the albino rats (Fukuda, Sugimoto,

& Shirokawa, 1982; Lund, 1965; Steininger, Rye, Gilliland, Wainer, & Benca, 1993) contribute to aptitude in visual discrimination tasks (Mohn & Russell, 1983).

However, the genetic background of a laboratory rat, like the genetic background of a human, is not the sole determinant of the size, structure and function of brain areas, and therefore is not the only factor that can affect behaviour. In addition to intrinsic differences in the size and structure of brain areas, the same experience or intervention can have differential effects, dependent on the strain of rat used. For example, stressors will produce ulcers of different severity in the LER and WR strains (Pare, 1989). Therefore, not only are there immediate differences between rat strains, but experience can differentially shape their physiology and behaviour.

The sex of the individual can also contribute to behavioural differences. Indeed, aside from sex-specific behavioural characteristics, such as parturition in female mammals, male and female mammals will exhibit different behavioural strategies in a large number of tasks. One highly studied example is the behaviour of male and female rats and humans in spatial learning tasks (Astur, Ortiz, & Sutherland, 1998; Clint, Sober, Garland, & Rhodes, 2012; Jonasson, 2005). In multiple mammalian species, including rats and humans, females preferentially navigate using non-Euclidean-based strategies, using landmarks to spatially navigate whereas males will preferentially navigate using spatial-mapping strategies (Astur, Tropp, Sava, Constable, & Markus, 2004; Blokland, Rutten, & Prickaerts, 2006; Rodriguez, Chamizo, & Mackintosh, 2011; Rodriguez, Torres, Mackintosh, & Chamizo, 2010; Saucier et al., 2002). These differences are thought to be mediated through both structural (Galea, Perrot-Sinal, Kavaliers, & Ossenkopp, 1999; Keeley, Tyndall, Scott, & Saucier, 2013; Woolley, 1998; Woolley et al., 2010) and biochemical (Mizuno & Giese, 2010) differences between the brains of

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males and females. Although spatial learning has been identified as one behavioural difference between males and females, there are multiple other tasks that show sex differences (Vanhaaren, Vanhest, & Heinsbroek, 1990), including but not limited to fear-conditioning (Maren, De Oca, & Fanselow, 1994; Markus & Zecevic, 1997; Stark et al., 2006), anxiety-based behaviours (Johnston & File, 1991; Toufexis, 2007) and locomotor activity (Imhof, Coelho, Schmitt, Morato, & Carobrez, 1993; Mitsushima, Takase, Takahashi, & Kimura, 2009).

The basis for sex differences in behaviour is not as simple as differences in circulating sex hormones. Sex hormones can have both organizational and activational effects, both of which can have direct consequences on brain (Parducz et al., 2006) and behaviour (Vanhaaren et al., 1990). Organizational effects of sex hormones include the generally more static effects that typically occur during developmental epochs and confer long-term changes in the size and circuitry of some brain areas. Activational effects of sex hormones include the transient, often reversible, effects of sex hormones. The organizational effects of sex hormones during specific developmental periods can alter the response to the activational effects of sex hormones at other developmental periods (for example, Mitsushima, Takase, Takahashi, et al., 2009). Regardless, both the organizational and activational effects of sex hormones can alter sex-specific behavioural strategies (Williams, Barnett, & Meck, 1990). An example of an organizational effect is exemplified in an experiment where exposure to testosterone during development in females can result in male-specific sexual postures as well as a trend towards the adoption of more male-like strategies in a spatial learning task (Roof & Havens, 1992). A classic example used to study the activational effects of sex hormones involves observing changes in behaviour throughout the natural fluctuations of sex hormones during the

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female reproductive cycle. Changes in the structure and activity of the brain and behaviour have been observed throughout the human menstrual cycle (Hampson, 1990; Hausmann, Slabbekoorn, Van Goozen, Cohen-Kettenis, & Gunturkun, 2000; Protopopescu et al., 2008; Rosenberg & Park, 2002; Schoening et al., 2007) and the rat estrous cycle (Korol, Malin, Borden, Busby, & Couper-Leo, 2004; Stackman, Blasberg, Langan, & Clark, 1997). For example, non-landmark-based strategies are preferentially adopted during cycle phases with high estrogen (Korol & Kolo, 2002) and are correlated with differential activation of specific brain areas (Korol, 2004). Therefore, sex differences in behavioural strategies are not simply due to the genetic differences between male and female rats but are also based on the long-term and acute effects of sex steroids on multiple organs including the brain.

Not only can strain and sex individually contribute to differences in behaviour, but these two factors can interact such that strain differences are observed in one sex and not another or sex differences are observed in one strain and not another. This has been observed both intrinsically and in response to specific experience (Keeley, Wartman, Hausler, & Holahan, 2010). Therefore, when considering individual differences in behaviour, it is important to consider not only the effect of genetic background but also the sex of the individual.

How each of these factors contributes to the shaping of individual differences in behaviour is of general interest in scientific research. However, individual differences still persist beyond those that can be explained by strain and sex. Therefore, although both of these factors may contribute to some of the variance observed in data sets, they still do not entirely explain differences between individuals. Indeed, a recent study examining differences between inbred strains of rats noted a very high level of individual

variability within rat strains (Richards et al., 2013). This phenomenon is not observed

exclusively in rats either. Indeed, there are few human diseases that can be explained

entirely by the genetic background of the person, and individual differences can account

for some of the risk factors involved in disease etiology. The source of individual

differences can be varied, from sex to prenatal experience, with the mechanisms varying

from pure allelic differences to epigenetic modifications.   The understanding of

phenomenon or intervention, either positive or negative, altering behaviour robustly, is

vital to determining which factors can have large effects on behaviour.

The use of laboratory rodents to study the etiology, progression and treatment of

multiple diseases is a common practice. One example of their use has been for the

understanding of drug-addiction and drug-seeking behaviour (for example, Deroche-

Gamonet, Belin, & Piazza, 2004). The use of rodents has aided in the understanding of

multiple endogenous systems that drugs of abuse highjack for their behavioural effects,

including the endocannabinoid system.

The endocannabinoid system

The endocannabinoid system was first identified through the study of exogenous

cannabinoids found in marijuana (Devane, Dysarz, Johnson, Melvin, & Howlett, 1988).

The endogenous cannabinoid system is made of two main endogenous cannabinoids,

anandamide and 2-arachidonoylglycerol, the enzymes that break down endocannabinoids,

such as fatty acid amide hydrolase and monoacylglycerol lipase, and two, with a possible

third, endogenous cannabinoid receptors (Mackie, 2005). The first subtype of

cannabinoid receptor, the CB1R, is the most widely expressed G-protein coupled receptor

in the mammalian brain and is expressed throughout *Animalia,* from *Cnidaria* to

Mammalia (Berry & Mechoulam, 2002). CB1Rs are present in the hippocampus, amygdala, prefrontal cortex and hypothalamus, among many other areas (Herkenham et al., 1991; Mailleux & Vanderhaeghen, 1992b). CB1Rs are located presynaptically and are activated by both endogenous and exogenous cannabinoids (Kano, Ohno-Shosaku, Hashimotodani, Uchigashima, & Watanabe, 2009). Endogenous cannabinoids, such as anandamide and 2-arachidonoylglycerol, are cleaved on demand postsynaptically following depolarization and action potential propagation (Piomelli, 2003). Once produced, they reach presynaptically located CB1Rs, bind and activate the CB1R which results in the inhibition of vesicle docking and neurotransmitter release from that particular synapse (Marsicano & Lutz, 2006; Piomelli, 2003). They are broken down by enzymes to maintain biologically relevant levels of endocannabinoids. Therefore, CB1Rs act to inhibit neurotransmission at specific synaptic sites. CB1Rs are found at both excitatory and inhibitory synapses throughout multiple areas of the brain including those related to learning, memory, anxiety and executive functions (Marsicano & Lutz, 2006).

The endogenous cannabinoid system plays a regulatory role for multiple systems, including learning and memory, pain modulation, epileptiform activity, stress and feeding (Marsicano & Lutz, 2006). Numerous reviews have been written on these topics and are beyond the scope of this introductory section. In short, given its ubiquitous presence in the brain as well as across species, the endogenous cannabinoid system plays an important modulatory role on multiple systems, including those related to complex behaviours. For example, endogenous cannabinoids are essential for the habituation of the stress response, including both the learning and expression of stress habituation (Hill et al., 2010).

As mentioned above, the discovery of the endogenous cannabinoid system first began through the study of the effects of cannabis. Cannabis plants are used throughout assorted cultures and in various forms for both recreational and medicinal purposes (Mechoulam, 1986), and archaeological evidence has traced the use of cannabis by humans as early as 2,000 BCE (Mallory & Mair, 2000). Cannabinoids are produced by the female cannabis plant as a naturally-occurring insecticide or deterrent for other non-insect herbivores (Pate, 1994). Cannabis contains upwards of 60 different cannabinoids (Gaoni & Mechoulam, 1971), and the main psychoactive component of cannabis, Δ^9-tetrahydrocannabinol (THC), accounts for a high proportion of the psychoactive properties of marijuana (Gaoni & Mechoulam, 1971; Razdan, 1986). THC is a partial agonist of the CB1R (Pertwee & Ross, 2002) through which it mediates its psychoactive effects (Razdan, 1986), including slowing of the perception of time, learning and memory deficits, giddiness and increased perception of hunger, among other things (Kaufmann et al., 2010; Martin-Santos et al., 2012). THC is not the sole compound used to understand the effects of marijuana. Indeed, other compounds that act both as partial agonists as well as complete agonists at CB1Rs, such as WIN 55,212-2 and CP55,940, have been extensively studied in similar paradigms (for example, Biscaia et al., 2003). As mentioned, THC acts as a partial agonist and is contained in marijuana whereas the abovementioned compounds are not found endogenously in the marijuana plant. Therefore, a large proportion of the discussion and cited research includes only studies using THC, due to its unique pharmacology and high concentrations in marijuana.

Currently, marijuana use is popular in multiple countries, and there is a high prevalence of its use specifically in Canada, with 41.5% of Canadians reporting having tried it at least once (Health Canada, 2013). Not only is it a popular recreational drug, but

it is often first consumed during adolescence, and a recent report from Health Canada confirmed that the average age when cannabis is first experienced is during adolescence (Health Canada, 2013). Indeed, adolescence itself is a critical point for the initiation of drug use as most commonly, drugs such as cannabis and alcohol are frequently first experienced during this period of development.

Adolescence: a period of critical brain development

Adolescence has been identified as a critical period for brain development (Sisk & Foster, 2004). From childhood to adolescence, there is a shift in behavioural manifestations, such that adolescence is highly associated with risk-taking and novelty-seeking as compared to both childhood and adulthood (Casey, Jones, & Hare, 2008). The unique patter of brain development during this period may explain some of the characteristic behaviour of adolescents.

Adolescence is typically defined starting at the beginning of puberty onset and encompasses the period up to adulthood. Although the exact definition for the termination of adolescence is grey, it is widely accepted that adolescence starts with the initiation of puberty. The onset of puberty is determined through a cascade of permissive hormonal signals that trigger the development of secondary sexual characteristics (Lee & Styne, 2013). In mammals, including humans, this is triggered through the release of gonadotropin-releasing hormone (Harris & Levine, 2003; Ojeda et al., 2003). Following initiation of multiple signal cascades (Ebling & Cronin, 2000; Sisk & Zehr, 2005), the result is the sexual maturation of many physiological systems, including the ovaries and testes, as well as the brain (Sisk & Zehr, 2005). Indeed, there is evidence of changes in activity of cortical and limbic areas in addition to grey and white matter changes from

adolescence to adulthood (Casey et al., 2008; Galvan et al., 2006; Markham, Morris, & Juraska, 2007). Further, some sex differences in behaviour require the organizational effects of sex hormones during this critical period (De Bellis et al., 2001). Therefore, certain interventions or experiences can have differential effects depending on whether they are experienced during childhood or adolescence.

One example of these effects includes modulation of the hypothalamic-pituitary-adrenal (HPA) axis, which mediates our physiological and psychological experience of stress (De Kloet, Joëls, & Holsboer, 2005). Experimental research in humans and rodents has shown that the adolescent period is a critical period for the development of appropriate responding to stressful stimuli (Romeo, 2010), and as discussed above, there is a link between the endogenous cannabinoid system and the stress response.

The endocannabinoid system & adolescence: sex and strain differences

As mentioned above, adolescence is characterized by both the initiation of puberty but also increased risk-taking and novelty-seeking behaviour (Casey et al., 2008). This has been shown across species, including humans and rats. Initiation of drug abuse is a reasonable expression of these increased propensities, as they can be considered both risky and novel to a drug-naïve individual. Numerous lines of research have identified the adolescent period as especially risky not only for the initiation of drug use but also for the potential to develop addictive behaviour (as discussed in, Bernheim, Halfon, & Boutrel, 2013). Cannabis has been identified as one possible drug of abuse that when used during adolescence, may result in the initiation and further use of more sociologically and physiologically harmful drugs of abuse (Fergusson, Boden, & Horwood, 2006a; Fergusson, Boden, & Horwood, 2006b; Hall, 2006; Kandel, 1975;

Kandel, Yamaguchi, & Klein, 2006; Maccoun, 2006; Rubino, Zamberletti, & Parolaro, 2012; Schneider, 2008). Indeed, pre-exposure to THC can increase morphine self-administration in rats and has long-term physiological consequences on the endogenous opiod system (Ellgren, Spano, & Hurd, 2007; Morel, Giros, & Dauge, 2009).

Not only has cannabis been postulated to increase the risk of addiction to other substances of abuse, but adolescent cannabis use may alter brain organization and neurobiological mechanisms leading to the development of affective disorders. There is a link between early-onset cannabis use and the risk for the development of psychiatric disorders, including depression, anxiety and psychosis (Moore et al., 2007). However, it does not appear that all groups are equally at risk for the development of affective disorders following adolescent cannabis use. A longitudinal study in Australia demonstrated that women are 5 times more likely to self-report anxiety and depression as adults if engaged in cannabis use in adolescence, even when controlling for these symptoms in childhood (Patton et al., 2002). Therefore, women may be particularly at risk for the development of affective disorders given cannabis use during adolescence. In addition to human research, there is some evidence that in rats, females may be sensitive to modulation of the endocannabinoid system, as some short- and long-term consequences of THC exposure are only apparent in female subjects and not males (Borcel et al., 2004; Craft, 2005; McGregor & Arnold, 2007; Navarro, Rubio, & Defonseca, 1994; Rubino, Realini, Braida, Alberio, et al., 2009; Rubino, Vigano, et al., 2008; Tseng & Craft, 2001; Tseng, Harding, & Craft, 2004). One possible explanation for this sex difference lies in the differential metabolism of THC, such that females will preferentially metabolize THC to an active metabolite that will also bind to CB1Rs whereas males will metabolize THC to an inactive form (Narimatsu et al., 1992;

Narimatsu, Watanabe, Yamamoto, & Yoshimura, 1991). Additionally, endogenous and exogenous cannabinoids can interact with both estrous cyclicity (Bonnin, Ramos, Rodriguez de Fonseca, Cebeira, & Fernandez-Ruiz, 1993; Craft & Leitl, 2008; Craft, Marusich, & Wiley, 2013; Fattore & Fratta, 2010; Fattore et al., 2007; Hill, Karacabeyli, & Gorzalka, 2007; Nir, Ayalon, Tsafriri, Cordova, & Lindner, 1973; Rawitch, Schultz, Ebner, & Vardaris, 1977; Riebe, Hill, Lee, Hillard, & Gorzalka, 2010; Rodriguez de Fonseca, Cebeira, Ramos, Martin, & Fernandez-Ruiz, 1994) and stress (Eldridge & Landfield, 1990; Gorzalka, Hill, & Hillard, 2008; Hill & Gorzalka, 2004; Hill et al., 2005; Reich, Taylor, & McCarthy, 2009), both of which are sex-dependent (Conrad et al., 2004; Critchlow, Liebelt, Bar-Sela, Mountcastle, & Lipscomb, 1963; Panagiotakopoulos & Neigh, 2014; Stark et al., 2006). Without a doubt, it appears that females may be especially at risk to the consequences of cannabis exposure.

Not only are there sexually dimorphic responses to the short- and long-term consequences of cannabis, but the strain of the laboratory rat can also alter the consequences. Strain differences in response to other drugs of abuse have been reported (Aulakh, Hill, & Murphy, 1988; Onaivi, Maguire, Tsai, Davies, & Loew, 1992; Woolfolk & Holtzman, 1995), including amphetamine (Camp, Browman, & Robinson, 1994; Fujimoto et al., 2007). Similarly, strain differences in rats have been observed in the endocannabinoid system (Chen, Paredes, Lowinson, & Gardner, 1991; Coria et al., 2014; Deiana et al., 2007; Hoffman, Macgill, Smith, Oz, & Lupica, 2005; Ortiz, Oliva, Perez-Rial, Palomo, & Manzanares, 2004), in response to stress (Abel, 1992; Tohei, Mogi, Kon, Hokao, & Shinoda, 2003; Wu & Wang, 2010), and in learning and memory (Andrews et al., 1995; Holahan, Rekart, Sandoval, & Routtenberg, 2006; Keeley et al., 2010; Pare, 1996; Pryce et al., 1999; Tinius et al., 1989; van der Staay et al., 2009). Despite these

reported strain differences among rat strains, both LER and WR are used in studies of adolescent exposure to THC or other CB1R agonists, and the possibility that males and females of these two strains showing differential responses to the long-term effects of THC in adolescence has not been fully explored.

Purpose of the present study

With all of these issues in mind, this project sought to determine the long-term consequences of THC exposure following puberty onset in male and female LER and WR rats. First, differences in development and adult behaviour in male and female rats of these two rat strains were assessed. This was followed by investigation directed at understanding the short-term consequences of post pubertal exposure to THC on brain development. In a separate group of rats, the long-term consequences of postpubertal administration of THC were examined on adult cognitive functioning. Rats were assessed in a suite of behavioural tasks assessing anxiety behaviour, motor learning, spatial learning and contextual fear-conditioning. Brains were examined for gross morphological changes in brain areas related to the assessed behaviours. A final group of rats was observed for their sensitivity to amphetamine in a conditioned place preference task. As mentioned above, despite the use of multiple CB1R agonist compounds in other research, we chose to use a compound found in marijuana. Despite the differences in pharmacology, it was not expected that THC would produce effects that were entirely different than those seen in previous studies. Overall, it was assumed that LER would consistently outperform WR, and that males would outperform females in spatial tasks. No *a priori* assumptions were made regarding differential effects of THC in LER and WR, although it was predicted that females would be more sensitive to these effects than

males. Finally, it was assumed that any group that was identified as being particularly sensitive to the pubertal administration of THC would be more sensitive to the rewarding properties of a low dose of amphetamine in the conditioned place preference task.

This project will help to determine if exposure to THC following puberty onset will result in effects in addition to those originating from strain and sex differences. It will also help determine if exposure to THC during this developmental period is robust enough to overcome intrinsic differences between strain and sex groups. This project seeks to determine if there are long-term consequences associated with marijuana use during adolescence, as well as to identify potential subgroups of individuals who are particularly sensitive or resilient to the consequences of cannabis exposure. With this concept in mind, this project could help inform the public as to which individuals could be particularly sensitive to the long-term consequences of marijuana so as to develop targeted intervention and treatment strategies.

CHAPTER 2 - STRAIN & SEX DIFFERENCES

CHAPTER 2.1 – STRAIN AND SEX DIFFERENCES IN PUBERTY ONSET AND BRAIN OF JUVENILE RATS

Introduction

Sex-specific environmental pressures have led to sexually dimorphic physiological and behavioural traits. Sexual dimorphisms in rat brain and behaviour are well documented, ranging from volumetric differences in specific brain areas (Hines, Allen, & Gorski, 1992; Noonan, Smith, Kelleher, & Sanfilippo, 1998) to differences in strategies in a spatial learning task (Blokland et al., 2006; Kanit et al., 1998; Keeley et al., 2013; Korol et al., 2004). However, volumetric differences in brain and behaviour are not the only sexually dimorphic traits, and sex differences exist at the microstructural level in many brain areas, such that sex-dependent activation of second messenger pathways and transcription factors following membrane depolarization have been reported (Mizuno & Giese, 2010). Specifically, the hippocampus and its subregions are sensitive to sexual dimorphisms, as both coarse- (Galea et al., 1999; Roof, 1993) and fine-scale differences (Parducz et al., 2006; Woolley, 1998; Woolley & McEwen, 1992) have been reported extensively.

Sexual dimorphisms in brain and behaviour occur not only because of genetic differences between the sexes and are thought to originate primarily from either the activational or organizational effects of sex hormones (McEwen, 2002; McEwen & Alves, 1999). Activational effects of sex hormones in the brain describe those effects that are acute, changing local circuitry directly in response to sex hormone surges, for example during the estrous cycle in female rats (for example, see Parducz et al., 2006). Organizational effects of sex steroids confer the long-term changes in brain circuitry

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during certain developmental epochs, such as in mammalian gestation when sexual differentiation first occurs, or during puberty. A large pool of research to date has described and characterized brain structure and specific behaviours dependent either on the activational or organizational effects of sex hormones (for example, Roof & Havens, 1992; Williams et al., 1990) although specific emphasis has been placed on the organizational effects of these hormones during the perinatal period.

One specific perinatal period associated with changes in hormone levels is puberty onset. Puberty onset is signalled through the increased release of gonadotropin-releasing hormone (Harris & Levine, 2003; Ojeda et al., 2003) and is elicited through the complex interaction of multiple stimuli, including both hormonal and environmental factors (Ebling & Cronin, 2000; Sisk & Foster, 2004). Like the organizational effects of sex hormones during the prenatal and perinatal period, which have long-term consequences on a variety of sexually dimorphic brain areas and behaviour (for example, Roof & Havens, 1992; Williams et al., 1990), the adolescent and post-pubertal period are considered periods of brain development that, if disrupted, can have long-term consequences on brain development and behaviour (for review, see Schulz, Molenda-Figueira, & Sisk, 2009; Sisk & Zehr, 2005). However, the exact timing of environmental influence can have an effect on the outcome, with certain critical periods sensitive to specific interventions but not others (see Andersen, 2003, for review). For example, hippocampal plasticity can be affected by interventions both during the prenatal (Isgor & Sengelaub, 1998b), perinatal (Isgor & Sengelaub, 2003), pubertal and post-pubertal periods (Hebbard, King, Malsbury, & Harley, 2003). Interestingly, interventions during the pubertal period alone can have long-term consequences on anxiety behaviours in the male and female rat (Brand & Slob, 1988; Primus & Kellogg, 1989). The hippocampus,

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prefrontal cortex and amygdala are all centrally involved in the regulation of anxiety

behaviour, and maturation of these pathways occurs during pubertal development (Giedd

et al., 2006; Neufang et al., 2009; Romeo et al., 2006). Therefore, maturation of anxiety

behaviour and the brain areas responsible for the expression of anxiety behaviours may be

a critical component of pubertal development. Indeed, specifically in human populations,

puberty onset itself has been associated with increased risk for mental illness, including

depression and anxiety (Ge, Conger, & Elder, 2001; Kaltiala-Heino, Marttunen,

Rantanen, & Rimpela, 2003; Koff & Rierdan, 1993; Stice, Presnell, & Bearman, 2001).

The influence of gonadal-hormones during puberty and adolescence are sex-specific,

and early-life experience can have differential effects in adulthood, such that pre-

experience in the Morris water task facilitates adult learning (Wartman et al., 2012) in a

sex-specific fashion (Keeley et al., 2010). In Keeley et al. (2010), two different rat strains

were also used, based on previous research that had observed strain-dependent

innervation patterns in hippocampal subregions in conjunction with differential

behavioural strategies in a spatial learning task (Holahan, Honegger, & Routtenberg,

2007; Holahan et al., 2006). Indeed, the two rat strains used, Long-Evans hooded (LER)

and Wistar (WR) rats have been shown to exhibit differences in multiple behavioural

tasks and to exhibit differences in specific brain areas (Bauer, 1990; Fujimoto et al., 2007;

Pare, 1989; Sachs, 1996; Tohei et al., 2003). Yet, these two strains are used

interchangeably in the literature, with many individuals not considering which strain to be

most appropriate or directly comparing data obtained from the two strains. Not only

should initial differences between these two rat strains be considered, but the possibility

of strain-dependent responses to any experimental manipulation must be posited. If LER

and WR reach puberty at different time points as well as display differences in

development following puberty onset, this may offer an explanation of adult behavioural differences and establish the possibility that any interventions or experimental manipulations may result in differential responses in these two rat strains.

With all these considerations, this study examined two rat strains (LER and WR) for multiple developmental metrics, including age of onset of puberty, weight gain following the pubertal period as well as volumetric differences in brain areas related to learning and anxiety after a 2 week handling period following puberty onset. We attempted to characterize intrinsic differences between LER and WR males and females apparent following puberty onset.

Methods

Subjects

Female and male LER and Wistar WR rats were obtained from Charles River (Semmeville, QC; N = 9/group). Rats were allowed to acclimate to the University of Lethbridge animal housing rooms for approximately one week. Rats were paired and allowed to breed. Approximately one to two days before parturition, females and males were separated. Litters were culled to approximately 12 per litter (6 female and 6 male) for all groups. All pups were weaned at postnatal day 21 (PND21) and placed into sex-matched pairs or triplets. All rats were housed in standard laboratory conditions (21°C and 35% relative humidity; 12D:12L) Plexiglas tubs (46cm x 25cm x 20cm) with *ad libitum* access to food and water unless otherwise indicated. All rats handling and procedures were done in accordance to the University of Lethbridge's Animal Welfare Committee and the Canadian Council on Animal Care guidelines.

Puberty Onset & handling

All handling began following the determination of puberty onset. Females (LER: N = 84; WR: N = 85) and males (LER: N = 82; WR: N = 84) began monitoring for puberty onset at PND28. Puberty onset was determined using the external features of the genitalia, which are highly correlated with hormone levels (Korenbrot, Huhtaniemi, & Weiner, 1977; Parker & Mahesh, 1976). Females were examined for vaginal opening while males were examined for preputial separation. Vaginal opening was characterized by the increased size of the opening of the vagina, and preputial separation was characterized as the separation of the skin surrounding the penis. Both of these phenomena have been discussed and extensively described, verbally and pictorially, elsewhere (Korenbrot et al., 1977; Parker & Mahesh, 1976). On weaning day, rats were separated into cages in pairs or triples. All animals were handled following puberty onset for 14 consecutive days.

Histology

Perfusion and Fixation - The following day after the last handling day, a subset of rats (LER female: N = 6; WR female: N = 5; LER male: N = 5; WR male: N = 6; remaining rats were used in other experiments) were euthanized with a single i.p. injection of sodium pentobarbital (120mg/kg) and transcardially perfused with approximately 150mL of 1x phosphate-buffered saline (PBS). Following decapitation, brains were removed from the skull. The left hemisphere was immersion fixed in another solution used for another set of experiments, and the right hemisphere was immersion fixed in 4% paraformaldehyde (PFA) in 1xPBS. Right hemisphere PFA was replaced the

day after perfusion with 30% sucrose and 0.2% Na azide in 1xPBS until sectioning.

Right hemispheres were sectioned in a series of 12 at 40µm using a cryostat (CM1900, Leica, Germany) and placed directly into Eppendorf tubes containing 0.2% Na azide in 1xPBS.

 Cresyl violet staining – Sections were float-mounted in 1xPBS onto 1% gelatin 0.2% chrom alum slides (VWR Canada) and allowed to dry overnight. Sections were rehydrated and placed in a 1% cresyl violet solution in dH_2O for 10min. Slides were then rinsed in dH_2O followed by 70% ethanol. Sections were placed in differentiator for 2min and were dehydrated and coverslipped with Permount.

Volumetric measurements

 Volumetric analysis of medial prefrontal cortex (mPFC), orbital frontal cortex (OFC), amygdala (AMYG), whole hippocampus (HP), and hippocampal subregions, including the dentate gyrus (DG), CA1 and CA3 subregions, were conducted using the Cavalieri method (Gundersen, Jensen, Kieu, & Nielsen, 1999a), as implemented in StereoInvestigator (Microbrightfield, Williston, VT) on a Zeiss Axio Imager MT (Carl Zeiss, MicroImaging GmBH, Germany). Section intervals and grid size were chosen to include roughly 200 counted points from 10 sections (Garcia-Finana, Cruz-Orive, Mackay, Pakkenberg, & Roberts, 2003; Gundersen, Jensen, Kieu, & Nielsen, 1999b). Every 12th section was quantified for HP. All other areas of interest were quantified using every 6th section. Grid size was 500µm for HP, 150µm for DG, CA1 and CA3, and 250µm for OFC, mPFC and AMYG. The 5X objective was used to quantify HP. The 10X objective was used to quantify DG, CA1 and CA3. mPFC, OFC and AMYG were quantified using the 2.5X objective. For all measures, the coefficient of error

(Gunderson, m = 1) was less than 0.044, which is within acceptable parameters. Tracings

of regions of interest (ROIs) for border definitions can be found in Appendix 4.

Statistical Analysis

All statistical analyses were conducted using SPSS software package (ver21,

IBM). Strain and sex were considered as between group factors. Weight gain during the

pubertal period was examined as a within subjects design with handling day as a repeated

measure. No *a priori* hypotheses were used to guide statistical analyses.

Results

Puberty Onset

Puberty onset was determined as previously described. Overall, LER reached

puberty at an earlier age than WR ($F_{(1, 331)}$ = 8.641, p = 0.004), and as expected, females

reached puberty earlier than males ($F_{(1, 331)}$ = 655.931, p < 0.001). A significant strain by

sex interaction ($F_{(1, 331)}$ = 82.323, p < 0.001; Fig 1A) indicated that these main effects

were not applicable to all individual comparisons. Analysis of these comparisons

revealed that LER females reached puberty significantly younger than LER males (p <

0.001) and WR females (p < 0.001). WR females reached puberty at a younger age than

WR males (p < 0.001). However, no differences were observed between males.

Weight gain during adolescence

Weight gain 14 days following puberty onset was examined in all strain and sex

groups (Fig 1B). One data point on Day 10 for a LER female, and two data points for

WR females on days 11 and 13 were missing due to recording errors. All animals gained weight over the course of 14 days ($F_{(2.713, 273.996)} = 1372.509$, $p < 0.001$), and WR gained relatively less weight than LER overall ($F_{(1, 101)} = 8.339$, $p = 0.005$). No differences were observed between the sexes nor was there a significant strain by sex interaction. However, interactions between day and strain ($F_{(2.713, 273.996)} = 6.165$, $p = 0.001$), day and sex ($F_{(2.713, 273.996)} = 4.704$, $p = 0.004$) and day by strain by sex ($F_{(2.713, 273.996)} = 3.309$, $p = 0.025$) indicated that the strain and sex groups gained weight at different rates over the course of the 2 weeks following puberty onset. Between group comparisons revealed that only LER and WR females gained weight at different rates over the course of the 14 days ($F_{(1, 50)} = 6.019$, $p = 0.018$), and no such difference was observed between males. Therefore, this strain difference in relative weight gain was only observed within females.

Individual day analysis revealed that LER females' relative weight was greater than WR females' on days 8 ($p = 0.018$), 9 ($p = 0.032$), 10 ($p = 0.027$), 11 ($p = 0.018$), 12 ($p = 0.002$), 13 ($p = 0.001$) and 14 ($p = 0.009$) following puberty onset (Fig 1B).

Volumetric measurements

Mean volume for all groups and each measurement of the right hemisphere brain area can be found in Table 1 including coefficients of error. No significant effects of strain or strain by sex interaction were observed for volumetric measurements of any brain area. However, significant sex differences were observed in all hippocampal subregions, such that males, regardless of strain, had larger volumes of total hippocampi ($F_{(1,19)} = 20.368$, $p < 0.001$), with larger DG ($F_{(1, 18)} = 10.588$, $p = 0.004$), CA1 ($F_{(1, 18)} = 10.376$, $p = 0.005$) and CA3 subregion ($F_{(1, 18)} = 5.059$, $p = 0.037$) volumes. Individual comparisons within each strain between the sexes revealed that LER males had larger

hippocampal volumes (p = 0.03) than LER females, and this effect was also observed in WR (p = 0.003). However, the main effect of sex was preserved only in WR in the DG and CA1 subregions, such that only WR males had larger DG (p = 0.015) and CA1 (p = 0.029) subregion volumes in comparison to their female counterparts. Individual comparisons revealed that neither LER nor WR had significant differences in the CA3 subregion. No differences were observed for any of the other brain areas examined.

Discussion

In this study, we discovered that LER females reach puberty sooner than their male counterparts and WR females (Fig 1A). Additionally, LER females gained weight at a faster rate than WR females during the post-pubertal period (Fig 1B). Finally, in all brain regions examined, the hippocampus displayed sexual dimorphisms such that hippocampal volumes were larger in males than females from both rat strains (Fig 2A). Further, these differences were preserved in all subregions given that overall, male DG (Fig 2B), CA1 (Fig 2C) and CA3 (Fig 2D) regions were larger in males than females. Individual comparisons within rat strains revealed that only WR males had larger DG and CA1 regions than their female counterparts. This is the first study of this kind to examine developmental differences between males and females of these two rat strains in brain volumetrics and post-pubertal development.

Females of either strain were found to reach puberty earlier than males. This is as expected, as female rats enter puberty sooner than males (Engelbregt, Houdijk, Popp-Snijders, & Delemarre-van de Waal, 2000). In addition to this, female LER entered puberty earlier than WR females and gained weight at a faster rate towards the end of the handling period, which was unexpected. Earlier puberty onset has been linked to

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improved prenatal and perinatal nutrition in humans, such that over the last 150 years, age of onset of puberty has decreased in most industrialized European countries (Lee & Styne, 2013). In contrast, early onset puberty has also been linked to multiple disorders, including hormone disorders, such as polycystic ovarian syndrome, and to psychiatric illness, such as anorexia or depression (Graber, Lewinsohn, Seeley, & Brooks-Gunn, 1997; Graber, Seeley, Brooks-Gunn, & Lewinsohn, 2004; Lee & Styne, 2013; Michaud, Suris, & Deppen, 2006). For the most part, factors such as heritability (Park et al., 2012; Phillip & Lazar, 2005), early-life exposure to environmental toxins (Bateman & Patisaul, 2008; Bourguignon et al., 2010; Hu et al., 2013; Ma et al., 2006; Shirota et al., 2006) and early-life nutrition (Engelbregt et al., 2000; Gereltsetseg et al., 2012; Iwasa et al., 2010; Li, Lin, Kinsey-Jones, & O'Byrne, 2012), have been identified for their effects on altered puberty onset. However, the results in the literature are varied, and the exact mechanisms involved in the timing of puberty onset are poorly understood (Ibanez & de Zegher, 2006; Kilic et al., 2012; Sisk & Foster, 2004; Sorensen et al., 2012; Yang et al., 2012).

Puberty represents a period of brain development where experience can shape future behavioural states, including emotional processing (Primus & Kellogg, 1990; Schulz & Sisk, 2006; Sisk, Schulz, & Zehr, 2003). The difference in puberty onset between the two rat strains under investigation in the present study may help explain behavioural and morphometric differences observed in adulthood, given the superior performance of LER as compared to WR in most cognitive behavioural tasks, including the Morris water task (see Chap 2.2; Holahan et al., 2006) and discriminative fear-conditioning to context (Chap 2.2).

It is debatable whether the earlier age of onset for LER represents an increased risk for the development of diseases or whether the later onset of puberty in the WR in fact

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represents a developmental flaw that will increase the impact of other risk factors. Whether this LER-biased advance in puberty onset is detrimental or beneficial remains to be seen. In addition, this strain difference in puberty onset could offer benefits earlier in life that predispose to deficits later, or vice versa. The difference in puberty onset may in fact be part of the mechanism for the observed behavioural differences between these two rat strains. Artificially inducing puberty in the WR strain may help reveal the level of influence puberty onset plays in adult behaviour. However, this may prove problematic given that the exact switch "turning on" puberty is not fully elucidated (Sisk & Foster, 2004). Only future research exploring the differences between these rat strains could help disentangle the mechanisms behind these differences and the exact mechanism behind the timing of puberty. Not only can this earlier age of onset lead to both immediate and long-term changes in brain development, it could also lead to differences in development throughout the adolescent period. Indeed, although LER females reached puberty at an earlier date than WR females, it is possible that WR females could rapidly reach the same developmental time point as LER females, potentially in a shorter period of time. This is possible given the lack of volumetric differences between LER and WR females following the 14-day monitoring period. Only further research examining LER and WR females following puberty onset will be able to address this issue.

In contrast to these physiological differences, no direct strain differences were observed in any volumetric measurement. This is surprising given the previous indication that these strains display different innervation patterns of the CA3 subregion (Holahan et al., 2007). However, sex differences were prevalent, such that males of both strains had significantly larger total hippocampal volume. These differences were expected, as a variety of species show male-biased hippocampal size (Burger, Saucier, Iwaniuk, &

Saucier, 2013; Jacobs, Gaulin, Sherry, & Hoffman, 1990; Jacobs & Spencer, 1994; Roof & Havens, 1992; Sherry, Galef, & Clark, 1996). Multiple factors may contribute to the this sexual dimorphism, including but not limited to changes in neurogenesis, sex hormone-specific effects on hippocampal volume (Fugger, Cunningham, Rissman, & Foster, 1998; Galea et al., 1999; Williams et al., 1990), and multiple evolutionary explanations (Jones, Braithwaite, & Healy, 2003). Sexual dimorphisms in the hippocampal subregions have been explained to cause the male-bias in spatial learning (Astur et al., 2004). A more thorough examination of the differences between male and female spatial navigation abilities (Astur et al., 1998; Astur et al., 2004; Gaulin & Fitzgerald, 1989; Jonasson, 2005) and its relationship to sex hormones (Hausmann et al., 2000; Keeley et al., 2013; Kimura, 1996; Korol, 2004; Luine, Jacome, & Maclusky, 2003; Parducz et al., 2006; Vanhaaren et al., 1990; Zuloaga, Puts, Jordan, & Breedlove, 2008) have been extensively investigated and will not be covered here. Regardless, the male-biased hippocampal volume observed here has been observed many other times and may reflect the adoption of alternative strategies as well as brain areas in order to solve spatial tasks.

Some subtle strain differences did appear as only the WR displayed sexual dimorphisms in the size of the dentate gyrus and CA1 subfields. No other sex or strain differences were observed for any other measure. The dentate gyrus is one of the sites of neurogenesis (Cameron & McKay, 2001), and sexual dimorphisms in this area have been observed previously in a variety of species (Burger, Gulbrandsen, Saucier, & Iwaniuk, 2014; Burger et al., 2013; Galea & McEwen, 1999; Galea et al., 1999; Roof, 1993), although volumetric analyses have not always been implemented.   The CA1 region has not been previously reported to show sexual dimorphisms in volume, however sex

differences in cytoarchitecture in CA1 have been reported (for example, Shors, Chua, & Falduto, 2001). This region has been implicated in the temporal encoding of memories (Gilbert, Kesner, & Lee, 2001; Kesner, Hunsaker, & Gilbert, 2005), and we are unaware of any strain or sex differences in this reported hippocampal function. Alterations in dentate gyrus and/or CA1 through lesions or temporary inactivations, can affect spatial learning, temporal encoding and novel object recognition (Gilbert et al., 2001; Hoge & Kesner, 2007; Lee, Hunsaker, & Kesner, 2005; Lee, Jerman, & Kesner, 2005; Lee & Kesner, 2004; Ridley, Timothy, Maclean, & Baker, 1995; Spanswick, Epp, Keith, & Sutherland, 2007; Spanswick & Sutherland, 2010; Zola-Morgan, Squire, & Amaral, 1986). These regions are thought to mediate differential aspects of learning, with the dentate gyrus being more important for pattern separation and the CA1 region for pattern completion (Kesner, 2013; Rolls, 2013; Yassa & Stark, 2011). A discussion of the functional differences between these hippocampal subregions is beyond the scope of this discussion. However, we are not aware of any strain or sex differences in the functional aspects of these different hippocampal regions. However, based on the results reported here, it could be that in the Wistar rat strain, males may display superior performance in both spatial navigation tasks as well as temporal encoding. Only future behavioural research examining sex differences within WR will help elucidate whether the sexual dimorphisms in these brain areas translates to a functional behavioural difference.

Both CA1 and the dentate gyrus contain sex hormone receptors (Kerr, Allore, Beck, & Handa, 1995; McEwen, Luine, Plapinger, & Dekloet, 1975). The observed differences between male and female WR could be a function of sex hormone alterations in connectivity as a result of sex hormone exposure in the perinatal or pubescent period as the dentate gyrus can be altered with early-life exposure to testosterone in male and

female rats (Roof & Havens, 1992). Whether these differences persist to adulthood and alter behavioural strategies between males and females of these two rat strains remains to be seen. Perhaps the sex differences observed only in the WR strain will result in larger sex differences as well as sensitivity of strategies in response to fluctuating sex hormones within this rat strain and not in the LER strain.

One possible confound of this experimental design was the lack of distinction between dorsal and ventral hippocampus. Dorsal and ventral hippocampus are functionally distinct areas, with dorsal portions involved in spatial learning and ventral portions regulating emotional processing and inhibitory learning processes (Fanselow & Dong, 2010; Ferbinteanu & McDonald, 2000, 2001; McDonald, Jones, Richards, & Hong, 2006). Additional studies examining sex differences separately for dorsal and ventral hippocampus may help relate sex differences in spatial learning and fear-conditioning to anatomical differences in the hippocampus.

No strain differences were observed in brain volumetrics for any area examined. This is of interest, since behavioural and morphometric differences have been observed in both juveniles and adult LER and WR (Cameron & McKay, 2001; Holahan et al., 2007; Holahan et al., 2006; Keeley et al., 2010). Perhaps, given the short window of observation following the post-pubertal period, adult differences were not able to fully develop. Only careful examination of adult brain and behaviour will help determine whether these differences persist.

Conclusions

This study is the first of its kind to identify early physiological differences between LER and WR rats. In addition, it has further supported the male-bias of larger

hippocampal volume in the rat species, pointing to a possible influence of strain in the differences between the sexes in hippocampal subregions. Future research should examine whether these post-pubertal differences are responsible for possible differences in adult behaviour, anatomy and functionality of the different brain regions. This research supports the rationale of carefully choosing rat strains as any experimental manipulation could induce differential results.

CHAPTER 2.2: STRAIN AND SEX DIFFERENCES IN THE BRAIN AND BEHAVIOUR OF ADULT RATS

Introduction

There are a myriad of factors that can alter behaviour, most of which fall into the categories of heredity or experience. How each of these components contributes to individual differences in behaviour varies. For example, a recent study (Richards et al., 2013) examined the heritability of impulse control, i.e. the ability to suppress a behavioural response, in different rat strains. Many behavioural traits were found to be heritable, although this varied from behaviour to behaviour. Regardless, this study demonstrated how genetic differences between rat strains can alter behavioural strategies and performance on a variety of tasks.

The effect of rat strain has been discussed since not long after the first use of rats for behavioural research. With the development of multiple outbred rat strains, many of which were derived from an original stock from the Wistar Institute (Clause, 1998; Krinke, 2000), rats have been selected and bred for multiple factors, including docility (Keeler, 1948), anxiety (Liebsch, Montkowski, Holsboer, & Landgraf, 1998) or seizure susceptibility (Racine, Steingart, & McIntyre, 1999), to name a few. Unplanned strain differences between rats are not a recent discovery. For example, strain differences have been studied in a variety of behavioural tasks, including those related to impulse control (Hamilton et al., 2014), emotionality (van der Staay et al., 2009) and learning and memory (Andrews et al., 1995; Pare, 1996; van der Staay et al., 2009). In addition to intrinsic differences, differential responses to experience have been observed between different rat strains, including in response to stress (Pare, 1989; Tohei et al., 2003; Wu & Wang, 2010) or certain drugs (Aulakh et al., 1988; Deiana et al., 2007; Fujimoto et al.,

2007; Gordon & Watkinson, 1995; Ortiz et al., 2004; Woolfolk & Holtzman, 1995). This has been quantified not only in behavioural outcomes but also with specific alterations in neurological circuits in the brain. For example, LER showed superior performance in the Morris water task (MWT) in comparison to WR, and this experience in LER and WR resulted in differential innervation patterns in the hippocampus (Holahan et al., 2006), an area of the brain integral to learning and memory, that also shows strain-dependent developmental trajectories (Holahan et al., 2007). In addition to this differential response to experience as adults, juvenile pre-training in the MWT facilitated performance in LER but not in WR (Keeley et al., 2010).

This same study also investigated sex differences, and the facilitation in behaviour occurred exclusively in LER males and not females (Keeley et al., 2010). Intrinsic and experience-dependent sex differences are well documented. For example, throughout a variety of species, males typically outperform females in spatial memory tasks (Jonasson, 2005). Not only are there sex differences in behaviour, but sexual dimorphisms are observed in a variety of brain areas, including but not limited to areas related to learning and memory (Galea & McEwen, 1999; Galea et al., 1999; Mizuno & Giese, 2010; Toufexis, Myers, Bowser, & Davis, 2007). Indeed, a male-bias towards a large hippocampus and some of its sub-regions has been observed in a variety of species (Burger et al., 2013; Galea et al., 1999) and is thought to mediate the enhanced male performance in spatial learning tasks (Clint et al., 2012; Cox, Skelly, & John-Alder, 2003; de Vries & Sodersten, 2009; Gaulin & Fitzgerald, 1989; Iaskin, 2011; Jones et al., 2003; Spritzer, Meikle, & Solomon, 2004). Other behavioural measures show sexual dimorphisms, such as those measuring fear conditioning or anxiety (Toufexis, 2007). However, locomotor activity can be a confounding variable in many of these measures, as

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females will vary in their locomotor activity dependent on regularly fluctuating hormone levels (Brobeck, Wheatland, & Strominger, 1947; Slonaker, 1924), although this result is not consistently observed (for example, Marcondes, Miguel, Melo, & Spadari-Bratfisch, 2001). Strain can also influence locomotor activity (Bauer, 1990; Nakajima, 2014), and sexual dimorphisms in certain measures can be apparent in some rat strains and not others, such as nicotine sensitivity (Pryce et al., 1999). Therefore, the interactions between strain and sex often have an important influence on behaviour.

The purpose of this study was two-fold. First, we wanted to examine the effect of strain and sex in a variety of behavioural tasks in order to assess strain and sex differences on these tasks, some of which have been conducted before, and some that are completely novel. Both LER and WR are the most widely used strains for behavioural assessments. We used skilled reaching (SRT) to examine motor learning, the Morris water task (MWT) to examine spatial memory performance, discriminative fear-conditioning to context (DFCTC) in order to examine contextual fear-based learning and the elevated plus maze (EPM) to examine anxiety behaviours. These tasks are widely used throughout the literature, and a direct comparison of strain and sex differences has never been conducted in any of these variants of these tasks in the same rats. It was predicted that males and LER would outperform females and WR respectively in all tasks related to spatial or contextual learning, and that there would be no differences in motor learning between either the sexes or strains, as that has not been observed previously in any strain comparison between LER and WR. It was predicted that females would be less anxious than males in the elevated plus maze (Johnston & File, 1991). The effects of strain on anxiety are not immediately obvious in the literature. Furthermore, it was predicted that males would display enhanced conditioned freezing behaviour in comparison to females,

as this has been observed previously in other fear-conditioning tasks (Toufexis et al., 2007). Secondly, we examined the effect of strain and sex on volumetric differences in brain areas related to these behaviours, including the hippocampus (HP) and its associated subregions (DG, CA1 and CA3), the medial prefrontal cortex (mPFC), the orbital frontal cortex (OFC) and the amygdala (AMYG). These areas were chosen as they directly relate to task performance and could help explain behavioural differences between or within strains. It was predicted that superior performance in males and LER, as described above, would confer larger volumes of associated brain areas. For example, superior performance in the MWT was assumed to be the product of a larger hippocampus and its associated subregions. This study highlights differences between males and females of two different rat strains, demonstrating the importance and the influence of rat strain and sex on behavioural outcomes. Careful comparison between two rat strains in the literature as well as choosing the appropriate rat strain for research in Behavioural Neuroscience will be emphasized.

Methods

Subjects

     Female and male LER and WR rats were obtained from Charles River (Semmeville, QC; N = 9/group). All procedures were conducted as previously described (Chap 2.1). For all behavioural tasks, N = 8 per group. One group was used for MWT, SRT and DFCTC, and a separate group was used in the EPM. All rat handling and procedures were performed in accordance to the University of Lethbridge's Animal Welfare Committee and the Canadian Council on Animal Care guidelines.

Handling

Handling was done as previously described (Chap 2.1). Rats were aged to 90 days before the start of behavioural assessments as PND90 was considered a standard "adult" age.

Vaginal cytology and determination of estrous cycle

Female estrous cycle was collected using the lavage technique (Goldman, Murr, & Cooper, 2007; Marcondes, Bianchi, & Tanno, 2002). Sterile Q-tips were dipped in sterile dH_2O and inserted into the vagina and rotated. The Q-tip was wiped onto a glass slide (VWR Canada) and placed in a slide box for qualification. To ensure comparable handling procedures between males and females, for males, a Q-tip dipped in sterile dH_2O was brushed gently in the scrotal area.

Estrous cycle determination and qualification was conducted using brightfield microscopy on a Zeiss Axio Imager MT (Carl Zeiss, MicroImaging GmBH, Germany) using the 20X objective. Cell types and qualifications were done in reference to previous research (Goldman et al., 2007; Marcondes et al., 2002). *Proestrous* was characterized as the presence of granular cells presented in clumps or strands. *Estrous* was characterized as the presence of keratinized, needle-like and/or jagged-edged cells in clumps. *Metestrous* was characterized as the combination of multiple cell types including larger rounded cells, needle-like cells and leukocytes. Characteristic images of these different phases can be found in Appendix 2.

Vaginal smears were collected during all handling days as well as on all days of behavioural testing. The only exception being that vaginal smears were only collected for the first 10 days of SRT, and estrous cycle was extrapolated for days 11-21 based on the

observed results from the first 10 days. Representative images of the different phases can be found in Appendix 2.

Skilled reaching task (SRT)

Apparatus – A similar apparatus is previously described (Metz & Whishaw, 2000). Briefly, the reaching chamber consisted of a clear Plexiglas rectangular chamber (40cm high, 45cm long and 13cm wide) with a 1.3cm wide vertical opening in the middle of the front wall. A Plexiglas shelf was placed at the front of the opening, 4cm from the floor, measuring 13cm long and 3cm wide. Two small indentations (5mm in diameter, 1.5mm deep) for the placement of pellets were made in the shelf and were placed 1.5cm from the vertical opening.

Training – Five days before training, rats were food restricted to 95% of their *ad libitum* body weight. All training trials lasted a maximum of 10min and were conducted as previously described (Metz & Whishaw, 2000) for 21 days. Briefly, rat behaviour was shaped in order to train a rat to reach for a pellet at the front of the apparatus, and then run to the back of the apparatus before attempting another reaching trial. Pellet placement in the left or right indentation was determined through the initial training trials and was placed corresponding to the rats' paw preference. Number of pellets eaten, number of reaches, type of reach and total time taken to reach for 20 pellets was recorded every day. Analyses were conducted exclusively for the last day of training in order to simplify the interpretation. For the last day, two measures were analyzed: success relative to the number of attempts or reaches and success relative to the total number of pellets eaten. A success was scored as a trial with a retrieved pellet through reaching and not using other methods, such as with their tongue. The first measure, success relative to the number of

attempts, gave a conservative estimate of the efficiency of the reaching, as low ratios would indicate a large number of attempts with few successful trials. The second measure, the success relative to the total number of pellets eaten gave an indication of alternative strategies, as a ratio smaller than 1 would indicate that higher numbers of pellets were eaten using other means than reaching. Essentially, we were interested in examining metrics of skilled motor learning not necessarily differences in fine motor dexterity.

Morris water task (MWT)

Apparatus - A large circular pool with a diameter of 1.4m was filled to a depth of 40cm with 21˚C water rendered opaque using white non-toxic poster paint (Crayola, PA, USA). A clear Plexiglas platform (13cm X 13cm) was placed in the pool approximately 3cm below the water surface. Extra maze cues, such as posters, the computer and the experimenter placement during training remained stationary throughout the experimental period.

Data collection – Data were collected using a computer rat tracking system (Ethovision 3.1, Noldus, USA) and a camera located above the pool. WR were marked using a non-toxic Sharpie marker in order to be identified by the tracking system. The tracking system recorded latency to reach the platform and distance travelled.

Training - MWT training was done as described previously (Amtul et al., 2014; Keeley, Zelinski, Fehr, & McDonald, 2014). Starting positions were determined in a quasi-random fashion such that all starting positions were used every day and no two adjacent starting positions were used on consecutive trials. For all trials, rats were placed in the pool facing the wall and allowed 60s to reach the submerged platform. If they did

not reach the platform after 60s, they were guided to it by the experimenter. Once rats

had reached the platform, they remained there for 10s. For all trials, there was an

intertrial interval of at least 60s. On days 1-4, the platform was located in the same

quadrant. All rats were trained for 8 trials a day. On day 5, the platform was removed for

a probe trial, and rats were allowed to freely swim for 60s before being removed from the

pool. On the 6th day, the platform was moved to another quadrant for a mass training day

to a new location. On day 6, rats were given 16 trials. On day 7 and 8, a black visible

platform was placed in the pool approximately 4cm above the water surface. The

platform was located in two different quadrants for each day, and rats were given 4 trials

per day. A pictorial representation of the MWT can be found in Figure 3.

Discriminative fear-conditioning to context (DFCTC)

Apparatus – A similar apparatus was used as described previously (Amtul et al.,

2014; Antoniadis, Ko, Ralph, & McDonald, 2000; Antoniadis & McDonald, 1999, 2000;

Craig & McDonald, 2008). Briefly, an opaque black triangular shaped box (61cm X

61cm X 30cm) and an opaque white square box (41cm X 41cm X 29cm), both with metal

bars as the floor (0.32cm in diameter) spaced roughly 1.5cm apart, were used as the two

contexts. Both contexts had pill bottles inset into the walls containing cotton balls

infused with a scent cue. The black triangle context was always paired with amyl acetate

and the white square with eucalyptus. Contexts differed in shape (triangle versus square),

colour (black versus white) and odour (amyl acetate versus eucalyptus). Every day, the

scent cues were reloaded with the appropriate scent. The contexts were connected with a

grey alleyway (11cm X 11cm X 16.5cm**)** and could be separated via Plexiglas doors.

Both contexts and the alleyway were placed upon a clear Plexiglas table. Underneath the

table was a mirror inclined at a 45˚ angle that allowed for viewing by both an observer and a video camera (where noted).

Pre-exposure – On the pre-exposure day, all animals were acclimated to the testing apparatus. The doors of both contexts were opened and rats were placed in the grey alleyway and allowed to freely explore either context for 10min. An observer recorded dwell time in both chambers. The presence of urine and the number of boli were also recorded. Following the 10min pre-exposure, animals were removed and returned to a transport tub, following which they were returned to their home cage. The maze was cleaned using soap and water to remove any scent cues.

Following pre-exposure, rats were assigned to either be shocked in the black triangle or white square context in a quasi-random fashion. All groups were counterbalanced such that half of the animals in each group were shocked in the white context and half were shocked in the black. The groups were further counterbalanced such that half started training in the shock context (paired) while half started in the no shock context (unpaired).

Training – Training lasted over the course of 8 days. Briefly, rats were exposed to either the shock (paired) context or the no shock (unpaired) context on a given day. Rats also alternated the contexts they were exposed to such that on day 1, a given rat would be exposed to their paired context, and on day 2, they would be exposed to their unpaired context and so on for 8 days and 4 pairings per context. Training consisted of placing the rat in one or the other context with the door to the alleyway closed for 5min. In the paired context, at minutes 2, 3 and 4, rats would be exposed to a 0.6mA shock for 2s. In the unpaired context, no shock was administered, and rats were allowed to freely explore. Number of boli and the presence of urine were recorded.

Test days – All rats were exposed to 2 test days where the relative time spent freezing was assessed in the paired context on one day and the unpaired context on the other. Groups were counterbalanced such that only half were assessed in the unpaired context on the first test day and the other half were observed in the paired context and vice versa on the second day. Relative time freezing was observed, which included the animal remaining completely motionless with upper and lower limbs rigid and was recorded over the course of 5min. Number of boli and the presence and absence of urine was also recorded. All test days were recorded on video.

Preference test – Following the second test day, animals were assessed in exactly the same procedure as the pre-exposure day such that they were allowed to freely explore both contexts connected by a grey alleyway for 10min. Dwell time in each context as well as the presence of urine and the number of fecal boli were recorded. The preference test was recorded on video, and results were verified by an observer. A pictorial representation of the DFCTC task apparatus and procedure can be found in Figure 4.

Elevated plus maze (EPM)

A separate group of rats, with exactly the same early life experience but no exposure to other behavioural tasks, was observed in the EPM.

Apparatus – The apparatus was similar to that previously described (Muhammad et al., 2013). Briefly, the base of the apparatus was 94cm high off the floor and was made of black Plexiglas. Both open and closed arms measured 10cm in width and 40cm in length. The walls of the closed arms were 40cm in height. All procedures were conducted with the lights on. A camera was placed at the front of the apparatus at a 30˚

angle above the maze and all behaviour in the maze was recorded. The experimenter remained in the room for the duration of the testing.

Testing – Rats were placed in the apparatus either facing the right or left closed arm and allowed to freely explore for 5min. Which arm they faced was determined in a quasi-random fashion. Following testing, rats were removed from the maze and placed back in their home cage. The apparatus floors and walls were wiped down with detergent between rats. Quantification of behaviour was conducted through video monitoring after the experiment. The number of arm entries and the dwell time for open and closed arms were recorded.

Perfusion and Fixation

The day after the last behavioural testing day, rats were euthanized and perfused, and brain extraction and fixation were conducted as previously described in (Chap 2.1). Right hemispheres were collected and sectioned in a series of 12 at 40μm using a cryostat (CM1900, Leica, Germany) and placed directly into Eppendorf tubes containing 0.2% Na azide in 1xPBS.

Histology – Cresyl violet staining

Sections were float-mounted in 1xPBS onto 1% gelatin 0.2% chrom alum slides (VWR Canada) and allowed to dry overnight. Sections were rehydrated and placed in a 1% cresyl violet solution in dH_2O for 10min. Slides were then rinsed in dH_2O followed by 70% ethanol. Sections were placed in differentiator for 2min and were dehydrated and coverslipped with Permount.

Volumetric estimates using unbiased stereology

Volumetric analysis of total HP, DG, CA1, CA3, mPFC, OFC and AMYG were conducted using the Cavalieri method (Gundersen et al., 1999a) as implemented in StereoInvestigator (Microbrightfield, Williston, VT) on a Zeiss Axio Imager MT (Carl Zeiss, MicroImaging GmBH, Germany) as described in Chap 2.1. Section intervals and grid size were chosen to include roughly 200 counted points and 10 sections (Garcia-Finana et al., 2003; Gundersen et al., 1999b). For hippocampal estimates, every 12th section was quantified. For all other areas of interest, every 6th section was quantified. Grid size was 500µm for HP and AMYG, 150µm for DG, CA1 and CA3, and 300µm for mPFC and OFC. HP was quantified using the 5X objective. DG, CA1 and CA3 were quantified using the 10X objective. AMYG, mPFC and OFC were quantified using the 2.5X objective. The rat (*R. norvegicus*) brain atlas (Paxinos & Watson, 2007) was used as a reference guide. Representative tracings for ROI border definitions can be found in Appendix 4. Mean values and coefficients of error for each brain region for each group can be found in Table 2.

Statistical analysis

Statistical analyses were conducted with a set of *a priori* hypotheses in mind. First, for all measures, analyses were conducted between LER and WR male and female controls in order to determine significant strain and sex differences. No comparisons were made between LER males and WR females nor WR males and LER females as this comparison was considered irrelevant in terms of justifiable and interpretable comparisons. All statistical analyses were conducted using SPSS ver 21 (IBM, USA). Estrous cycle was examined as a covariate for individual day analysis for all measures.

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SRT - A between subject design ANOVA was conducted with strain and sex as between subjects factors. The number of successful attempts and the number of successful trials were both analyzed for between subjects effect. No *a priori* hypotheses were tested.

MWT - For the first 4 days of training, a mixed design ANOVA was conducted with day as a repeated measure, and with strain and sex as the between subjects measures. For the probe day, a mixed design ANOVA was conducted for all measures with quadrant as a repeated measure and when appropriate, strain and sex as between subjects measures. For the 6th day (mass training), a mixed design ANOVA was conducted with trial block as a repeated measure and strain and sex as between subjects measures. Finally, for the 7th and 8th day of training, a mixed design ANOVA was conducted with day and trial as repeated measures and strain and sex as between subjects measures.

Comparisons were established *a priori* for all probe test analyses such that differences within a group were examined for dwell time and distance travelled in the target quadrant in comparison to the other quadrants. Further, *a priori* comparisons were conducted for the first time bin on the 6th day (mass training day) in order to examine perseverative behaviour once the platform had been moved to a new location.

DFCTC - A mixed design ANOVA was conducted with strain and sex as between subjects factors and context (paired versus unpaired). For test and preference days, *a priori* hypotheses were tested to determine within each strain and sex group whether freezing (for test days) or dwell time (for preference day) differed between the paired and unpaired context.

EPM - A mixed design ANOVA was conducted for the number and the percent dwell time in open versus closed arms. For the ratio of open to closed arms, a between subjects ANOVA was conducted. There were no *a priori* hypotheses for any comparison.

Volumetric estimates - For volumetric measurements, some rats were included who had no behavioural experience. This factor was used as a covariate in all volumetric analyses, but experience never significantly contributed to the observed results in any analyses, so all subjects, regardless of behavioural testing paradigm, were included in all volumetric analyses.

For volumetric estimates, inter-rater reliability was estimated using two-way mixed consistencies, average-measures intra-class correlation (ICC), as described in (Hallgren, 2012), to assess the degree of consistency between one potentially biased observer and a completely blind observer from a subset of 10 randomly selected subjects. An ICC of 1 indicates perfect relatedness whereas an ICC of <0.4 is considered poor inter-rater reliability (Hallgren, 2012). The achieved ICC of 0.982 suggests that volumetric differences were similar between observers, therefore the first set of observations, conducted by the non-blind observer, were considered to be non-biased as they were highly similar to that of a completely blind observer.

Results

A summary of all results in all behavioural tasks, with specific emphasis on strain and sex differences can be found in Table 3.

SRT

SRT was trained over the course of 21 days. All subsequent analyses only examined success on the last day of testing. Two measures of success were used: success relative to attempts and success relative to total numbers of pellets eaten. These two metrics gave an indication of motor learning performance after a set amount of training. Analyses were restricted to the final day of testing (day 21) in order to examine the differences between groups given training and not during acquisition.

Successful attempts - The number of pellets eaten and the number of reaching attempts ratio were analyzed as a measure of success. No significant differences were observed for strain or sex and there was no strain by sex interaction (Fig 5A). Estrous cycle did not have any effect on successful attempts.

Successful trials - The number of pellets eaten and the number of trials were used as a ratio and metric of success. No significant differences were observed for strain or sex, nor was there any strain by sex interaction (Fig 5B). Estrous cycle did not have any effect on female performance.

MWT

Acquisition – Day 1-4 - Rats decreased their latency to reach the platform over the course of the first four days of training ($F_{(3,84)} = 46.706$, p <0.001). Further, LER took significantly less time to reach the platform overall than WR ($F_{(1,28)} = 44.297$, p<0.001; Fig 6A). No significant main effects of sex were observed. No significant interaction effects were observed. Latency to reach the platform decreased over days, such that the latency to reach the platform was significantly longer on day 1 in comparison to day 2 (p

< 0.001), day 2 in comparison to day 3 (p = 0.001) and on day 3 in comparison to day 4 (p = 0.048).

On the first day of training, all groups took roughly the same time to reach the platform. On the 2nd day of training, WR took significantly longer to reach the platform ($F_{(1,28)}$ = 27.876, p < 0.001), and there were no differences observed between either sex. *Post hoc* comparisons indicated that both WR females and males took significant longer to reach the platform in comparison to LER females (p = 0.017) and males (p < 0.001), respectively. On day 3, males took significantly less time to reach the platform ($F_{(1,28)}$ = 4.893, p = 0.035), and LER overall took less time to reach the platform ($F_{(1,28)}$ = 52.874, p < 0.001). LER females took significantly less time than WR females to reach the platform (p = 0.001); this difference was paralleled in LER and WR males (p < 0.001). Further, LER males were significantly faster at finding the hidden platform in comparison to LER females (p = 0.02). Male and female WR took the same amount of time to reach the platform. On the 4th day of training, LER took significantly less time to reach the platform than WR (p < 0.001). No differences were observed between the sexes. LER took significantly less time to reach the platform in comparison to WR for both males and females (females: p = 0.004; males: p = 0.003).

Estrous cycle did not have any effect on the latency to reach the platform for the first 4 days of training.

Retention : Day 5 probe - Probe trials were examined in time bins, such that behaviour in the first 10s, 30s and the entire 60s of the probe were examined. However, for the present analysis, only the 30s time bin will be discussed as the other time bins showed similar results. Both dwell time and distance travelled were examined. One LER

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female and one WR female were not included in the analysis due to experimental error using the tracking system.

For total distance travelled during the first 30s of the probe, all rats travelled significantly further in the target quadrant as compared to the other quadrants ($F_{(1,26)}$ = 18.155, $p < 0.001$), indicating retention of the previous days' platform location. However, LER travelled significantly further than WR overall ($F_{(1,26)}$ = 8.562, $p = 0.007$), indicating improved retention of LER over WR. There was a significant quadrant by strain interaction ($F_{(1,26)}$ = 4.697, $p = 0.04$; Fig 6B), and no differences were observed between the sexes.

Within the target quadrant, LER travelled significantly further than WR ($F_{(1,26)}$ = 8.71, $p = 0.007$), and there was no significant effect of sex. Specifically, LER males demonstrated better retention than WR as shown by increased distance travelled in the target quadrant ($p = 0.034$). No such difference was observed between females of either strain. Within the other quadrants, there were no significant differences between groups for distance travelled.

LER and WR males travelled significantly further in the target quadrant in comparison to the other quadrant (LER males: $p = 0.004$; WR males: $p = 0.018$). This difference was not observed in females of either strain. Although no main effects of sex were observed, this is an indication that a sex difference may exist such that males of either strain displayed differential distance travelled in the target quadrant in comparison to the other quadrants and females did not.

However, when examining dwell time in the quadrants, all rats spent significantly more time in the target quadrant in comparisons to the other quadrants ($F_{(1,26)}$ = 24.817, $p < 0.001$; Fig 6C). No other significant differences were observed. All groups except for

LER females spent significantly more time in the target quadrant in comparison to all other quadrants (LER females: p = 0.105; LER males: p = 0.011; WR females: p = 0.039; WR males: p = 0.032).

Estrous cycle had no effect on either distance travelled or dwell time.

Mass training to a new location - On the 6th day, all rats were trained over 16 trials to find the location of the submerged platform after it had been moved. One data point was missing for one trial for a LER female due to experimental error. Trials were averaged in consecutive groups of 4 trial blocks in order to simplify the analysis (Fig 6D). Therefore, over the course of 16 trials, there were 4 trial blocks (trial block 1 = trial 1-4; trial block 2 = 5-8, etc.).

All groups took significantly less time over the course of trials ($F_{(1.844, 51.623)}$ = 37.657, p < 0.001). LER rats took significantly less time than WR ($F_{(1, 28)}$ = 18.199, p < 0.001). There were no significant effects of sex, and only a trial block by strain by sex interaction was significant ($F_{(1.844, 51.623)}$ = 3.358, p = 0.046). Female rats decreased their latency to reach the platform over all trial blocks ($F_{(1.642, 22.992)}$ = 27.033, p < 0.001) but there was no significant difference between females of either strain. There was a trial block by strain interaction ($F_{(1.642, 22.992)}$ = 3.855, p = 0.043), which indicated the possibility of a difference in the learning curves between females of different strains. Males, like females, found the platform faster over the course of the training ($F_{(3, 42)}$ = 14.062, p < 0.001), and LER males were significantly faster than WR males in that respect ($F_{(1, 14)}$ = 18.372, p = 0.001).

Because of the difference between the strains overall, each individual trial block was examined for differences. During the first trial block, there were no main effects of strain or sex but there was a significant strain by sex interaction ($F_{(1,28)}$ = 6.403, p =

0.017). Within group comparisons revealed that LER males were significantly faster during the first four trials as compared to WR males (p = 0.016) and LER females (p = 0.044).

During the second and third trial blocks, the same patterns of behaviour were observed. LER overall were faster than WR to reach the platform (trial block 2: $F_{(1, 28)}$ = 10.487, p = 0.003; trial block 3: $F_{(1, 28)}$ = 14.622, p = 0.001), and no differences between the sexes were observed. Both LER females (trial block 2: p = 0.027; trial block 3: p = 0.021) and LER males (trial block 2: p = 0.044; trial block 3: p = 0.012) were faster than their WR counterparts. A similar pattern of behaviour emerged on the fourth trial block such that LER continued to be faster than WR overall ($F_{(1, 28)}$ = 12.742, p = 0.001), with no statistical differences between the sexes. However, only LER females proved to find the platform significantly sooner than WR females (p = 0.009), and the difference between the males of either strain did not persist to the fourth trial block. These differences indicated a large effect of strain such that LER outperformed WR on the mass training day. Further, a sex difference with the LER strain existed only during the initial training trials to the new platform location.

Estrous cycle did not affect latency for any of the time bins measured.

Visible platform training : Day 7 & 8 - All rats improved at finding the platform over the course of the two days of visible platform training ($F_{(1,28)}$ = 13.546, p = 0.001), and a day by trial ($F_{(2.468, 69.117)}$ = 5.935, p = 0.002) interaction was observed (Fig 6E). No significant main effect of strain, sex or trial was observed. Estrous cycle did not have an effect on either day.

DFCTC

Pre-exposure - All animals were allowed to freely explore either context in order to determine if any pre-existing preference was present as well as to aid in counterbalancing groups for the paired and unpaired context assignments. All groups spent the same amount of time in either context whether it was to-be paired or to-be unpaired, and no between group differences were observed for sex or strain (Fig 7A). Using estrous cycle as a covariate did not alter the results among females.

Test - Test days were used to assess whether rats had learned to associate the fearful stimuli (shock) with the features of the paired context. This was measured through freezing behaviour when placed in the paired context as compared to baseline freezing behaviour in the unpaired context. Overall, all animals froze more in the paired versus unpaired context ($F_{(1, 28)} = 8.412$, $p = 0.007$). Further, LER exhibited more freezing behaviour overall as compared to WR ($F_{(1, 28)} = 7.885$, $p = 0.009$), and females froze more overall than males ($F_{(1, 28)} = 4.318$, $p = 0.047$). No strain by sex interaction was observed.

Individual contrasts revealed that LER females froze more in the paired context than LER males ($p = 0.034$) and WR females ($p = 0.006$). However, only LER females demonstrated a differential freezing response between the two contexts ($p = 0.041$); all other groups showed non-discriminative freezing behaviour (Fig 7B).

Estrous cycle did not affect the results for females.

Preference - The preference test was used to measure whether rats could discriminate between the paired and unpaired context through active avoidance. Therefore, given a choice between both the paired and unpaired contexts, rats that had learned the shock-context association would actively avoid and spend less time in the paired context. Overall, all rats spent less time in the paired context as compared to the

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unpaired context ($F_{(1, 28)} = 15.363$, $p = 0.001$). No strain or sex differences were

observed, but there was a context by strain interaction ($F_{(1, 28)} = 4.963$, $p = 0.034$). The

significant context by strain interaction indicated that dwell time in either context likely

differed by strain, despite the non-significant strain difference. Indeed, individual

comparisons revealed that LER males spent significantly more time in the unpaired

context as compared to WR males ($p = 0.04$). Additional individual comparisons in each

context revealed no significant differences between groups. No other significant

interactions were observed.

Planned comparisons within each strain and sex group revealed that only LER

demonstrated active avoidance of the paired context and spent more time in the unpaired

context when given the choice (LER females: $p = 0.014$; LER males: $p = 0.004$); no such

pattern was observed in WR, regardless of sex (Fig 7C).

Estrous cycle did not alter any of the achieved results for preference day.

EPM

Two measures were used to assess anxiety behaviour in the EPM: number of arm

entries and dwell time. Rats who entered open arms less frequently as well as spent less

time in open arms were considered to be more anxious, as has been previously described

(Pellow, Chopin, File, & Briley, 1985). Dwell time was assessed as the percentage of

total time spent in the elevated plus maze. For arm entries, both the raw number of arm

entries as well as the ratio of open to closed arm entries was used. This ratio controls for

increased activity in the EPM, which has been reported to be affected by estrous state

(Morgan, Schulkin, & Pfaff, 2004). All of these values were considered as they generated

differential results.

Dwell time in open and closed arms - Dwell time in the open and closed arms relative to the total time was assessed for all rats. Overall, all rats spent significantly more time in the closed arm relative to the open arm ($F_{(1, 28)} = 276.622$, $p < 0.001$; Fig 8A). No significant differences were observed between the strains or the sexes. However, there was a significant interaction between the arm choice and strain ($F_{(1, 28)} = 4.746$, $p = 0.038$) indicating that different strains may have spent more or less time in the open or closed arm. However, analyses within the sexes between the strains revealed no significant strain effects. No other significant interactions were observed.

All groups spent significantly more time in the closed arm as compared to the open arm, regardless of strain or sex (LER females: $p < 0.001$; LER males: $p < 0.001$; WR females: $p < 0.001$; WR males: $p = 0.001$; Fig 8A). Therefore, no overall strain or sex differences were observed for dwell time, and no one strain or sex showed more anxiety-related behaviour than the other using this metric.

When examining only females using phase in the estrous cycle as a covariate, there was a significant interaction between the arm choice and estrous cycle, but no significant effect of strain. When examining group means, rats in estrous spent more time in the open arm in comparison to metestrous and proestrous rats, who were equal, and spent more time in the open arm than diestrous rats. In the closed arm, dwell time in descending order was: diestrous > proestrous > metestrous > estrous. From this, given that diestrous rats spent the least amount of time in the open arms and the most amount of time in the closed arms, it can be concluded that they exhibited the most anxiety.

Arm entries : Raw values - Overall, all rats entered the closed arms more than the open arms ($F_{(1, 28)} = 32.194$, $p < 0.001$; Fig 8B). There were significant strain and sex differences, such that WR entered the arms more than LER ($F_{(1, 28)} = 11.295$, $p = 0.002$),

and females entered the arms more than males ($F_{(1, 28)} = 5.069$, p = 0.032). No significant interaction between any of these values was observed. Post hoc comparisons revealed that WR entered both the open (p = 0.008) and closed arms (p = 0.007) significantly more than LER. Overall, females entered the closed arms more than males ($F_{(1, 28)} = 4.47$, p = 0.044), and no such difference was observed for the number of open arm entries. This could be accounted for by increased locomotor activity of females as compared to males and may not be due to sex differences in anxiety.

Specific comparisons in the open arm revealed that LER females were more anxious than WR females since WR females entered the open arms more frequently than LER females (p = 0.037). However, this same pattern was observed for the number of closed arm entries (p = 0.021), which may indicate that WR females were not less anxious than females. Rather, it is more likely that WR females displayed increased locomotor activity in comparison to LER females.

Within strain and sex comparisons revealed that LER females (p = 0.015), LER males (p = 0.028) and WR females (p = 0.005) entered the closed arms more often than the open arms (Fig 8B). This was not observed in WR males, which may indicate that WR males were less anxious than all other groups.

Since estrous cycle can affect locomotor activity, it was used as a covariate in an analysis for only female rats. Despite this, there was no effect of estrous cycle on number of open or closed arm entries.

Arm entries: Open relative to closed - The number of open arm entries relative to the number of closed arm entries was examined in order to account for any alterations in locomotor activity, which may have confounded results when examining only the raw values for number of arm entries. Overall, there was no effect of strain, sex or a strain by

sex interaction (Fig 8C). These results aligned with relative dwell time which indicated no overall group differences in anxiety. Similarly, estrous cycle did not affect the ratio of open to closed arm entries within females.

Therefore, any differences observed between groups in the EPM were not preserved when locomotor activity was accounted for. Given these results, no strain or sex differences were observed in anxiety, but estrous cycle did alter relative dwell time. Among females, low estrogen states were associated with increased anxiety-like behaviour.

Volumetric estimates

HP volume - A significant strain effect was observed such that WR had larger hippocampal volumes in comparison to LER overall ($F_{(1,49)} = 4.105$, p = 0.048; Fig 9A). There were no significant sex or strain by sex interactions, and comparisons within the sexes revealed no significant difference between LER and WR. No strain differences were found between females when you accounted for estrous cycle.

DG volume - There were no significant effects of strain, sex or strain by sex interactions (Fig 9B). When estrous cycle was used as a covariate for females, no strain differences were observed.

CA1 volume – Females had significantly larger CA1 volumes overall ($F_{(1,47)} = 5.36$, p = 0.025; Fig 9C), and there were no significant differences between the strains or a strain by sex interaction. Individual comparisons within the strains between the sexes revealed no significant differences. No strain differences between females were observed when estrous phase was accounted for.

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CA3 volume - There were no significant effects of strain, sex or strain by sex interactions (Fig 9D). Within females, no strain differences were observed when estrous cycle was used as a covariate.

OFC volume - There were no significant effects of strain, sex or a strain by sex interaction (Fig 9E). When estrous cycle was used as a covariate, however, there was a significant effect of strain within females, such that LER females had a significantly larger OFC volume in comparison to WR females ($F_{(1,18)}$ = 7.663, p = 0.013).

mPFC volume - There were no significant effects of strain, sex or strain by sex interactions (Fig 9F). When estrous cycle was used as a covariate, there was no significant effect of strain within females.

AMYG volume – There was a significant effect of strain such that WR had larger amygdalar volumes overall ($F_{(1, 46)}$ = 6.881, p = 0.012; Fig 9G). There were no significant effects of sex or a strain by sex interaction. Individual comparisons within strains revealed that WR females had larger amygdala volume in comparison to LER females (P = 0.011). However, this strain difference was not observed when estrous cycle was used as a covariate.

Discussion

Across multiple behavioural tasks and strikingly in some brain areas, differences within and interactions between strain and sex were discovered. Each of these effects will be discussed in turn and how this might relate to the consequences and interpretation of other research.

Effect of strain

Results from multiple tasks indicated significant strain differences. Both tasks

assessing learning and memory, the MWT and DFCTC, showed significant strain effects,

such that LER consistently outperformed WR. This was evident across acquisition,

retention and re-acquisition of a new spatial location in the MWT (Fig 6). Additionally,

LER outperformed WR during both test phases of DFCTC (Fig 7). WR did not show

discriminative freezing or active avoidance on either test day. Multiple factors could

contribute to these significant strain differences. One possibility is the ability of WR, an

albino strain, to use visual cues. Albinos across multiple species have altered optic tracts,

showing differential decussation at the optic chiasm (Guillery, 1974; Lund, 1965;

Steininger et al., 1993). Both the MWT and DFCTC rely on the use of visual cues, using

distal environmental cues (Morris, 1981; Sutherland & Dyck, 1984) and proximal

contextual cues (Antoniadis & McDonald, 1999), respectively. Therefore, a lack of visual

acuity observed in albinos due to differential neuroanatomy of the visual system could

have resulted in alterations in behaviour. Despite this neuroanatomical difference, this is

likely not the case, as the differential neuroanatomy of albinos only results in worse

visual acuity and not in blindness (Dyer & Swartzwelder, 1978; Mohn & Russell, 1983).

In addition to this, all rats were exposed to a visible platform in the MWT in order to

ensure that any alterations in swim latencies or path lengths were not due to motor or

visual impairments. Both LER and WR reached the visible platform in the same amount

of time (Fig 6E), therefore, despite poor visual acuity in WR, this did not disrupt

performance reliant on the use of visual cues. Furthermore, in the DFCTC task, multiple

cue modalities were used. The shape of the context, which could also be explored via

tactile exploration, and the odour of each context differed. Therefore, superior performance of LER is not likely explained by the poor visual acuity of WR.

Another possible mechanism behind these behavioural differences includes differential size of associated brain areas, specifically the hippocampus and amygdala, as the hippocampus is associated with spatial learning and the amygdala with conditioned fear responses. Hippocampal lesions will disrupt spatial learning and retention (Sutherland, Whishaw, & Kolb, 1983), and lesions to both amygdala and hippocampus will disrupt learning in the DFCTC task (Antoniadis & McDonald, 2000). However, contrary to our *a priori* hypotheses, WR overall had larger hippocampal and amygdalar volumes (Fig 9A&G). This demonstrates that larger size does not always confer heightened function. This has been observed with hippocampal volume and function across species (Roth, Brodin, Smulders, LaDage, & Pravosudov, 2010), demonstrating that larger volume is not always associated with enhanced ability. Indeed, volumetric differences do not take into account cell density, therefore, despite larger total volumes, it is possible that fewer cells are present. Additionally, earlier research has demonstrated strain-specific development and experience-based alterations of the hippocampus in LER and WR (Holahan et al., 2007; Holahan et al., 2006; Keeley et al., 2010), which may provide some insight into these behavioural differences. It is possible that despite a larger volume, WR do not show adequate connectivity that allows them to excel at spatial and associative learning tasks. Only additional research examining the cytoarchitectural differences in hippocampus and amygdala between these two rat strains could help elucidate these possibilities.

One method for eliminating this behavioural difference could include the addition of training trials for both MWT and DFCTC. Regardless of the mechanism behind the

behavioural difference, it is possible that additional training trials could help clarify the mechanism behind this strain difference in learning and memory tasks. For example, measuring activation patterns or the formation of place cells in these two rat strains may help determine exactly how these strains learn at different rates.

In addition to differences in learning and memory tasks, WR overall were more active than LER in the EPM (Fig 8B). This is contrary to previous research, which showed increased running wheel activity in LER as compared to WR (Bauer, 1990). However, running wheel activity is a metric of baseline activity, whereas EPM behaviour can monitor activity levels in response to a fear-eliciting situation, which in this study, included being exposed to a novel and anxiety-eliciting apparatus in a brightly lit room. Underlying the design of the EPM task is the assumption that a more anxious rat will exhibit more fearful behaviour, in this case freezing and remaining in the closed arm. WR and LER did not differ in their measures of dwell time in the open and closed arms (Fig 8A), therefore it is assumed that they did not differ in levels of anxiety. If you examine the number of arm entries, WR females entered both open and closed arms significantly more than their LER counterparts (Fig 8B). This may be indicative of increased anxiety, if this was the only measure considered. However, when you normalize the number of open arm entries relative to the number of closed arm entries, no significant differences emerged between groups (Fig 8C). When normalizing relative to closed arm entries, thus accommodating for increased locomotor activity, WR exhibit increased activity levels. The source of this difference is unknown, but could reflect differential levels of hyperactivity in response to a stressful situation

Despite differences in locomotor activity and learning and memory tasks, no strain differences in the SRT task were apparent (Fig 5). This was expected. Additionally,

visual acuity did not affect performance in the SRT, as it does not rely on a functional

visual system (Whishaw & Tomie, 1989). However, this does not discount differences in

the qualities of reaching, as strain differences in the implemented movements in SRT with

no changes in success rate have been observed (Whishaw, Gorny, Foroud, & Kleim,

2003). On the other hand, given the lack of strain differences in visible platform training

in the MWT (Fig 6E), it can be concluded that no gross differences in motor function

were observed between the strains. From the strain differences observed, it can be

concluded that WR represent an inappropriate animal model for experiments requiring

fast and effective learning and memory due to poor task performance across spatial and

contextual learning tasks, but they perform adequately in measures of motor learning.

Caution should be implemented when interpreting results achieved in WR in similar

behavioural tasks.

Effect of sex

The effects of sex on MWT performance were strain-specific, as only LER males and

females displayed differences in behaviour. LER males outperform their female

counterparts in late acquisition and during the mass training day (Fig 6D). No differences

were observed in retention during the probe (Fig 6B&C) nor were there any differences in

motor or visual performance when using the visible platform (Fig 6E). Sex differences in

spatial learning in the MWT have been reported previously (Jonasson, 2005) and are

mediated through interactions between spatial and non-spatial strategies (Blokland et al.,

2006; Korol & Kolo, 2002; Korol et al., 2004; Rodriguez et al., 2010), which are believed

to be due to the effects of female sex hormones on associated brain regions (Korol, 2004;

Luine et al., 2003; Vanhaaren et al., 1990; Zurkovsky, Brown, Boyd, Fell, & Korol,

2007). Females will preferentially adopt non-spatial strategies when at low estrogen

phases of their regularly fluctuating cycle. Here, in the MWT, the effects of estrous cycle

are partially compensated for, as each day, female rats have transitioned to another phase

of their cycle and perform the task. Typically, this simply introduces noise into the

system, and multiple training trials over multiple days, or even pretraining with a visible

platform can help overcome sex differences (Jonasson, 2005).

In addition to differences in behavioural strategies, sex differences in spatial learning

specifically in the MWT could potentially be mediated through differential stress

responses in females. When stressed, female subjects will have higher circulating

corticosterone (CORT; Dunn, Scheving, & Millet, 1972; Heinsbroek, Van Haaren,

Feenstra, Endert, & Van de Poll, 1991; Kitay, 1961), which can enhance, in the short term

(Roozendaal, 2002), and disrupt, in the long term (Bodnoff et al., 1995), spatial learning.

However, with a heightened CORT response, it is assumed that females would display

improved spatial learning and memory in the stressful MWT, which was not the case.

However, stressing females prior to MWT spatial memory testing can also enhance

spatial memory formation, whereas the opposite effect is observed in males (Conrad et

al., 2004).

Another finding contrary to our predictions demonstrates that females outperform

males overall in the DFCTC paradigm, and this was shown to be strain-specific (Fig

7B&C). Strain-specific sexual dimorphisms in both cued and contextual conditioning

have been observed previously (Pryce et al., 1999), although WR and LER have yet to be

directly compared. Females will show an enhanced startle reflex as compared to males

(Vanhaaren et al., 1990), as well as display estrogen-dependent expression of fear

responses (Markus & Zecevic, 1997). However, many of the studies examining the

effects of female sex hormones on fear-learning tend to implement training protocols throughout the course of one day, only showing the effects of differing hormone levels on the acquisition and expression of fear learning (for example, Toufexis, 2007). In our study, we trained and tested rats in the DFCTC apparatus over the course of 11 days, which would encapsulate at least one and a half full estrous cycles. Therefore, regardless of the effect of endogenous sex hormone levels, females would have had compensatory days wherein the levels of sex hormones would have been ideal for learning and some days that were not. In addition to the training paradigm, estrous cycle did not affect discriminative freezing or active avoidance, indicating that estrous cycle did not alter the expression of fear learning in this DFCTC paradigm.

Sex differences in contextual fear, like MWT, may be sensitive to the effects of stress hormones. As mentioned above, females and males differ in their baseline levels of CORT (Critchlow et al., 1963; Dunn et al., 1972; Griffin & Whitacre, 1991; Weinberg, Gunnar, Brett, Gonzalez, & Levine, 1982) as well as in response to stress (Dunn et al., 1972; Heinsbroek et al., 1991; Kitay, 1961). Although stress hormones, including CORT, were not recorded in the present experiment, it is possible that the altered levels of CORT between males and females, and the enhancing effects of CORT on learning and memory, could have resulted in sexually dimorphic learning in the DFCTC paradigm. This is the first study of its kind to evaluate males and females in this behavioural task, so only future research examining both sex and stress hormones in this behavioural task will help determine the mechanism behind this sex difference.

In addition to alterations in behaviours, the volume of the CA1 region (Fig 9C) and the amygdala (Fig 9G) showed a female-biased sexual dimorphism. Earlier research did not find this effect, as overall, males have been found to have larger CA1 volumes in

other species (Burger et al., 2013), which is dependent on the organizational effects of testosterone and estrogen (Isgor & Sengelaub, 1998a). Although no clear explanation as to the origin of this inconsistency, perhaps this is the result of breeding in house from rats received from Charles River, and we are viewing founder effects. This is not likely as we used 9 different parents over the course of this research design. Only further research will elucidate the mechanism behind this anomalous sexual dimorphism.

Females overall also demonstrate significantly larger amygdalar volumes, and this difference between males and females was only significant within WR (Fig 9G). This is contrary to what was expected, as WR overall were impaired in the DFCTC task (Fig 7B&C), which has been shown to be dependent on the amygdala (Antoniadis & McDonald, 2000). In addition to this, LER demonstrated a sexual dimorphism in task performance (Fig 7B&C), with the absence of a sexual dimorphism in associated brain area, the amygdala (Fig 9G). One possible factor that would contribute to this is the absence of delineating specific amygdalar subregions in our volumetric estimates. It is possible that careful inspection of amygdalar subregions would help elucidate the exact mechanism behind this behavioural difference.

One final difference that was observed between males and females includes the activity observed in the EPM (Fig 8B). This has been observed previously (Pryce et al., 1999), as well as has been shown, as it was here, to be dependent on estrous cycle (Marcondes et al., 2001).

Here, no behavioural differences were observed between males and females for the SRT task (Fig 5) or in the EPM for anxiety behaviour (Fig 8A&C). The achieved results for SRT were expected as this is a simple motor learning task, and this has been observed previously (Whishaw, 1992). However, sex differences in EPM have been observed

previously (Marcondes et al., 2001). In addition to no sex differences in behaviours, no sex differences were observed in the size of the hippocampus, the DG, CA3 region, the OFC or the mPFC (Fig 9A, B, D, E & F, respectively). Sex differences in the size of the hippocampus (Galea et al., 1999), DG (Galea & McEwen, 1999; Roof, 1993; Roof & Havens, 1992) and the CA3 subregion (Burger et al., 2013) have been reported previously in multiple species, although not all of the abovementioned experiments implemented stereological estimates for volumes. Non-biased stereology is a useful tool for the evaluation of the volume of specific brain areas of interest, despite the fact that it is not as commonly employed.

Implications for behavioural research

Here, it is apparent that despite the oft-made conclusion in neuroanatomy and behavioural neuroscience circles that "bigger is better," a larger volume of an associated brain area did not always confer a behavioural advantage (as discussed in, Aboitiz, 1996). Therefore, careful consideration to the conclusions drawn with the use of stereological estimates of brain volumes in conjunction with behavioural assessment should be considered. Clearly here, sometimes bigger was associated with improved performance but not always. Evaluation of cytoarchitectural differences in conjunction with volumetric estimates and estimates of cell density would help clarify the exact mechanisms behind improved performance.

One clear conclusion of this study is the obvious deficits of WR in learning and memory based tasks. Given these behavioural results, it is obvious that WR are not adept at learning spatial and contextual conditioning tasks. Care should be taken to interpreting

results achieved in previous studies using similar behavioural tasks in WR as they are judged to display poor learning aptitude.

Finally, this study highlights the importance of including males and females across all behavioural analyses. Although the inclusion of females can be problematic and more time and resource consuming, especially if estrous cycle is taken into consideration, this study demonstrates clearly that males and females learn spatial tasks at different rates as well as females more easily express fear-conditioned behaviours. Although these results were strain-specific, it occurred in a strain that was deemed more appropriate for learning- and memory-based behavioural analyses. Inclusion of females in all future behaviour and neuroscience research can only help elucidate which mechanisms or neurological correlates of function are sex-specific.

CHAPTER 2.3: STRAIN AND SEX DIFFERENCES IN CPP BEHAVIOUR AND BRAIN ACTIVATION IN RESPONSE TO D-AMPHETAMINE

Introduction

Addictive behaviour, as defined by the continued and pathological ingestion of one or multiple drugs of abuse (O'Brien, 2011), has well defined molecular (Nestler & Aghajanian, 1997) and neural circuits (Kauer & Malenka, 2007), which includes the specific contributions of various neurotransmitter systems and brain areas (Koob, 2006). Addiction is a multifaceted state, and its etiology includes contributions from genes and environment. For example, alcoholism, a subtype of addiction, has an estimated heritability of 50% (Enoch, 2006; Goldman, Oroszi, & Ducci, 2005). However, addiction in humans is not based solely on genetic risk and results from the interaction between multiple factors (Kreek, Nielsen, Butelman, & LaForge, 2005). One possible factor includes individual variability, which stems from multiple sources, including both genetic and environmental. Response to novelty (Klebaur, Bevins, Segar, & Bardo, 2001), other drugs of abuse (Harrod, Lacy, & Morgan, 2012), rearing environment (Schenk et al., 1986) and stress (Anisman & Cygan, 1975), among other factors, have all been shown to contribute to the development of addictive behaviour in animal models and can be partially mediated through heritability (Kreek et al., 2005). The interaction between environmental factors and genetic risk contribute to the development of an individual that is particularly sensitive to the addictive properties of drugs and the development of behavioural patterns of an addicted state.

Animal models can provide insight into the mechanisms and the contributions of multiple factors, including genetics and individual variability, to the development of addiction. One factor that has been identified to contribute to the development of

addiction is strain of rat used (George, Porrino, Ritz, & Goldberg, 1991). For example, the Lewis rat displays characteristic responses and ability to easily form context-pairings with multiple drugs of abuse (Kosten & Ambrosio, 2002), as well as greater response impulsivity (Hamilton et al., 2014) and anxiety (Wu & Wang, 2010) . Yet other rat strains have been identified as having intrinsic and experience-dependent differences. For example, rat strains differ on measures of learning (Andrews et al., 1995; Hort, Brozek, Komarek, Langmeier, & Mares, 2000; Mohn & Russell, 1983; Pare, 1996; van der Staay et al., 2009), multiple metrics of anxiety-related behaviour (Schmitt & Hiemke, 1998; van der Staay et al., 2009) and novelty seeking (Camp et al., 1994). In addition to this, rat strains differ in their response to drugs (Camp et al., 1994; Deiana et al., 2007; Fujimoto et al., 2007; Onaivi et al., 1992; Ortiz et al., 2004) and stress (Pare, 1989; Tohei et al., 2003; Woolfolk & Holtzman, 1995; Wu & Wang, 2010), both of which have been linked to the development of an addicted state. Although, to date, much work has focused on inbred rat strains, such as Lewis and Fischer 344 rats, there are multiple innate differences between outbred strains of rats, such as LER and WR. Although the use of inbred strains is thought to determine the genetic correlations of behavioural variation, the use of outbred strains can also help identify differences in the neural circuitry of addiction, as well as the specific contributions to individual variability. For example, differences observed in outbred strains, as determined by both genetics and experience-driven modulations, can help identify the interaction between genetics and experience. Although they lack the genetic homogeneity observed in inbred lines, outbred strains can be argued to closely resemble human populations with a greater degree of genetic heterogeneity between individuals. Additionally, both LER and WR are used extensively throughout animal research in addiction. Indeed, these two rat strains differ on multiple metrics,

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including developmental differences (Chap 2.1; Keeley, Trow, & McDonald, in submission), volumetric differences in multiple brain areas, and multiple measures of learning and memory and anxiety (Chap 2.2). Therefore, it was of interest to determine if these two rat strains differ in their responsiveness to drugs of abuse, as that has not yet been fully explored.

An additional factor that can both alter behaviour in a myriad of tasks as well as responsiveness to drugs of abuse is sex. Though many studies narrow their focus to only males, there is evidence that males and females can have differential responses to multiple drugs of abuse as well as will show altered sensitivity depending on the time frame of exposure (see Fattore, Altea, & Fratta, 2008, for review). In addition to differences between males and females, strain can interact with sex differences, such that differences between strain can be observed in one sex and not the other (for example, Pryce et al., 1999). This is best exemplified by LER males who benefit from juvenile pre-experience in the Morris water task, whereas LER female and WR of either sex do not (Keeley et al., 2010). Therefore, there is scientific justification to include not only two different rat strains to determine their initial responsiveness to drugs of abuse but also to determine if these differences are the same in either sex.

There are multiple behavioural tools that can be used to study addiction in animal models. For example, self-administration paradigms, in which a rodent learns to lever press to receive a dose of a particular substance, can be trained with most drugs of abuse (Schuster & Thompson, 1969). In addition to this paradigm, conditioned place preference has also been used, where rats learn to associate a particular context or environment with a substance of abuse and another context with no drug (Bardo & Bevins, 2000; Rossi & Reid, 1976; Van der Kooy, Mucha, O'Shaughnessy, & Bucenieks, 1982). Following a

training schedule, rats are then allowed to choose between either context. A rewarding drug will induce conditioned place preference such that rats will preferentially spend time in the context paired with the rewarding drug, in the absence of the drug presentation. Many drugs have been shown to have highly rewarding properties using this paradigm (Tzschentke, 2007), including d-amphetamine (AMPH). AMPH is a potent stimulant that has been shown to be highly rewarding to rodents, including rats (Esposito, Perry, & Kornetsky, 1980; Robbins, Watson, Gaskin, & Ennis, 1983). In addition to this, amphetamines are presently abused in the Canadian population (Health Canada, 2013), which necessitates discovering how individual differences may contribute to the development of an addiction to this particular drug of abuse.

With these premises in mind, the present study investigated the responsiveness of LER and WR males and females to two doses of amphetamines in order to establish a sub threshold dose in a conditioned place preference study to be used in experiments reported in a later chapter (Chap 3.3). Rats were ordered from Charles River or bred in house. Rats ordered from Charles River were tested using two different doses of amphetamines. Also, following the investigation, we discovered a possible effect of rearing environment, so the highest dose not inducing conditioned place preference was used to test whether there were significant effects of rearing environment in males and females of these two rat strains. Therefore, rats from Charles River were compared to rats bred in-house at the University of Lethbridge. No *a priori* assumptions were made regarding the outcome, as this study is the first of its kind to compare the interaction between strain and sex and its effect of amphetamine sensitivity using a conditioned place preference paradigm.

Methods

Subjects

Two different subject groups were used for this study. One was purchased from Charles River as adults and shipped to the University of Lethbridge (LER female: N = 16; LER male: N = 16; WR female: N = 16; WR male: N = 16). This group was used to determine a sub threshold dose of AMPH. The second group was bred in house using breeding pairs purchased from Charles River Laboratories (Laval, QC) and, using the sub threshold dose of AMPH established in the previous experiment, strain and sex differences were observed (LER female: N = 8; LER male: N = 8; WR female: N = 8; WR male: N = 8). Further, the effect of in-house breeding was examined *post hoc* due to recent experiments showing the differences between rats purchased and shipped versus rats bred in house.

Vaginal cytology and determination of estrous cycle

Vaginal cytology and determination of estrous cycle was done as previously described (Chap 2.2). Vaginal smears were collected during all behavioural testing days.

Conditioned place preference (CPP) to a sub threshold dose of AMPH: Apparatus & Training

Apparatus - A similar apparatus and procedure to that used for both appetitive conditioning (Keeley et al., 2014; Ralph et al., 2002) and discriminative fear-conditioning (Chap 2.2; Antoniadis & McDonald, 1999, 2000) to context tasks used in our lab was implemented for this procedure. Briefly, an opaque black triangular shaped box (61cm X

61cm X 30cm) and an opaque white square box (41cm X 41cm X 29cm) both with metal bars as the floor (0.32cm in diameter) spaced roughly 1.5cm apart were used as the two contexts. Both contexts had pill bottles inset into the walls containing cotton balls infused with a scent cue. The black triangle context was always paired with amyl acetate and the white square with eucalyptus. Both contexts differed in shape (triangle versus square), colour (black versus white) and odour (amyl acetate versus eucalyptus). Every day, the scent cues were reloaded with the appropriate scent. The contexts were connected with a grey alleyway (11cm X 11cm X 16.5cm) and could be separated via Plexiglas doors. Both contexts and the alleyway were placed upon a clear Plexiglas table. Underneath the table was a mirror inclined at a 45˚ angle that allowed for viewing by both an observer and a video camera (where noted). For all behavioural procedures, N = 8/strain and sex group.

Pre-exposure – On the pre-exposure day, all rats were acclimated to the testing apparatus. The doors of both contexts were opened, and rats were placed in the grey alleyway and allowed to freely explore either context for 10min. An observer recorded dwell time in each chamber. The presence of urine and the number of fecal boli were also recorded. Following the 10min pre-exposure, animals were removed and returned to a transport tub before being returned to their home cage. The contexts were cleaned using soap and water to remove any scent cues.

Following pre-exposure, animals were assigned to their paired, i.e. be injected with AMPH, in the black triangle or white square context in a quasi-random fashion. All groups were counterbalanced such that half of the animals in each group were given AMPH in the white context and half were given amphetamine in the black. The groups were further counterbalanced such that half started training in the context where they

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received AMPH (paired) while half started in the context where they received saline (unpaired).

Training – Following assignment to the groups, rats were given 6 consecutive training days, as per (Tzschentke, 2007) where they were give an injection of either saline or amphetamine and placed in the one of the contexts for 30min. Injection type alternated each day. All rats were weighed every day of training.

Preference test – Rats were assessed using the same procedure as the pre-exposure day such that they were allowed to freely explore both contexts connected by a grey alleyway for 10min. Dwell time in each context as well as the presence of urine and the number of fecal boli were recorded. The preference test was also recorded on video.

Drug dosages

Different drug dosages were used for each experiment. An initial experiment was conducted in order to determine a sub threshold dose of AMPH in experience-naïve rats. Two sets of rats were ordered from Charles River. One was exposed to a 0.5mg/kg dose of AMPH and run through the behavioural procedure. Another group of rats was exposed to a 0.7mg/kg dose of AMPH. These two doses of amphetamine were chosen as they were found to be lower than the dose of 1mg/kg of AMPH which has been shown to induce CPP behaviour (Tzschentke, 2007). Both doses used a 0.49mg/ml AMPH solution dissolved in saline.

In the first part of the experiment, given that there were no significant differences between dwell time in the paired and unpaired contexts for either dose, 0.7mg/kg was considered a sub threshold dose of AMPH, as 1mg/kg has been shown to induce CPP (Tzschentke, 2007). Rats were bred in-house at the University of Lethbridge and were

examined for strain and sex differences in the induction of conditioned place preference to a sub threshold dose of AMPH.

Perfusion & fixation

One week after the final day of CPP training, rats were injected with a single 1mg/kg dose of AMPH and allowed to sit for 1hr in the same room as CPP training and testing. This injection/delay procedure was used because it has been shown that AMPH is found in brain tissue within 5min following intraperitoneal (i.p.) injection in rats, and levels of AMPH remain stable for roughly 1hr (Kuhn & Schanberg, 1978). Furthermore, *cfos* protein is present in neurons that were active 20-30min after a particular experience (Dragunow & Faull, 1989). Therefore, it was assumed that any *cfos* protein signal detected 1hr after AMPH injection would represent the population of neurons active 30min after AMPH injection, while AMPH was still present and active in the brain. Therefore, rats were euthanized with a single i.p. injection of sodium pentobarbital (120mg/kg) and transcardially perfused with approximately 150mL of 1xPBS followed by 4% PFA in 1xPBS. Following decapitation, brains were removed from the skull and immersion fixed in 4% PFA in 1xPBS. PFA was replaced 24hr after the perfusion with 30% sucrose and 0.2% Na azide in 1xPBS until sectioning. Brains were sectioned in a series of 12 at 40µm using a cryostat (CM1900, Leica, Germany) and placed directly into Eppendorf tubes containing 0.2% Na azide in 1xPBS until immunohistochemical staining.

Cfos immunohistochemistry & quantification

The amount of *cfos* protein was stained as previously described (Blum, Lamont, Rodrigues, & Abizaid, 2012). Briefly, free-floating tissue was washed in 1xPBS. This

was followed by a 30min quenching step in 0.3% H_2O_2 in 1xPBS to remove any endogenous peroxidises. After washing in 1xPBS, the tissue was blocked in 1.5% goat serum in 0.3% triton-X 1xPBS for 30min. Following this step, the tissue was incubated in 1˚ antibody (Santa Cruz, California) at a concentration of 1:1000 in 0.33% triton-X in 1xPBS with 1.5% goat serum for 24hr. The following day, the tissue was washed, which was followed by a 24hr incubation in 2˚ antibody (1:1000, anti-rabbit; Vector Labs, Canada). On the third day, tissue was washed then placed in AB Complex (Vector labs, Canada) for 45min. Tissue was washed then bathed for 5min in a 0.5% 3,3'-diaminobenzidine (DAB) solution in 1xPBS with $NiCl_2$-$6H_2O$ in order to turn the solution purple and 0.05% H_2O_2. Sections were washed then mounted on 1% gelatin coated slides and let to dry for 24hr. Slides were dehydrated and coverslipped with Permount.

Representative images from nucleus accumbens and dorsal hippocampus were taken and quantified using particle analysis in Image J (NIH, US). Regions of interest were traced in consultation with (Paxinos & Watson, 2007), and particles were counted per unit area of the region of interest. Example regions of interest can be found in Appendix 4.

Statistical Analysis

For all statistical analyses of CPP behaviour, repeated measures ANOVA was conducted for dwell time in either context with strain and sex as between subjects factors. Since we were interested in whether a preference for one context over another had occurred, *a priori* comparisons were conducted within each strain and sex group comparing dwell time in each context. When examining for the effects of the rearing environment, cohort was used as a between-subjects factors. For *cfos* quantification,

strain and sex were used as between-subjects factors. Estrous cycle phase was considered as a covariate for all analyses, but it did not significantly alter any statistical analyses, so it will not be discussed.

Results

Determination of a sub threshold dose of AMPH

0.5mg/kg AMPH - The first attempted dose was 0.5mg/kg of AMPH for all strain and sex groups ordered from Charles River. There was no initial preference of one context over the other during the pre-exposure trials, or any effect of strain, sex or any interaction effect. *A priori* comparisons within groups for an effect of context revealed no significant difference between groups (Fig 10A). During the training period, rats were monitored for weight gain. All groups gained weight over the course of training ($F_{(2.416, 67.655)}$ = 16.809, p < 0.001), and males weighed significantly more than females, regardless of strain ($F_{(1, 28)}$ = 815.972, p < 0.001; data not shown). There was a significant strain by sex interaction which was explained by LER males initially weighing more than WR males (p = 0.026; data not shown). However, this difference was only present on the first day of training. Following training, there was no preference of the paired context in terms of dwell time nor was there any effect of strain, sex or any interaction effects (Fig 10B). Therefore, it was concluded that 0.5mg/kg of AMPH did not induce CPP in male and female WR and LER purchased from Charles River.

0.7mg/kg AMPH - The next attempted dose was 0.7mg/kg of AMPH for all strain and sex groups ordered from Charles River. There was no initial preference for the paired or unpaired context in terms of dwell time on the pre-exposure day of training (Fig 11A).

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Neither was there any effect of strain, sex or any interaction. *A priori* comparisons within strain and sex groups revealed no significant difference between dwell-time in the paired or unpaired contexts. During training, females always weighed less than males ($F_{(1, 28)}$ = 329.921, p < 0.001), and all rats gained weight as the trials progress ($F_{(5, 140)}$ = 2.761, p = 0.021; data not shown). There was no effect of strain or any other interaction observed on weight gain during the training period. On the preference test, all strain and sex groups spent equal amounts of time in the paired and unpaired contexts (Fig 11B). There was no significant effect of strain or sex or any interaction. Therefore, 0.7mg/kg did not induce CPP and was used as a sub threshold dose of AMPH for rats bred in house (discussed below) and in a later experiment (Chap 3.3).

CPP to a sub threshold dose of AMPH

For this experiment, rats bred in house at the CCBN, and a dose of 0.7mg/kg of AMPH was used.

Pre-exposure - All animals were allowed to freely explore both contexts in order to determine if there was a pre-established preference to either context as well as aid in counterbalancing across groups for the paired and unpaired contexts. No differences were observed between the strains or sexes for time spent in either context nor were there any differences between time spent in the paired or unpaired contexts (Fig 12A).

Weight during training period - During the training period, all animals were weighed to determine appropriate volumes of AMPH and saline. Weights during this injection period showed consistent strain and sex differences over all days such that WR consistently weighed more than LER ($F_{(1, 28)}$ = 10.025, p = 0.004; data not shown) and males consistently weighed more than females ($F_{(1, 28)}$ = 194.424, p < 0.001; data not

shown). No effect of day or any interaction effect on weight was observed. Individual

comparisons between groups showed that over all days, LER females weighed

significantly less than LER males ($p < 0.001$) and WR females ($p = 0.001$). WR males

weighed significantly more than both LER males ($p = 0.033$) and WR females ($p <$

0.001). No effects of day were observed for any of these comparisons nor were any

interaction effects observed.

Preference - This test day determined whether there was a preference to a context

previously associated with a sub threshold dose of AMPH. Overall, all rats spent equal

amounts of time in the paired and unpaired contexts, showing no preference. However,

despite a lack of significant effects of strain or sex on dwell time, there was a significant

strain by sex interaction ($F_{(1, 28)} = 4.568$, $p = 0.041$; Fig 12B). This indicated that

different strain and sex groups were spending differential time in either context. No other

interaction effects were observed. Individual contexts revealed that regardless of strain or

sex, all groups spent the same amount of time in either the paired or unpaired contexts.

In light of our *a priori* assumptions, the dwell time in the paired versus unpaired

context was tested in each strain and sex group. Only LER females showed a preference

for the paired context over the unpaired context ($p = 0.04$). All other groups had no such

preference, as demonstrated by no difference between dwell times in either context.

Therefore, for LER females reared at the CCBN, 0.7mg/kg of AMPH was a sufficient

dose to produce CPP.

Effect of rearing environment

To ensure that rearing environment did not affect the behavioural response to

AMPH, a separate analysis using rearing environment (Charles River versus University of

Lethbridge, CCBN) as a factor was conducted including all rats that received the 0.7mg/kg dose of amphetamine.   For pre-exposure, no differences were observed between cohorts, strain, sex or any interaction. There was no difference between dwell time in the paired and unpaired contexts for any group (Fig 13A). However, there was a significant effect of cohort on the preference test day ($F_{(1, 56)}$ = 12.193, p = 0.001; Fig 13B). Further, there was a strain by sex interaction ($F_{(1, 56)}$ = 7.617, p = 0.008). There was also an overall effect of context such that rats overall spent more time in the paired in compared to the unpaired context ($F_{(1, 56)}$ = 7.105, p = 0.01). Comparisons between groups within the paired or unpaired contexts revealed no effect of strain, sex, cohort or any interaction. However, individual comparisons within strain and sex groups revealed that only in LER males was there a significant effect of cohort (p = 0.037), such that LER males raised in the CCBN showed differential dwell time versus the equal dwell time in either context seen in LER males from Charles River. No effect of context or a context by cohort interaction was observed. In LER females, however, when the data was pooled across rearing environments, dwell time in the paired context was significantly longer than in the unpaired context (p = 0.015). Therefore, once you doubled the number of rats used with LER females, the 0.7mg/kg dose was no longer a sub threshold dose of AMPH.

Cfos quantification

The amount of *cfos* protein found in rats raised at the CCBN and exposed to 0.7mg/kg dose of AMPH for CPP from representative images of the nucleus accumbens and the dorsal hippocampus following a single dose of 1mg/kg of AMPH prior to euthanasia was quantified. No effect of strain, sex or any interaction for strain and sex was observed on number of cfos-positive particles per area in the nucleus accumbens (Fig

14A). However, a near significant ($F_{(1,22)} = 4.074$, p = 0.056) effect of strain was observed on cfos-positive particles per area in dorsal hippocampus. No effect of sex or a strain by sex interaction was observed. Therefore, individual comparisons within each sex were conducted, and it was observed that WR females had significantly more *cfos*-positive staining in the dorsal hippocampus as compared to LER females (p = 0.034; Fig 14B). No such difference was observed within males.

Discussion

These results are the first to identify differential responses between LER and WR to two sub threshold doses of AMPH that are dependent on rearing environment. For all groups, 0.5mg/kg of AMPH was not sufficient to induce CPP (Fig 10B). However, 0.7mg/kg of AMPH induced CPP in LER females bred at the CCBN (Fig 12B). This effect was almost significant in the rats obtained from Charles River, and when these two groups were pooled, overall, LER females regardless of rearing environment showed CPP to 0.7mg/kg of AMPH (Fig 13B). However, despite this difference between rearing environments, the effect of rearing environment was statistically significant only for LER males, indicating that only LER males were significantly affected by their rearing environment. WR were immune to such differences and never showed CPP to either dose of AMPH (Fig 10-13). However, female-specific activation in the dorsal hippocampus occurred with WR females such that WR females had more *cfos* activation in a representative image of dorsal hippocampus as compared to LER females (Fig 14B). In short, LER and WR had different thresholds not only for the response to AMPH but also showed strain-dependent rearing effects on AMPH sensitivity.

Strain differences

Strain differences in response to other drugs of abuse have been reported previously (Chen et al., 1991; Deiana et al., 2007; Horan, Smith, Gardner, Lepore, & Ashby, 1997; Onaivi et al., 1992; Ortiz et al., 2004; Woolfolk & Holtzman, 1995), including AMPH (Anisman & Cygan, 1975; Camp et al., 1994; Fujimoto et al., 2007; George et al., 1991). However, to date, much of the focus for studying these differences have been on the difference between the Lewis and Fisher 344 inbred strains of rats (for example, Coria et al., 2014; Hamilton et al., 2014; Kosten & Ambrosio, 2002). Lewis rats are more sensitive to most drugs of abuse, but the exact mechanism behind this difference has been only briefly explored (Camp et al., 1994; Coria et al., 2014; Hamilton et al., 2014; Horan et al., 1997; Kosten & Ambrosio, 2002). In short, the interplay between the hypothalamic-pituitary-adrenal (HPA) axis and monoaminergic function may be responsible for the differential response between Lewis and Fisher 344 (Kosten & Ambrosio, 2002; Wu & Wang, 2010). It is possible that differences in these metrics may also occur in LER and WR, however we have no direct measure of these systems. We do have a proxy measure however, given the differential responding of cells in the dorsal hippocampus of LER and WR females to a higher dose of AMPH (Fig 14B). Differential responses in one sex and not the other across strains are not uncommon (for example, Stohr, Schulte Wermeling, Weiner, & Feldon, 1998), however, most studies examining strain differences to drugs of abuse typically only use males (as discussed in Kosten & Ambrosio, 2002). Regardless, the difference in the activation of cells in the dorsal hippocampus is a potential indicator of differential firing in response to a dose of AMPH. Only further research examining, in detail, differential responses of these two strains to AMPH and other drugs of abuse will help elucidate the mechanism behind this difference.

Indeed, it may also help determine why LER but not WR females demonstrated CPP to the 0.7mg/kg dose of AMPH (Fig 12B & 13B).

Another possible mechanism of action behind strain differences heralds to early research in drug abuse. This work examined how responding to novelty could predict behavioural responses to other drugs, such as AMPH. Rats placed in a novel environment were classified as either high or low responders (HR and LR, respectively; Piazza, Deminiere, Le Moal, & Simon, 1989). HR rats more readily self-administer many substances of abuse, including AMPH, whereas LR rats are more resilient to these effects (Piazza et al., 1989). Given the behavioural results observed in the earlier chapter, that demonstrated that WR females were more active in the EPM (Fig 8B; Chap 2.2), we would expect that WR females would be more responsive to AMPH. This was not what was observed. Kosten and Ambrosio (2002) have suggested that the sensitivity to drug of abuse may lie on an inverted U distribution, where responsiveness to drugs of abuse is dependent on HPA axis activity, where both low and high HPA axis activity can be protective against the development of an addictive state. Indeed, LER have higher baseline CORT in comparison to WR (Tannahill, Dow, Fairhall, Robinson, & Fink, 1988), which may explain their sensitivity to AMPH as compared to WR. Only future research examining baseline CORT, CORT in response to stress and CORT in response to AMPH will help to elucidate the interplay between the HPA axis and AMPH responding in these two strains to determine if difference in HPA axis function determines their differential response to the same dose of AMPH.

One major caveat, however, may explain the lack of ability of WR females to display CPP with a dose of 0.7mg/kg AMPH. This could be a result of a lowered aptitude of WR females to learn this associative contextual task. As seen in Chap 2.2, WR, regardless of

sex, are impaired in their ability to associate a context with a foot shock (Fig 7).

Therefore, it may be that WR required more training trials in order to properly associate

the drug reward with its corresponding context. This is likely not the case, as previous

research has shown significant differences as well as large effect sizes with the trial

duration and the number of training trials used here for WR for CPP with higher doses of

AMPH (Bardo, Rowlett, & Harris, 1995; Spyraki, Fibiger, & Phillips, 1982).

Additionally, this difference is not likely due to differential responses to AMPH as WR

did not form a conditioned place aversion, as has been seen with other rat strains in

response to other drugs of abuse (Horan et al., 1997).

Another observed strain difference included differential weight dependent on the

origin of the rat. Of the rats purchased from Charles River, LER males were always

larger than WR males, whereas the opposite was true from rats bred at the CCBN. LER

from the CCBN weighed less than LER rats born at Charles River, and WR males from

born at the CCBN weighed more than WR originating from Charles River. However,

given this strain difference in weight was only found in males, it likely does not indicate a

possible mechanism behind the observed strain differences in response to the 0.7mg/kg

dose of AMPH observed in females.

The final observed strain difference was that the only group to show statistically

significant effects of rearing environment on CPP behaviour were LER males (Fig 13B),

as LER males reared at the CCBN showed an approaching significant place preference

(Fig 12B), whereas those raised at Charles River did not (Fig 11B). This identifies this

strain and this sex as particularly vulnerable to rearing effects, something that has not

been reported previously.

Sex differences

Sex differences were observed only within LER, such that only females displayed CPP in response to the highest dose of AMPH used, 0.7mg/kg (Fig 12B & 13B). This strain-dependent sex difference is not the first observation of a sex difference in response to AMPH, as this has been seen in Fisher 344 but not Lewis rats (Kosten & Ambrosio, 2002). However, this effect has never been shown in LER. There is a tendency for females to be more sensitive to drugs of abuse, such as amphetamines (Beatty & Holzer, 1978; Klebaur et al., 2001; Kosten & Ambrosio, 2002; Tseng & Craft, 2001). Part of this effect is thought to be mediated through the endogenous hormonal rhythms in females, where high estrogen phases are associated with more pronounced locomotor responses to acute administration of AMPH as compared to low estrogen (Becker, 1990; Becker & Beer, 1986; Peris, Decambre, Coleman-Hardee, & Simpkins, 1991). Additionally, AMPH-stimulated catecholamine release is found to be both sex- and hormone-dependent *in vitro* (Compton & Johnson, 1989) and *in vivo* (Savageau & Beatty, 1981). Here, the training days covered the extent of at least one and a half estrous cycles as well as there was no significant effect of cyclicity. The lack of effect of cyclicity may be simply a reflection of the small numbers of individuals in each phase of the estrous cycle, as rats were not cycle synchronized. Further research with cycle-synchronized rats should be able to determine if estrous cycle would significantly alter this course of behavioural training over multiple estrous cycles. More likely, estrous cycle simply introduced more variability into the data set. Therefore, the observed sex difference is likely mediated through organizational effects of sex hormones, with potentially a small influence of activational effects of estrous cycle. Only additional research will be able to address this issue.

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Conclusions

This research is the first to identify differential sensitivity to AMPH between LER and WR in a CPP task, as well as amplifies the conclusions of earlier research identifying sex differences exclusive to some rat strains. This project has salient implications for the study of the neurobiological correlates of addiction in relation to AMPH-sensitivity, as this study clearly shows that rearing environment, in the form of strain or location, and sex can alter AMPH-sensitivity. LER rats, in comparison to WR, were more sensitive to AMPH as where they were raised (in house or Charles River) and their sex altered AMPH sensitivity, whereas neither sex nor rearing environment altered AMPH sensitivity in WR. This may further identify WR as less than optimal research subjects to use in associative learning tasks and LER as more appropriate, in addition to their heightened sensitivity to AMPH. Only additional research examining differences in catecholamine release between these two strains as well as differential activation in reward circuitry will help answer these questions. Therefore, care must be taken when interpreting studies across these rat strains specifically in the realm of AMPH-based studies, addiction research in general, as well as associative learning research. This research has potential implications for the study of addiction and sensitivity to drugs of abuse in humans because it identifies factors specific to individuals that contribute to drug sensitivity. It can help with the identification of at-risk individuals. Differences between LER and WR in terms of neurobiology may help identify what makes one individual more sensitive to the effects of drugs of abuse as compared to another. Given that humans have varied genetic background, and heredity is not the sole determinant of risk of addiction, it is important to determine other risk factors, such as sex or early-life experience that may increase the

likelihood of developing an addiction. In many cases of addiction, relapse is high, therefore it is of societal interest to develop early screening, whether behaviourally or genetically, in order to help prevent the development of an addicted individual. The cost of addiction to society is high, although it varies by drug type (Nutt, King, Saulsbury, & Blakemore, 2007). Here, we identified some genetic and environmental backgrounds as having a higher risk and/or sensitivity, which in this case, included LER and females. This study is a stepping-stone along the path for understanding the contributing factors for the development of an addictive state.

CHAPTER 3 – EFFECTS OF THC WITHIN STRAIN AND SEX GROUPS

CHAPTER 3.1: ACUTE EFFECTS OF THC ADMINISTRATION ON BRAIN AND HISTOLOGICAL MARKERS

Introduction

Marijuana is an abundantly and commonly used drug among a variety of age groups, including adolescents (Health Canada, 2013). The main psychoactive component of marijuana is Δ^9-tetrahydrocannabinol (THC; Mechoulam, 1970), which binds to endogenous cannabinoid receptors, one of which is the most highly expressed G-protein coupled receptor in the mammalian brain (Elphick & Egertova, 2001). Endogenously, cannabinoids are synthesized on demand at the synaptic cleft, resulting in inhibition of neurotransmission (Berry & Mechoulam, 2002; Kano et al., 2009; Piomelli, 2003; Wilson & Nicoll, 2002). They are present at both excitatory and inhibitory synapses (Piomelli, 2003), with concentrations varying according to the brain area in which they are expressed (Marsicano & Lutz, 2006; Rey, Purrio, Viveros, & Lutz, 2012). Cannabinoid signalling is crucial for the regulation of seizure propagation (Monory et al., 2006) and learning and memory (Hampson & Deadwyler, 1998; Lichtman, Dimen, & Martin, 1995; O'Shea, Singh, McGregor, & Mallet, 2004; Rubino, Realini, Braida, Guidi, et al., 2009; Yim, Hong, Ejaredar, McKenna, & McDonald, 2008), and cannabinoid receptors are highly expressed in the hippocampus, the amygdala and cortical areas, all of which have roles in spatial learning and memory, fear learning and anxiety, executive functions, and the regulation of emotional and cognitive control (Mackie, 2005; Mailleux & Vanderhaeghen, 1992b; Marsicano & Lutz, 2006; Yim et al., 2008). Importantly, these areas all undergo critical periods of development and maturation during the adolescent

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period (Casey et al., 2008), making them susceptible targets for disruption following puberty.

The distinction between puberty and adolescence is more than semantic. Puberty is defined as a period of hormonal surges, whose onset is signalled by gonadotropin-releasing hormone (Harris & Levine, 2003; Ojeda et al., 2003), which results in the sexual maturation of reproductive organs as well as the brain (Sisk & Foster, 2004). The adolescent period is defined as the period post-puberty wherein brain, behaviour and physiology are shaped to their adult state (Sisk & Foster, 2004). The post-pubescent or adolescent period is considered an additional period of brain development, like certain prenatal and perinatal periods (Ebling & Cronin, 2000; Sisk & Foster, 2004). Following puberty onset, in addition to sexual maturation of reproductive organs, extensive synaptic modifications occur, which have long-term consequences for specific forms of learning and memory as well as social behaviour (Schulz et al., 2009; Schulz & Sisk, 2006; Sisk et al., 2003; Sisk & Zehr, 2005). Adolescent rats and humans are predisposed to seeking novelty, and novelty seeking in adolescence is partly a result of differential maturation of prefrontal and limbic areas, such that limbic areas develop to adult signalling levels earlier in development (during adolescence) before prefrontal regions complete development (in adulthood; Casey et al., 2008). Enhanced novelty seeking could put adolescents at particular risk for engaging in drug use. In support of this, in Canada alone, roughly 40% of individuals report marijuana use, with the average age of onset occurring within the teenage years (Health Canada, 2013). This statistic highlights a particular risk for adolescents to engage in the consumption of this specific drug of abuse. Given the highly plastic nature of the brain, and ultimately behaviour, during the post-pubescent period and the concordant high levels of marijuana use among adolescents, it is

important to understand the short- and long-term consequences of marijuana use on both the brain and behaviour.

Females may be at particular risk to behavioural consequences on marijuana use. In a longitudinal study, women were five times more likely to display anxiety and depressive symptoms in their early twenties following adolescent marijuana use (Patton et al., 2002). In addition to this, there is evidence in rodent models that females preferentially metabolize THC into a psychoactive component (Narimatsu et al., 1992; Narimatsu et al., 1991) and may be more sensitive to the long-term consequences of adolescent THC exposure (Rubino, Realini, Braida, Alberio, et al., 2009; Rubino, Vigano, et al., 2008). Sexually dimorphic responses to experience are not a novel phenomenon, as this has been observed previously in response to sex hormones (Mitsushima, Takase, Funabashi, & Kimura, 2009; Mitsushima, Takase, Takahashi, et al., 2009; Roof & Havens, 1992; Stewart & Kolb, 1994; Vanhaaren et al., 1990; Williams et al., 1990) as well as experience (Conrad et al., 2004; Keeley et al., 2010; Roof, Zhang, Glasier, & Stein, 1993).

Not only does sex alter the response to experience, but in animal models, rat strain also plays a significant role. This has been observed in multiple metrics, including early-life experience (Holahan et al., 2007; Holahan et al., 2006; Keeley et al., 2010). Our previous study (Chap 2.1; Keeley et al., in submission) examined the development of two rat strains following the post-pubescent period. LER and WR rats differed in the age of puberty onset as well as demonstrated differences in metrics of physiological development following the start of puberty. For example, female LER gained weight at different rates during the post-pubertal period. In addition to this, strain-specific sexual dimorphisms were observed in hippocampal areas, such that only WR showed sexually

dimorphic dentate gyrus and CA1 subregion volumes, with a male bias. Given the differential developmental time course of these two rat strains, it is possible that experience, such as adolescent drug exposure, would shape the brain and behaviour of males and females differently, both in the short- and long-term. Results of this nature would clarify any discrepancies found in the literature and refine any future research to include multiple strains in analyses, to examine how robust effects are, and/or to distinguish which experiences will have strain-dependent effects.

To this end, this study examined the physiological development of WR and LER rats exposed to a daily dose of THC following puberty onset for 2 weeks. Rats were either handled or injected daily with THC or vehicle. Following this 14-day period, rats were euthanized and the effects of THC on volumetric measurements in the AMYG, OFC, mPFC, HP and its subregions (DG, CA1 and CA3) were examined. All comparisons were maintained within a strain and sex group given the differential developmental time course of males and females of these two rat strains.

Methods

Subjects

All procedures were conducted as described in Chap 2.1. Female and male LER and WR rats were obtained from Charles River (Semmeville, QC; N = 9/group). Rats were allowed to acclimate to the University of Lethbridge animal housing rooms for approximately one week. Rats were paired and allowed to breed. Approximately one day before parturition, females and males were separated. Litters were culled to approximately 12 per litter (6 female and 6 male). All pups were weaned at postnatal day

21 (PND21) and placed into sex-matched pairs or triplets. All rats were housed in standard laboratory conditions (21˚C and 35% relative humidity, 12D:12L) Plexiglas tubs (46cm x 25cm x 20cm) with *ad libitum* access to food and water unless otherwise indicated. All rat handling and procedures were done in accordance to the University of Lethbridge's Animal Welfare Committee and the Canadian Council on Animal Care guidelines.

Puberty Onset & Drug Administration

Puberty onset was determined, as previously described (Chap 2.1), and all injections began following the determination of puberty onset. Females and males began monitoring for puberty onset at PND28. Females were examined for vaginal opening while males were examined for preputial separation.

On weaning day, rats were randomly assigned to their experimental groups: handled control (CON), vehicle (VEH) or 5mg/kg THC (THC). Rats were assigned to experimental groups such that a maximum of two cagemates were in the same cage when in triplets. For the most part, in a cage of 3, each rat was in a different experimental group. However, an exception was made for the THC, such that only one rat per cage was exposed to THC. All injections were conducted during the last third of the dark cycle. On the day of determination of puberty onset, rats were removed from their cages, placed in a light-blocking transport tub and brought to an injection room that was lit with a red incandescent bulb. All rats were weighed before treatment. CON rats were handled for approximately 2min. VEH rats were given an intraperitoneal (i.p.) injection of vehicle (1:1:18 ethanol:cremaphor:saline). THC rats were given an i.p. injection of 5mg/kg Δ^9-tetrahydrocannabinol (THC; 1:1:18 ethanol:cremaphor:saline; Fisher Scientific, USA).

Following injections, females were assessed for vaginal cytology using the lavage technique (Goldman et al., 2007; Marcondes et al., 2002). Following injection, rats were returned to their home cages. All rats received treatment for 14 consecutive days following determination of puberty onset. For VEH and THC rats, injection site varied daily in order to eliminate any damage or irritation due to multiple injections at the same cite. For a pictorial representation of the injection sites, see Appendix 1.

Determination of Estrous Cycle & Vaginal Cytology

Vaginal smears were taken on every injection day. Female estrous cycle was determined using the lavage technique (Goldman et al., 2007; Marcondes et al., 2002) as previously described (Chap 2.2). Estrous cycle determination was conducted using brightfield microscopy on a Zeiss Axio Imager MT (Carl Zeiss, MicroImaging GmBH, Germany) using the 20X objective as described (Chap 2.2). Characteristic images of these differences phases can be found in Appendix 2.

Histology

Perfusion and Fixation - Following the last handling day, a subset of CON rats (LER female: N = 6, LER male: N = 5, WR female: N = 6, WR male: N = 6) VEH rats (LER female: N = 5, LER male: N = 7, WR female: N = 7, WR male: N = 7) and THC rats (LER female: N = 8, LER male: N = 5, WR female: N = 5, WR male: N = 5) were euthanized with a single i.p. injection of sodium pentobarbital (120mg/kg i.p.) and transcardially perfused with approximately 150mL of 1xPBS. The remaining rats were used for additional experiments in which we evaluated the effects of these post-pubertal manipulations on adult brain and behaviour (Chap 3.2 & 3.3). One WR male was not

included in the study because of improper perfusion. Following decapitation, brains were removed from the skull. The left hemisphere was immersion fixed in another solution used for another set of experiments, and the right hemisphere was immersion fixed in 4% PFA in 1xPBS. Right hemisphere PFA was replaced the day following perfusion with 30% sucrose and 0.2% Na azide in 1xPBS until sectioning. Right hemispheres were sectioned in a series of 12 at 40μm using a cryostat (CM1900, Leica, Germany) and placed directly into Eppendorf tubes containing 0.2% Na azide in 1xPBS and stored at 4˚C.

Cresyl violet staining – Sections were float-mounted in 1xPBS onto 1% gelatin 0.2% chrom alum slides (VWR Canada) and allowed to dry overnight. Sections were rehydrated and placed in a 1% cresyl violet solution in dH_2O for 10min. Slides were then rinsed in dH_2O followed by 70% ethanol. Sections were placed in differentiator for 2min and were dehydrated and coverslipped with Permount.

Volumetric measurements

Volumetric estimates were conducted as previously described (Chap 2.1). Mean volume and maximum coefficient of error for each area of interest can be found in Table 4.

Statistical Analysis

All statistical analyses were conducted using SPSS software package (ver21, IBM). All comparisons were done within strain and sex groups to examine the effects of THC. Group (CON, VEH or THC) was considered as a between subjects factor. Weight gain during the pubertal period was examined as a within subjects design with handling

day as a repeated measure. Length of estrous cycle was examined for all females during the injection period. No a priori hypotheses were used to guide statistical analyses, so all group comparisons were conducted post hoc only when main effects were detected using Bonferroni corrections.

Results

Weight gain during injection period

Rat weight was recorded throughout the duration of the injection period. It was normalized relative to the rat's weight on the first injection day. All rats regardless of strain and sex gained weight over the course of the injection period. For all groups, there was a significant effect of group (LER females: $F_{(2, 82)} = 10.334$, $p < 0.001$; LER males: $F_{(2, 79)} = 28.159$, $p < 0.001$; WR females: $F_{(2, 79)} = 10.493$, $p < 0.001$; WR males: $F_{(2, 80)} = 31.312$, $p < 0.001$; Fig 15). All strain and sex groups showed a significant group by day interaction, indicating that rats were gaining weight at different rates over the 14 day injection period (LER females: $F_{(5.47, 224.276)} = 4.249$, $p = 0.001$; LER males: $F_{(4.439, 175.36)} = 7.267$, $p < 0.001$; WR females: $F_{(5.317, 210.018)} = 4.233$, $p = 0.001$; WR males: $F_{(4.119, 164.772)} = 21.345$, $p < 0.001$).

Individual comparisons revealed that all groups showed the same effects. Relative to controls and vehicles, THC exposed rats, regardless of strain and sex group showed lowered weight gain over all days. This was observed in LER females (CONT vs. THC: $p = 0.001$; VEH vs. THC: $p < 0.001$; Fig 15A), LER males (CON vs. THC: $p < 0.001$; VEH vs. THC: $p < 0.001$; Fig 15B), WR females (CON vs. THC: $p < 0.001$; VEH vs.

THC: p = 0.001; Fig 15C) and WR males (CON vs. THC: p < 0.001; VEH vs. THC: p < 0.001; Fig 15D).

Length of Estrous cycle

Estrous cycle was the same length regardless of the group in both WR and LER females (data not shown).

Stereology

Hippocampal size varied significantly by group for females of both strains (LER: $F_{(2,16)} = 4.736$, p = 0.024; WR: $F_{(2, 15)} = 4.472$, p = 0.03; Fig 16). Specifically, overall hippocampal volume in females was greater in THC exposed rats as compared to handled controls (LER: p = 0.042, Fig 14A; WR: p = 0.028, Fig 16C). No other significant volumetric differences were observed for any group for any other brain area measured (Fig 17-22).

Discussion

Overall, administration of THC following puberty onset had effects on weight gain and hippocampal size. All groups, when administered THC, demonstrated significantly less weight gain following puberty onset as compared to both the VEH and CON groups (Fig 15). Additionally, hippocampal size was larger in females of both LER (Fig 16A) and WR (Fig 16C) strains who had been exposed to THC as compared to the CON, but not VEH, group. Possible mechanisms of these effects will be discussed.

Effect of THC on weight gain

Across all groups, regardless of strain and sex, THC disrupted relative weight gain throughout the entirety of the injection period (Fig 15). This was taken as a indication that the dose administered here, 5mg/kg, is likely to be considered a "medium" to "high" dose, as biphasic responses to cannabinoids have been shown on feeding behaviour; low doses will induce and high doses will halt feeding behaviour (Berry & Mechoulam, 2002). This can be the result of CB1R activation in central hypothalamic areas as well as chronic CB1R activation in the periphery, specifically in the gastrointestinal tract (Craft, 2005). This biphasic relationship may also be a by-product of the psychoactive effects and not of the effects on feeding behaviour. Perhaps the THC exposed rats were experiencing anxiety and other fear-related symptoms, as has been observed in humans who smoke marijuana (Thomas, 1996) or are exposed to exclusively THC (Carlini, 2004).

In this experiment, however, weight gain was only a proxy measure, so other factors, besides feeding, could have contributed to this lack of weight gain. One possible example is anxiety, as mentioned above. Additionally, the effects of THC on motor coordination and locomotor activity (Mallet & Beninger, 1998; McGregor, Arnold, Weber, Topple, & Hunt, 1998a) could have deterred access to the food hopper in the home cage following injection. Additionally, another factor may have been mediated through the social interactions between cagemates. Each cage consisted of subjects from different groups, such that no cage contained two rats exposed to THC. Unpublished work examining play behaviour in similarly treated rats demonstrated altered sociality and play fighting as a result of THC exposure in LER rats (see Appendix 3). Decreased weight gain could be partially mediated through disruptions in social interactions during this critical period in

social development, which could have prevented either access to food or altered the amount of food eaten.

An additional factor that could have affected weight gain is stress and the adaptation of the stress response to the injection. THC is necessary for learning and expressing stress habituation (Hill et al., 2010). In this case, the stress of the injection would have resulted in increases in corticosterone (CORT), which can decrease food intake (Calvez et al., 2011; reviewed in Maniam & Morris, 2012) in the short term. However, given that the injection occurred at the same time every day, as well as was of the same type and duration, stress habituation would have occurred, such that the CORT response would have decreased over time (De Boer, Van der Gugten, & Slangen, 1989). In the case of the THC exposed rats, the physiological habituation to the chronic predictable stress of injection might not have occurred, and the continued daily administration of injections could have resulted in elevated levels of CORT over the entirety of the THC injection period. This could have resulted in lowered weight gain over the course of that period.

However, chronic stress is also associated with increased food intake (Rostamkhani, Zardooz, Zahediasl, & Farrokhi, 2012) and preference for palatable (high fat or high sucrose) foods in mice, rats and humans (Dallman et al., 2003; Pecoraro, Reyes, Gomez, Bhargava, & Dallman, 2004; Warne, 2009). In rodents, these chronic stressors are unpredictable as well as more ethologically relevant (such as chronic social defeat and/or the scent of a predator) and are often maintained for a longer time than the two weeks of injections administered here (for example, Rygula, Abumaria, Domenici, Hiemke, & Fuchs, 2006). Only through daily monitoring of CORT pre- and post-injection in these injected animals one would be able to address this issue. However, chronic blood

collection can itself induce stress, depending on the method of collection, which was not entirely possible given our research parameters.

Regardless, in this injection paradigm, THC administration following puberty onset interferes with weight gain in the adolescent period. Future research examining the stress response as well as food intake or metabolism during this injection period will help to determine the exact mechanisms behind this alteration in weight gain. Since it was observed across all groups, regardless of strain and sex, clearly this effect is robust and, has been observed using similar injection protocols using other cannabinoid compounds (Biscaia et al., 2003).

Sex-specific effect of THC on HP volume

A sex-specific effect of THC was observed in both rat strains such that females exposed to THC had significantly larger hippocampi than those exposed to daily handling (Fig 16A&C). One important note to consider is that VEH and THC groups were not significantly different and neither were VEH and CON groups. This may in fact be, as discussed above, an effect of adaptation to the injection that was not able to occur in the presence of THC. In other words, the stress of the injection itself caused a non-significant increase in hippocampal volume that was more pronounced when the injection contained THC. The hippocampus has a high concentration of CB1Rs (Mailleux & Vanderhaeghen, 1992b), which may be partially responsible for the volumetric changes observed in this location. Despite this effect in overall hippocampal size, no specific subregions showed any alterations in volumetric estimates (Fig 17-19). This could be an indication that the changes in hippocampal size were a result of alterations in white matter connections within the hippocampus and not due to changes in cell number or in

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neurogenesis specifically in the DG. This is in line with *in vitro* and *in vivo* work that demonstrates that THC administration can increase brain-derived neurotrophic factor (BDNF) mRNA in CA1 and CA3 (Derkinderen et al., 2003), and elevations in hippocampal BDNF increases dendritic branching (Horch, Kruttgen, Portbury, & Katz, 1999). However, THC acutely disrupts memory formation at the synaptic (Tzavara, Wade, & Nomikos, 2003) and behavioural (Lichtman et al., 1995) level as well as decreases the number of synapses in the hippocampus (Scallet et al., 1987). Therefore, although the increase in hippocampal volume in female rats may be a result of increased white matter, which may arise through increased BDNF, it is possible that these connections were not facilitating communication within the hippocampus and therefore not facilitating behaviour. BDNF application, although it increases the number of dendritic processes, decreases the number of functional dendritic spines (Horch et al., 1999). Again, as demonstrated in the earlier chapter (Chap 2.2), increased volume does not necessarily heighten function, and with post-pubertal administration, THC may have induced a BDNF-dependent increase in white matter in the hippocampus specifically in females.

However, of interest is why this increase in volume in the hippocampus occurred in females and not in males. One possible mechanism of action is that females preferentially metabolize THC to an active form, which also acts as CB1R agonists (Narimatsu et al., 1992; Narimatsu et al., 1991). This differential metabolism of THC could allow for sex-specific prolonged effects of THC and its metabolites. In addition to the sex-specific metabolism of THC, endogenous and exogenous cannabinoids can interact with circulating estrogens and may mediate its sex-specific effects through interactions with the estrous cycle (Bonnin et al., 1993; Craft & Leitl, 2008; Craft et al.,

2013; Fattore & Fratta, 2010; Fattore et al., 2007; Hill et al., 2007; Nir et al., 1973;

Rawitch et al., 1977; Riebe et al., 2010; Rodriguez de Fonseca et al., 1994). Estrous

cycle phase can alter the hippocampus and its subregions (Galea & McEwen, 1999; Galea

et al., 1999; Woolley, 1998), which could account for these effects. Additionally, the

endocannabinoid system interacts with the HPA axis (Eldridge & Landfield, 1990;

Gorzalka et al., 2008; Hill & Gorzalka, 2004, 2005; Hill et al., 2009; Reich et al., 2009),

which can also alter hippocampal size (McEwen & Milner, 2007; Shansky, Hamo, Hof,

McEwen, & Morrison, 2009). In addition, sex differences in baseline and stress-induced

HPA activity have been observed previously (Conrad et al., 2004; Critchlow et al., 1963;

Panagiotakopoulos & Neigh, 2014; Reich et al., 2009; Stark et al., 2006). Therefore, the

exact processes behind the sex-dependent effects of THC on hippocampal volume are

complex and remain to be elucidated.

No strain differences

One striking effect was the observation of no strain-specific effects of THC on brain

volumes or weight gain through the injection period. All effects occurred either across all

groups or in one sex, regardless of strain. This is a surprising result, given strain-specific

differences in the behavioural effects of THC (Chap 3.2) as well as CB1R localization

and concentrations (Coria et al., 2014; Ortiz et al., 2004). However, LER and WR have

never been compared in this context for differences in response to THC administration for

weight gain and volumetric estimates. Indeed, Chap 2.1 and 2.2 are the first descriptions

of strain differences between LER and WR in brain volumes, as most studies examining

LER and WR have maintained a microscopic view of hippocampal morphology to

distinguish differences (for example, Holahan et al., 2007; Holahan et al., 2006).

However, despite no strain differences in response to THC, it still remains to be seen whether behavioural differences in adulthood following post-pubertal THC administration will result in strain-dependent effects. These effects will be explored and discussed in Chap 3.2.

Conclusions

Here, marked effects of THC on weight gain and sex-dependent effects on hippocampal volume were observed. This study demonstrates that THC does alter some aspects of brain morphology acutely following a long-term exposure. Whether this confers any long-term effects on brain morphology and behaviour remains to be seen. It does highlight the altered sensitivity of females to the effects of THC as well as may interact with other factors, such as social development, that could produce adverse effects later on in life.

CHAPTER 3.2: LONG-TERM EFFECTS OF THC ON ADULT BRAIN AND BEHAVIOUR

Introduction

Marijuana is one of the most commonly used drugs of abuse. In a recent Canadian survey, 42% of adults reported having used marijuana in their life time, and of these adults, most report the first instance of use during the adolescent period (Health Canada, 2013). Adolescent marijuana use is common and popular, and there have been detailed discussions regarding whether adolescence is a particularly vulnerable period for adolescent drug-use, resulting in long-term consequences on adult brain health and behaviour (Rubino et al., 2012; Schneider, 2008; Solowij & Battisti, 2008).

In addition, there are reports of sex-specific effects and consequences of adolescent marijuana use. In a long-term study, from childhood to early adulthood in Australia, women who reported regular consumption of marijuana during adolescence had a five times higher likelihood of self-reported anxiety or depression, irrespective of depression or anxiety measures recorded as a child (Patton et al., 2002). Although the exact mechanisms behind these sex differences are only speculative, it appears women are particularly vulnerable to the long-term consequences of marijuana use during the adolescent period.

Marijuana can contain upwards of 60 different cannabinoid compounds (Abood & Martin, 1992; Ashton, 2001). One of these compounds, Δ^9-tetrahydrocannabinol (THC), is thought to mediate many of the psychoactive and potentially addictive properties of marijuana (Razdan, 1986). THC binds to endogenously expressed cannabinoid receptors (Razdan, 1986). One subtype, the CB1R, is ubiquitously expressed throughout *Animalia*, ranging from *Cnidaria* to *Mammalia* (Berry & Mechoulam, 2002). In mammals, this

receptor is expressed throughout the peripheral and central nervous system and represents one of the most widely expressed G-protein coupled receptors in the brain (Herkenham et al., 1990; Mackie, 2005; Mailleux & Vanderhaeghen, 1992b). Cannabinoid receptors are found presynaptically, and endogenous cannabinoids are produced on demand following release of neurotransmitters at the synaptic cleft (Wilson & Nicoll, 2002). CB1R activity results in inhibition of additional neurotransmitter release from that particular synapse, dampening signal propagation (Marsicano & Lutz, 2006; Piomelli, 2003). CB1R are located at multiple synapses, including excitatory and inhibitory synapses (Freund, Katona, & Piomelli, 2003; Marsicano & Lutz, 1999), throughout multiple locations in the brain, including those related to anxiety, learning, memory, motor control, and fear (Herkenham et al., 1991; Herkenham et al., 1990; Mailleux & Vanderhaeghen, 1992a, 1992b; Moreira, Grieb, & Lutz, 2009; Ruehle, Rey, Remmers, & Lutz, 2012).

Animal models are often utilized to study the long-term consequences of adolescent THC exposure. These models support human studies demonstrating that the adolescent period is vulnerable to the long-term consequences of THC in comparison to perinatal or adult use (Cha, Jones, Kuhn, Wilson, & Swartzwelder, 2007b; O'Shea et al., 2004; Schneider, 2008), and studies have reported long-term effects ranging from deficits in learning and memory to increased anxiety (Cha, Jones, Kuhn, Wilson, & Swartzwelder, 2007a; O'Shea et al., 2004; Rubino, Guidali, et al., 2008; Rubino, Realini, Braida, Alberio, et al., 2009; Rubino, Realini, Braida, Guidi, et al., 2009; Rubino, Vigano, et al., 2008). However, many shortcomings and inconsistencies are apparent in the literature. For example, some studies distinguish any point after post-natal day 35 to be considered the adolescent period in rats (for example, Rubino, Realini, Braida, Alberio, et al., 2009; Rubino, Vigano, et al., 2008). However, an earlier study from our lab

demonstrated that male rats tend to enter puberty much later than that, approximately around postnatal day 40 (Chap 2.1; Keeley et al., in submission). CB1R are found in hypothalamic areas (Mackie, 2005) from which the permissive hormonal signal for the onset of puberty is released (Harris & Levine, 2003; Ojeda et al., 2003). Additionally, THC has been shown to delay the onset of puberty, albeit in female rats (Wenger, Croix, & Tramu, 1988), therefore this time frame for adolescent exposure may not accurately parallel that experienced in human adolescents and may cause unwanted physiological consequences on the maturational process of puberty. In addition to these potential flaws, some studies examining the long-term effects of THC have focused primarily on female subjects (for example, Rubino, Realini, Braida, Alberio, et al., 2009). Although the validity of using females is obvious, given the particular sensitivity of women and females of multiple species to the effects of marijuana and/or exogenous cannabinoid compounds, sex-differences in the frequency of marijuana use are not as clear, and men often report higher frequencies of lifetime use (Health Canada, 2013; Hall & Solowij, 1998). In addition to this, females preferentially metabolize THC to an active metabolite that will also bind to and activate CB1R (Narimatsu et al., 1992), whereas males primarily metabolize THC to an inactive form (Narimatsu et al., 1991). Additionally, as discussed in Chap 3.1, the endocannabinoid system and the application of exogenous cannabinoids can interact with the endogenous cyclical hormonal fluctuations in females (Bonnin et al., 1993; Craft & Leitl, 2008; Craft et al., 2013; Fattore & Fratta, 2010; Fattore et al., 2007; Hill et al., 2007; Nir et al., 1973; Rawitch et al., 1977; Riebe et al., 2010; Rodriguez de Fonseca et al., 1994), which may account for the sex-specific effects. Therefore, there is a need for the consideration and evaluation of both males and females in the same study.

Furthermore, there are conflicting results in the literature, where some groups show long-term consequences of adolescent marijuana use, and some do not. One possible explanation is the use of different laboratory rat strains. Rat strains can differ both naturally or in response to different treatments. For example, the LER strain outperforms the WR strain in the standard MWT (Holahan et al., 2006), displays differential cytoarchitecture in the hippocampus (Holahan et al., 2006), and benefits from juvenile MWT pretraining (Keeley et al., 2010). In addition to these differences, there are interactions between sex and rat strain, such that only LER males, and not females, benefit from juvenile pretraining (Keeley et al., 2010). Earlier research from our group has also shown that LER females reach puberty at an earlier time point than WR females, and this pattern is not observed in males (Chap 2.1; Keeley et al., in submission). Given the possibility for strain and sex to interact, it is important to consider all of these factors. Therefore, in order to determine if the effects of adolescent THC exposure are in fact robust and long-lasting, it is important to employ a drug administration schedule at a physiologically appropriate time in male and female rats of multiple rat strains, including LER and WR.

Given these considerations, this study was conducted to determine the long-term consequences of post-pubertal THC administration in male and female rats of two rat strains on cognition and brain volumetrics in adulthood. Rats were administered THC daily for two weeks following the determination of puberty onset. After the injection period, rats were aged to adulthood and assessed in behavioural tasks related to motor learning, spatial learning and memory, fear-based learning and anxiety. The skilled reaching task (SRT) was used to assess whether THC use had any long-term consequences on motor learning.   A variant of the MWT was used to assess spatial

learning and memory. The DFCTC paradigm was used to assess contextual fear learning. Finally, in a separate group of rats that had received no other behavioural testing, the EPM was used to assess basal anxiety. Following behavioural assessment, brain volumetrics in areas associated with all of these tasks, including the HP and its subregions (DG, CA1 and CA3), the OFC, the mPFC and the AMYG. This study attempted only to describe whether there were long-term consequences in LER and WR males and females that were observed across all strain and sex groups or whether one strain or sex group was particularly vulnerable to the effects of THC. This is the first study of its kind to evaluate the long-term consequences of marijuana in male and female LER and WR in one study.

Methods

Subjects

Subjects were housed and treated as previously described (Chap 2.1).

Puberty Onset & Drug Administration

Puberty onset and group assignment were conducted as previously described (Chap 2.1). Briefly, on weaning day, rats were assigned to their experimental groups: handled control (CON, N = 8/strain and sex group), vehicle (VEH; N = 8/strain and sex group) or 5mg/kg THC (THC; N = 8/strain and sex group) for a total of 96 rats for behaviour across all strain and sex groups. An additional 96 participated in the EPM separately.

Injection procedures and handling were conducted as previously described (Chap 3.1). Briefly, on the day of determination of puberty onset, rats were removed from their cages, placed in a light-blocking transport tub and brought to an injection room that was

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lit with a red incandescent bulb. All rats were weighed before treatment.   Following

injections, females were assessed for vaginal cytology using the lavage technique

(Goldman et al., 2007; Marcondes et al., 2002). Vaginal smears were taken on every

injection day. In order to control for stimulation during the puberty period, a sterile Q-tip

was dipped in sterile dH_2O and applied to the scrotal area of males. Following injection,

rats were returned to their home cages. All rats received treatment for 14 consecutive

days following determination of puberty onset. For VEH and THC rats, injection site

varied daily in order to eliminate any damage or irritation due to multiple injections at the

same site.   For a pictorial representation of the injection sites, see Appendix 1.

Determination of Estrous Cycle & Vaginal Cytology

Vaginal cytology and determination of estrous cycle was done as previously

described (Chap 2.2). Vaginal smears were collected during all handling days as well as

on all days of behavioural testing. The only exception was vaginal smears were only

collected for the first 10 days of SRT (as described in Chap 2.2).

Skilled reaching task (SRT): Apparatus & training

Apparatus and training were conducted as previously described (Chap 2.2). For

all skilled reaching, analyses were conducted exclusively for the last day of training in

order to compare groups on final performance and not on the rate of motor learning.

Morris water task (MWT): Apparatus & training

Apparatus, data collection and training were conducted as previously described

(Chap 2.2).

Discriminative fear-conditioning to context (DFCTC): Apparatus & training

Apparatus, training, testing and video recording were conducted as previously described (Chap 2.2).

Elevated Plus Maze (EPM): Apparatus & training

EPM was conducted in a separate group of rats (N = 96 total) naïve to any behavioural testing. These rats were, following EPM training, used in the experiments outlined in Chap 3.3. Apparatus and training were used and conducted as previously described (Chap 2.2).

Perfusion and Fixation

The day after the last behavioural testing day, rats were euthanized with a single i.p. injection of sodium pentobarbital (120mg/kg) and transcardially perfused with approximately 150mL of 1xPBS as previously described (Chap 2.1). Briefly, following decapitation, brains were removed from the skull. The left hemisphere was immersion fixed in another solution used for another set of experiments, and the right hemisphere was immersion fixed in 4% PFA in 1xPBS. Right hemisphere PFA was replaced 24h after the perfusion with 30% sucrose and 0.2% Na azide in 1xPBS until sectioning. Right hemispheres were sectioned in a series of 12 at 40µm using a cryostat (CM1900, Leica, Germany) and placed directly into Eppendorf tubes containing 0.2% Na azide in 1xPBS.

Histology – Cresyl violet staining

Sections were float-mounted in 1xPBS onto 1% gelatin 0.2% chrom alum slides (VWR Canada) and allowed to dry overnight. Sections were rehydrated and placed in a 1% cresyl violet solution in dH_2O for 10min. Slides were then rinsed in dH_2O followed by 70% ethanol. Sections were placed in differentiator for 2min and were dehydrated and coverslipped with Permount.

Volumetric Estimates Using Unbiased Stereology

Volumetric analysis of total HP, DG, CA1, CA3, mPFC, OFC and AMYG were conducted using the Cavalieri method (Gundersen et al., 1999a) as implemented in StereoInvestigator (Microbrightfield, Williston, VT) on a Zeiss Axio Imager MT (Carl Zeiss, MicroImaging GmBH, Germany). Section intervals and grid size were the same as described previously (Chap 2.2). A summary table of mean volume and coefficient of error (CE) can be found in Table 5.

Statistical Analysis

Statistical analyses were conducted with a set of *a priori* hypotheses in mind. First, for all measures, analyses were conducted within strain and sex groups such that drug effects were only considered within each strain and sex group. This was done because, despite the similar drug dose paradigm, the numerical ages of the rats were different between strain and sex groups for the first day of injection. Only analyzing for the effects of drug within strain and sex groups also simplified all statistical analyses. All

statistical analyses were conducted using SPSS ver 21 (IBM, USA). Estrous cycle was examined as a covariate for individual day analyses for all measures.

SRT - A between subjects design ANOVA was conducted with group as a between subjects factor. The number of successful attempts and the number of successful trials were both analyzed for between subjects effect. No a priori hypotheses were used.

MWT - For the first 4 days of training, a mixed design ANOVA was conducted with day as a repeated measure, and group as a between subjects measure. For the probe day, a mixed design ANOVA was conducted for all measures with quadrant as a repeated measure and group as a between subjects measure. For the 6th day (mass training), a mixed design ANOVA was conducted with trial block as a repeated measure and group as a between subjects measure. Finally, for the 7th and 8th day of training, a mixed design ANOVA was conducted with day and trial as repeated measures and group as a between subjects measure.

A priori comparisons were established for all probe test analyses such that differences within a group were examined for the data measured from the target quadrant in comparison to the other quadrants.

DFCTC - A mixed design ANOVA with group as a between subjects factor and context (paired versus unpaired) was conducted. For test and preference days, a priori hypotheses were tested to determine whether freezing (for test days) or dwell time (for preference day) differed between the paired and unpaired context for each treatment group within strain and sex groups.

EPM - Within strain and sex groups, mixed design ANOVAs were conducted with number of arm entries (open vs. closed) and total dwell time (open vs. closed). The ratio

of open to closed arm entries was run as a separate between subjects analysis with group as the between subjects factor. No a priori comparisons were conducted.

Volumetric estimates - For volumetric measurements, some rats were included who had no behavioural experience. This factor was used as a covariate in all volumetric analyses. In no analyses did behavioural experience significantly contribute to the observed results, so all subjects, regardless of behaviour, were included in all volumetric analyses. With the addition of subjects, a minimum of N = 11/strain and sex and drug treatment. A table containing the number of subjects from each group for each area of interest can be found in Table 6.

For volumetric estimates, inter-rater reliability was estimated using two-way mixed consistencies, average-measures intra-class correlation (ICC), as described in (Hallgren, 2012), to assess the degree of consistency between one potentially biased observer and a completely blind observer from a subset of 10 randomly selected subjects. An ICC of 1 indicates perfect relatedness whereas an ICC of <0.4 is considered poor inter-rater reliability (Hallgren, 2012). The achieved ICC of 0.982 suggested that volumetric differences were similar between observers, therefore the first set of observations, conducted by a the non-blind observer, were considered to be non-biased as they were highly similar to that of a completely blind observer.

Results

SRT

Skilled reaching was trained and assessed over the course of 21 days. All subsequent analyses only examined success on the last day of testing, as discussed in the

Methods section. Two measures of success were used: success relative to attempts and success relative to total numbers of pellets eaten.

Successful Attempts & Trials - Regardless of strain and sex group, there were no significant effects of THC on successful attempts of trials or any interaction effect (Fig 23A-D). Therefore, no significant effects of THC on skilled motor learning and performance were observed. For both LER and WR females, phase in the estrous cycle did not alter performance for the skilled reaching task.

MWT

Acquisition - Day 1-4 - All rats in all groups showed decreased latencies over the course of the trials (Fig 24A-D). There were no significant effects of drug or a day by drug interaction for any group. Estrous cycle did not alter performance on any of the acquisition days when analyzed as a covariate.

Retention : Day 5 Probe - Probe trials were examined for the full 60s of the probe as well as the first 10s and 30s. For the purpose of brevity, only the 30s probe will be presented, as results observed in the 30s probe are representative of both the 10s and 60s probes. For all probe trials, distance travelled in the pool and dwell times were measured. One CON LER female, one CON WR female and one THC WR female were excluded from the analysis due to experimental error using the tracking system.

LER females ($F_{(1, 20)}$ = 16.265, p = 0.001; Fig 25A), LER males ($F_{(1, 21)}$ = 35.812, p < 0.001; Fig 25B) and WR males ($F_{(1, 21)}$ = 14.644, p = 0.001; Fig 25D) all travelled further in the target quadrant indicating that they were preferentially searching that quadrant for the submerged platform. This was not observed in WR females (Fig 25C). Drug treatment had no effect on distance travelled nor was a drug treatment by quadrant

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interaction observed. Individual comparisons revealed that VEH (p = 0.003) but not

CON or THC LER females travelled significantly further in the target quadrant as

compared to the other quadrants (Fig 25A). All LER male groups spent significantly

longer in the target quadrant as compared to the other quadrants (CON: p = 0.004; VEH:

p = 0.019; THC: p = 0.014). Only CON WR males (p = 0.018) travelled significantly

further in the target quadrant as compared to the other quadrant (Fig 25D). This was not

observed in the THC or vehicle group among the WR males. No differences between the

target and other quadrants were observed for WR females.

Within all strain and sex groups, there was a main effect such that all rats spent

more time in the target quadrant than in the other quadrant, indicating increased search

time for the submerged platform in the quadrant where it had been previously placed

(LER females: $F_{(1,20)}$ = 20.335, p < 0.001, Fig 25A; LER males: $F_{(1,21)}$ = 26.935, p <

0.001, Fig 25B; WR females: $F_{(1,19)}$ = 8.01, p = 0.011, Fig 25C; WR males: $F_{(1,21)}$ = 7.064,

p = 0.015, Fig 25D). No effects of drug treatment or a drug by quadrant interaction were

observed for any group. Individual comparisons revealed that VEH (p = 0.032) and THC

(p = 0.009) but not CON LER females spent significantly more time in the target quadrant

as compared to the other quadrants (Fig 25A). All LER males groups spent significantly

more time in the target quadrant as well (CON: p = 0.011; VEH: p = 0.028; THC: p =

0.022). Only CON WR females (p = 0.039; Fig 25C) and CON WR males (p = 0.032; Fig

25D) spent significantly more time in the target versus the other quadrants. Overall LER

demonstrated retention of the old platform location, regardless of sex differences or drug

effects. WR, on the other hand, demonstrated poor retention regardless of drug effects.

Any effects of THC in WR were also observed in the VEH group.

When phase in the estrous cycle was used as a covariate, it did not alter any of the outcomes of the statistical tests. Therefore, it was not included in the final statistical model.

Mass training to a new platform location - LER females found the platform faster as they performed more trials ($F_{(1.531, 32.142)} = 115.266$, $p < 0.001$, Fig 26A). There was no significant effect of drug treatment or a drug by trial interaction. Like LER females, LER males found the platform faster as the trials progressed ($F_{(1.606, 33.727)} = 42.235$, $p < 0.001$), but there was no significant main effect of drug treatment or a drug by trial block interaction (Fig 26B). WR females all found the platform faster as the mass training day progressed ($F_{(2.637, 55.379)} = 4.905$, $p = 0.004$), but there was no effect of drug treatment or a drug by trial block interaction (Fig 26C). WR males found the platform faster as the trials progressed ($F_{(2.449, 51.431)} = 11.649$, $p < 0.001$), but no main effect of drug treatment or a drug by trial block interaction was observed (Fig 26D). Therefore, THC did not alter rapid acquisition of a new spatial location in the same training room in the MWT as all groups, regardless of drug treatment, learned to find the platform faster as the number of trials increased.

As was shown for all other days, estrous cycle had no effect on performance in the MWT for LER or WR females.

Visible platform : Day 7 & 8 - LER females improved their performance from one day to the next ($F_{(1,21)} = 20.84$. $p < 0.001$) and over the course of all 8 trials ($F_{(1.783, 37.445)} = 4.736$, $p = 0.018$), and a day by trial interaction was observed ($F_{(2.066, 43.38)} = 4.253$, $p = 0.02$). No significant main effects of drug treatment or any other interaction effect (Fig 27A) were observed. Like LER females, LER males found the platform faster over the course of the 2 days ($F_{(1,21)} = 14.818$, $p = 0.001$) and the 8 trials ($F_{(1.664, 34.939)} = 9.032$, $p =$

0.001), and a day by trial interaction was observed ($F_{(2.181,\ 45.791)} = 5.583$, p = 0.006). There was no significant main effect of drug treatment nor was any other interaction found to be significant (Fig 27A). WR females improved their performance over the course of days ($F_{(1,21)} = 8.144$, p = 0.01) and trials ($F_{(3,63)} = 7.635$, p < 0.001) and a significant day by trial interaction ($F_{(3,63)} = 5.596$, p = 0.002) was observed. No significant effect of drug treatment or any other interaction was found to be significantly different for latency to reach the platform on the visible platform days (Fig 27C). WR males also found the platform sooner over the course of the 2 days ($F_{(1,21)} = 16.258$, p = 0.001) and 8 trials ($F_{(3,63)} = 6.371$, p = 0.001), and a day by trial interaction was also observed ($F_{(3,63)} = 6.755$, p = 0.001). No effects of drug treatment or any other interaction were significantly different (Fig 27D). Therefore, THC had no significant effect on simple visual motor learning. Like THC, estrous cycle phase did not alter performance on the visible platform days.

DFCTC

Pre-exposure - Rats were examined for initial contextual bias as well as to aid in counterbalancing for assignment to paired and unpaired contexts. LER females, males and WR females showed no preference for the paired or unpaired context as well as no effect of drug or a context by drug interaction. WR males, however, showed no effect of context but did show a significant effect of drug on dwell time in the paired and unpaired contexts ($F_{(2,\ 21)} = 7.705$, p = 0.003; Fig 28D). However, analyses restricted to either the paired or unpaired contexts revealed no significant effect of drug. Therefore, regardless of strain or sex, there was no effect of THC on dwell time during the pre-exposure trial in

the DFCTC paradigm (Fig 28A-D). Dwell time on the pre-exposure day was not affected by estrous cycle.

Test Day - The test day assessed the effect of THC on discriminative freezing behaviour. Both LER males ($F_{(1, 21)}$ = 7.465, p = 0.012; Fig 29B) and females ($F_{(1, 21)}$ = 13.734, p = 0.001; Fig 29A) displayed significantly more freezing in the paired context overall. Only LER females, however, had a significant overall effect of drug administration on freezing behaviour ($F_{(2, 21)}$ = 8.335, p = 0.002), which was not observed in LER males. No interactions were observed between context and drug administration in either LER males or females.

Comparison between drug groups within LER females revealed that CON froze significantly more in the paired context than both VEH (p = 0.001) and THC (p = 0.002) groups (Fig 29A). No difference in freezing behaviour was observed in the paired context. For LER females, only CON (p = 0.041) and VEH (p = 0.015) groups showed discriminative freezing behaviour; this was not observed in the THC group. Therefore, in the long-term, THC altered the ability to express discriminative fear behaviour in LER females. However, this effect was confounded by the fact that the vehicle injection resulted in dampened fear responses in the paired context, as expressed by significantly less freezing in the paired context as compared to controls. Male LER VEH were the only group to display discriminative freezing behaviour (p = 0.01; Fig 29B).

Different patterns of behaviour were observed for the effects of THC in WR. Neither male nor female WR showed any discriminative freezing in the paired or paired context, and drug administration had no effect on this result (Fig 29C & D).

The inclusion of estrous cycle for LER and WR females demonstrated that estrous cycle did not alter performance on test days, as it did not significantly alter the amount of freezing in either context.

Preference - Preference day in the DFCTC paradigm assesses the ability to actively avoid the context previously associated with the shock stimuli. Appropriate learning behaviour is expressed as increased dwell time in the unpaired context and active avoidance and decreased dwell time in the paired context.

LER females overall spent significantly more time in the unpaired context ($F_{(1, 21)}$ = 10.292, p = 0.004). No significant effect of drug administration was observed. Individual analysis of dwell time within each group revealed that only CON (p = 0.014) and THC (p = 0.038) LER females displayed active avoidance of the paired context (Fig 30A). Therefore, LER females exposed to the VEH injection during the peripubertal period were unable to learn to actively avoid the context previously associated with shock.

LER males overall spent significantly more time in the unpaired context ($F_{(1, 21)}$ = 10.141, p = 0.004; Fig 30B). No effect of drug administration was observed. Individual group analysis revealed that only CON LER males (p = 0.04) spent significantly more time in the unpaired context. Therefore, active avoidance was not expressed in the VEH or THC group in LER males.

Like the LER strain, WR females overall spent significantly more time in the unpaired context ($F_{(1, 21)}$ = 7.887, p = 0.011; Fig 30C). No drug effects were observed. However, within group analysis revealed no significant difference between dwell times in either context for any group. Therefore, although overall, WR females spent significantly

more time in the unpaired context, within group analysis did not reveal active avoidance behaviour in any group.

WR males overall spent significantly more time in the unpaired context ($F_{(1, 21)}$ = 9.6, p = 0.005). No significant effect of drug or any interaction was observed. Within group analysis revealed that only the WR male THC group (p = 0.003) displayed discriminative freezing behaviour (Fig 30D). Therefore, THC exposure following puberty onset in WR males facilitated and allowed for the proper expression of active avoidance behaviour.

Estrous cycle did not alter performance on the preference test for either WR or LER females.

EPM

Dwell time in open and closed arms - Dwell time in the open and closed arms relative to the total time spent in the elevated plus maze was assessed for all rats. Regardless of strain or sex group, all animals spent significantly more time in the open relative to the closed arms (LER females: $F_{(1, 21)}$ = 126.45, p < 0.001; LER males: $F_{(1, 21)}$ = 285.521, p < 0.001; WR females: $F_{(1, 21)}$ = 81.779, p < 0.001; WR males: $F_{(1, 21)}$ = 142.285, p < 0.001; Fig 31). In all strain and sex groups, there was no effect of drug or a drug by open or closed arm interaction. Individual comparisons in each drug groups revealed that all drug administration groups spent significantly more time in the closed arm relative to the open arm in LER females (CON and VEH: p < 0.001; THC: p = 0.008), LER males (all groups p < 0.001), WR females (CON and VEH: p < 0.001; THC: p = 0.014) and WR males (all groups p ≤ 0.001). Therefore, all groups, regardless of drug treatment, showed the same pattern of dwell time behaviour on the EPM, indicating no

effect of THC on anxiety-related behaviours in the elevated plus maze as measured by dwell time.

Using estrous cycle as a covariate did not reveal estrous cycle as contributing to any of these values as well as did not alter the outcomes of the tests, therefore for LER and WR females, the original model was used.

Arm entries: Raw values - The complete number of open and closed arm entries was examined for every animal in each strain and sex group. In all groups, regardless of strain or sex, all animals entered the open arms more than the closed arms (LER females: $F_{(1, 21)} = 19.313$, $p < 0.001$; LER males: $F_{(1,2 1)} = 38.576$, $p < 0.001$; WR females: $F_{(1, 21)} = 35.746$, $p < 0.001$; WR males: $F_{(1, 21)} = 26.291$, $p < 0.001$). For all groups, there was no main effect of drug or any interaction effect. Individual comparisons within each strain and sex group revealed some THC effects specific to strain and sex groups. In LER females, all groups entered the closed arms more than the open arms (CON: $p = 0.015$; VEH: $p = 0.049$; THC: $p = 0.041$; Fig 32A). This exact pattern was also observed in LER males (CON: $p = 0.028$; VEH: $p = 0.007$; THC: $p = 0.003$; Fig 32B). However, in WR females, only the CON ($p = 0.005$) and VEH ($p = 0.002$) groups entered the closed arms significantly more times, and no difference between open and closed arm entries was observed for the WR female THC group (Fig 32C). An opposite pattern of behaviour was observed for WR males, such that the VEH ($p < 0.001$) and THC ($p = 0.013$) groups entered the open arms more (Fig 32D). This pattern was not observed in the WR male CON group, although this value approached significance ($p = 0.08$). Given these differences, THC had no effect on anxiety-related behaviour in the EPM in LER of either sex. However, it appears that for WR females, THC reduces baseline anxiety due to equal number of open and closed arm entries. For WR males, THC and VEH injections

result in differential open and closed arm entry, where CON WR males entered open and closed arms the same number of times.

Estrous cycle did not have any effect on the achieved statistical comparisons when included as a covariate, therefore it was not included in the final model.

Arm entries: Open relative to closed - The final measure of anxiety in the EPM looked at the ratio of open to closed arm entries in all groups. This helped to account for increased activity in all groups. All groups, regardless of strain or sex did not show any effect of THC on the ratio of open to closed arm entries (Fig 33). Therefore, it is assumed that post-pubertal exposure of THC to LER and WR males and females did not have any effect of anxiety-related behaviours in the EPM when locomotor activity was normalized.

Using estrous cycle as a covariate revealed that phase in the estrous cycle had no effect on this value nor altered the outcome of the effect of group on behaviour for females. Therefore, it was not included in the final statistical model.

Volumetric Estimates

For all brain areas examined, whether or not a rat had undergone behaviour or not was used as a covariate. For all brain areas examined, experience did not significantly alter the volume of an area of interest, therefore both groups were pooled in the analyses.

HP - Only LER females showed a significant effect of drug on the volume of the hippocampus ($F_{(2,40)}$ = 4.21, p = 0.022; Fig 34A). *Post hoc* Bonferroni comparisons revealed that VEH LER females had significantly larger hippocampal volumes in comparison to the THC group (p = 0.02). Estrous cycle did not affect the volume of the hippocampus for LER or WR females. No other groups demonstrated any effect of group on HP volumes (Fig 34B, C & D).

DG - Like hippocampal measurements, only LER females had a significant main effect of drug on DG volume ($F_{(2, 39)}$ = 4.747, p = 0.014; Fig 35A), and *post hoc* Bonferroni comparisons revealed that again, the VEH group had significantly larger DG volumes in comparison to the THC group within LER females. No other effects of drug treatment were observed in the other strain and sex group (Fig 35B, C & D).

Estrous cycle did have a significant effect on DG volumes in LER females only ($F_{(1, 33)}$ = 4.293, p = 0.046). However, despite the inclusion of estrous cycle as a covariate, there were no significant group effects. Individual comparisons revealed that, although none of these comparisons were significant despite the overall effect of estrous cycle, there was a pattern such that DG volume decreased in size from diestrous > metestrous > estrous > proestrous rats.

CA1 - Drug treatment had a significant effect on CA1 volume in LER females ($F_{(2, 39)}$ = 5.058, p = 0.011; Fig 36A) and WR females ($F_{(2, 33)}$ = 3.354, p = 0.047; Fig 36C); no such effects were observed in males (Fig 36B&D). *Post hoc* comparisons revealed that in LER females, the VEH group had significantly larger CA1 volumes in comparison to the THC group (p = 0.008; Fig 36A). *Post hoc* comparisons within WR females revealed no significant differences, and no differences were observed within male LER and WR.

When accounting for estrous cycle, there were no effects in LER females. However, for WR females, when estrous cycle was used as a covariate, there was no longer a significant effect of group. Therefore, estrous phase helped account for some of the variance in the CA1 volumes in WR females.

CA3 - No significant effects of drugs were observed in any strain or sex group on CA3 volumes (Fig 37). Using estrous cycle as a covariate demonstrated that there was no effect of estrous cycle.

OFC - Treatment group had no significant effect on OFC volume in any of the groups (Fig 38). Using estrous cycle as a covariate did not alter these results or show any effect of estrous cycle of OFC volumes in females.

mPFC - Treatment group altered mPFC volume in WR females alone ($F_{(2, 34)}$ = 4.107, p = 0.025; Fig 39C). *Post hoc* comparisons revealed that WR female VEH had significantly larger mPFC volumes in comparison to the THC group (p = 0.048). Estrous cycle had no effect on the data set, and group still had a significant effect of mPFC volumes in WR females when estrous cycle was included as a covariate. No other significant effects of drug treatment were observed within the other strain and sex groups.

AMYG - LER males had a significant effect of drug treatment group of amygdalar volume ($F_{(2, 37)}$ = 8.485, p = 0.001), and *post hoc* comparisons revealed that the THC group had significantly larger amygdalar volumes in comparison to the CON (p = 0.001) and VEH groups (p = 0.041; Fig 40B). There was no effect of estrous cycle in female rats. No other differences were observed in the other strain and sex groups.

Summary of results

A summary of the results can be found in Table 7.

Discussion

The results of the behavioural and volumetric analyses were mixed. THC was found to have strain-, sex- and measurement-specific effects. In the EPM, when only

examining the number of arm entries, THC decreased anxiety in WR females and increased it with WR males (Fig 32C). THC also disrupted the expression of discriminative freezing in LER females (Fig 29A). Finally, in LER females, THC decreased hippocampal (Fig 34A), DG (Fig 35A) and CA1 (Fig 36A) volume, and in WR females, decreased increased mPFC volumes, but only relative to the VEH group (Fig 39C). THC also increased the size of the amygdala in LER males (Fig 40B).

In addition to the effects of THC, some changes were observed in both VEH and THC groups and not CON groups, indicating an effect of the injection itself. For example, all strain and sex groups, except for LER males, showed impaired performance in the probe trial when they had been injected with either VEH or THC during the post-pubescent period (Fig 25). WR males also demonstrated injection effects on EPM behaviour, such that an injection increased anxiety-like behaviour that was dependent on the type of observed EPM behaviour (Fig 31D & 32D). Additionally, in LER males, both injected groups displayed indiscriminate freezing behaviour (Fig 29B). All of these effects will be discussed and their relationship to both the endocannabinoid system as well as the adolescent period will be discussed.

Effects of THC

Here, THC altered anxiety behaviour in the EPM for WR females. THC decreased anxiety behaviour, as shown through equal amounts of open and closed arm entries in WR females (Fig 32C). Contrary to our results, THC exposure has been shown to increase anxiety behaviour both acutely (Thomas, 1996) and after adolescent exposure, specifically in females (O'Shea et al., 2004). Both results are not surprising given the high density of CB1Rs in areas involved in both the expression and regulation of anxiety,

including the amygdala, hippocampus and prefrontal cortex (Mackie, 2005; Mailleux & Vanderhaeghen, 1992a). Indeed, CB1Rs agonists can have bi-phasic effects on anxiety behaviour, where high levels of exogenous or endogenous cannabinoids increase anxiety whereas low levels will cause decreased anxiety (Patel & Hillard, 2006; Viveros, Marco, & File, 2005). In addition, cannabinoids have site-specific effects, such that the biphasic effect on anxiety behaviour is observed in local application to the hippocampus and prefrontal cortex, but not to the amygdala (Rubino, Guidali, et al., 2008). Indeed, the endogenous cannabinoid system has a role in modifying anxiety behaviour through modifications of amygdalar signalling, partially through decreasing inhibitory actions of amygdalar interneurons thereby increasing their firing rate (Piomelli, 2003). However, due to the site-specific nature of cannabinoid effects in conjunction with our systemic application in adolescence, it is difficult to predict the directionality of the effects of THC on anxiety behaviour, with some studies showing increases (O'Shea et al., 2004), no change (Rubino, Vigano, et al., 2008) and decreases (Wegener & Koch, 2009) in anxiety following chronic application.

Adolescent application of THC has been shown to decrease CB1R densities, but the location of these changes in the brain were shown to be sex-specific (Rubino, Vigano, et al., 2008) and as discussed above, long-term changes on anxiety behaviour are dependent on the study conducted, but often are only apparent in females (for example, O'Shea et al., 2004). The aforementioned study also used WR females, therefore our effect of decreasing anxiety in female WR, which was specific to that strain and sex group, was surprising. Our results could be related to the decreased change in mPFC volume seen exclusively in THC-exposed WR females (Fig 39C), and the direction of change in mPFC volume is what you would expect with decreased anxiety behaviour.

Put simplistically, anxiety is regulated through interactions between amygdala, hippocampus and prefrontal cortex, where the prefrontal cortex, including the mPFC, and the hippocampus help regulate amygdalar signalling in order to identify and express context- and stimuli-appropriate fear responses and to suppress those that are not (Adhikari, 2014; Fanselow & Dong, 2010; Shin & Liberzon, 2010). Traditionally, a smaller volume confers decreased function, and here a larger mPFC was associated with enhanced function, i.e. less anxiety-related behaviour. However, no changes were observed in amygdalar volume, which would be expected as well. As discussed in other chapters, the shortcomings of using volumetric estimates could have contributed to this discrepancy. Although large-scale changes in cell densities and arborisations should be reflected in volumetric estimates, the macroscopic scale of this measure may not fully capture these differences. For example, if you have a loss of cell numbers but an increase in the complexity of the cytoarchitecture, these changes would not necessarily be reflected as any changes in volumetrics. However, volumetric estimates are a good starting point for identifying areas of interest where changes may have occurred. Given the widespread distribution of CB1Rs in the mammalian brain, volumetric estimates should be an essential tool for identifying key brain areas of interest.

Another question remains as to why our study found decreases in anxiety in female WR exposed to THC whereas other studies have found increases in anxiety, specifically in female rats. One difference includes the dose and timing of the injection. As mentioned previously, our injection paradigm is unique in that it only begins after the onset of puberty, which can be altered with CB1R agonists (Wenger et al., 1988). However, most injection paradigms instead choose to inject all rats, regardless of physiological maturation, starting at the same numerical age (for example, starting on

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postnatal day 35; Rubino, Vigano, et al., 2008). This does not take into consideration the possibility of all behavioural effects resulting from partially or entirely offsetting puberty. Additionally, other research paradigms gradually increase the dose of THC over the standard 2-week injection period (Rubino, Realini, Braida, Alberio, et al., 2009; Rubino, Realini, Braida, Guidi, et al., 2009; Rubino, Vigano, et al., 2008). This is believed to model human adolescent drug use, such that anecdotally, adolescents will increase the amount and frequency of drug use over time. However, this does bring into question the administration paradigm itself. Research using cocaine administration has demonstrated that self-administration of cocaine (i.e. lever pressing) results in heightened addictive states and relapse tendencies than yoked controls, i.e. rats administered the same doses at the same time as those being self-administrated (Markou, Arroyo, & Everitt, 1999). Therefore, the injection schedule administered both here and in other papers does not take into consideration the voluntary aspect of drug administration to induce a more addicted-like state. However, neither this study, nor those mentioned above, were interested in studying the long-term consequences of THC addiction, *per se*, but instead were interested in studying the long-term effects of exposure alone. Indeed, rats and mice do not easily self-administer cannabinoids, and often, pre-exposure to other drugs of abuse to induce lever pressing training is, at the very least, required before they exhibit this behaviour (Fattore, Cossu, Martellotta, & Fratta, 2001; Maldonado & Rodriguez de Fonseca, 2002; Takahashi & Singer, 1979).

Another caveat to our results is that WR females only showed an effect of THC on one metric of EPM behaviour. The EPM is a contentious behavioural testing procedure, and there have been some critiques of it as a metric of anxiety over the years (Carobrez & Bertoglio, 2005; Hogg, 1996; Wall & Messier, 2001; Weiss, Wadsworth, Fletcher, &

Dourish, 1998). Many of the metrics, like much of behavioural testing, are dependent on baseline activity levels. Here, when we normalized the number of arm entries relative to total arm entries, which should theoretically help normalize for baseline differences in activity levels, we saw no effects of THC in WR females, let alone any other group. Clearly, whatever effect of THC was achieved in WR females, it did not result in large changes in EPM behaviour, except for increasing the number of open arm entries such that there were no significant differences between open and closed entries.

In addition to alterations in anxiety, THC appears to disrupt the expression of discriminative freezing in LER females (Fig 29A). Again, we saw a female-specific effect of THC on behaviour. Discriminative freezing is a hippocampal-dependent behaviour in the DFCTC task (Antoniadis & McDonald, 2000). In conjunction with poor discriminative freezing behaviour, LER females exposed to THC also have smaller hippocampal (Fig 34A), DG (Fig 35A) and CA1 volumes (Fig 36A). Although much of this thesis has shown behavioural deficits without loss of volumes in specific brain areas, here, loss of function with THC administration occurred concomitantly with smaller volumes of associated brain areas. One reason for this loss of cells could be a direct result of CB1R activation on neurogenesis in the hippocampus. This is not likely as chronic application of CB1R agonists have been found to promote hippocampal neurogenesis (Jiang et al., 2005). DG volume did not change immediately following the THC administration period (Fig 17; Chap 3.1), but perhaps chronic activation of CB1Rs during the post-pubertal period resulted in alterations in neurogenesis, leading to decreased production of new cells throughout the rest of life. This is only speculation, as we have no indication of any changes in neurogenesis with our measures. One caveat to these results is that these differences in THC were only seen relative to the VEH group

and not relative to the handled control group. Since no significant differences were seen

between CON and VEH in these cases, it does affect the interpretation of the results.

Additionally, despite CON and THC groups in LER females displaying active avoidance,

VEH LER females did not show active avoidance (Fig 30A). This may simply be a by-

product of increased variability observed in females due to the effects of estrous cycle.

To minimize these effects, estrous cycle was used as a covariate to determine if it

significantly altered behaviour for these tasks. However, because estrous cycle was not

controlled for, we did not necessarily have enough subjects per group to be able to say

definitively whether estrous cycle did or did not affect behaviour. Only future research

using this fear-conditioning paradigm will be able to determine if this is the case.

One unexpected result in the DFCTC paradigm was the finding of improvement of

WR males exposed to THC (Fig 30D). CON and VEH WR males did not show active

avoidance on the preference test day, whereas THC exposed WR males did. This result is

entirely unexpected, but does highlight some strain- and sex-specific effects of this drug.

Although it is unclear why this may have occurred, this is the first case of WR males

performing active avoidance in the DFCTC task as well as improvement in cognitive

performance following adolescent THC exposure. Why exactly this result occurred only

in WR males and only in the DFCTC task remains to be seen.

One final effect of THC included the increase of amygdalar size in LER males

exposed to THC (Fig 40B), with no alterations in fear- (Fig 29B& 30B) or anxiety-related

(Fig 31B, 32B and 33B) behaviour. This result in amygdalar volumes is, as well,

completely unexpected but appears robust as THC exposed LER males were significantly

different from both the VEH and CON group. The amygdala does contain CB1R

(Mailleux & Vanderhaeghen, 1992b) and is responsive to local application of

cannabinoids (Rubino, Guidali, et al., 2008). However, because this injection schedule

was prolonged as well as systemic, this could have altered amygdalar signalling and

increased the complexity of dendritic arborisations in the amygdala, resulting in increases

in volume. This could have been a compensatory mechanism in response to the removal

of THC following cessation of treatment. Whether this increase in size is due to higher

number of cells, lower density or increased connections remains to be seen. Clearly,

however, this is a strike against "bigger is better," as larger amygdalar volume did not

confer alterations in amygdala-dependent behavioural tasks.

Effects of injection

Strikingly, some behavioural effects, as mentioned in Table 6, were observed in both

THC and VEH groups, but not in handled controls. This was observed in all groups

except LER males on the MWT probe trial, where both THC and VEH groups

demonstrated the same amount of distance travelled and dwell time in the target as

compared to the other quadrants, which was not observed in CON groups (Fig 25).

Additionally, this was observed in WR males for the number of arm entries in the EPM

(Fig 32D), in WR females for discriminative freezing (Fig 29C) and LER males in active

avoidance (Fig 30B) in the DFCTC task. The effect of injection could be the result of

multiple effects. One includes the effects of chronic predictable stress during

adolescence, in the form of an injection. Interestingly, as discussed in Chap 3.1, few

injection effects were observed immediately following the injection period. The effect of

THC could in fact be of a more plastic nature than that of stress, and prenatal exposure to

stress has been shown to alter adult brain and behaviour (Takahashi, Haglin, & Kalin,

1992; Takahashi, Turner, & Kalin, 1992; Weinstock, 2007, 2011). THC could be altering

circuits on the short term, but these changes are no longer detectable following either a 14-day exposure or following aging to adulthood. In addition to the effects of stress, since THC was dissolved in ethanol, the vehicle solution contained a very low dose of ethanol. Such a low dose (0.16mg/kg) of ethanol has not been studied for long-term effects, to my knowledge, but any effects of injection could be the result of a chronic low dose of ethanol, stress elicited by the injection or the combination of the two. However, many studies include only a vehicle group as a control, and clearly here, we have some confounding influences of the injection itself. Indeed, this aspect of the experiment clearly highlights the importance of including a vehicle and a handled group as controls.

Possible caveats

With the mixed results achieved in this behavioural experiment, multiple confounding factors and caveats require addressing. First and foremost, it is possible that running these behavioural tasks within subjects may have resulted in accommodation and enrichment, which could have helped ameliorate any effects of THC through the effects of enrichment, i.e. anything that removes the rats from their standard "shoebox" environment. One possible method for overcoming the possibility of these effects would be to run each behavioural task in separate groups of rats. However, we do not believe that this is the case, as all these tasks measure different elements and connectivity between brain areas. You would also predict that, if these tasks are related, deficits would be observed in tasks run early in the testing phase and few deficits in tasks run last. This was not the case, as many differences were observed in the task we ran last, DFCTC. Additionally, we are unsure whether performance in one task predicts performance in another. Finally, whether or not rats were exposed to behaviour did not alter volumetric

measurements, therefore, it is unlikely that behavioural experience in one task helped overcome performance deficits in another. Only further testing could help address this possible issue.

Another caveat includes the injection time frame. Since all rats were injected on the first day of puberty, which occurred roughly 10 days earlier in females and roughly 2 to 3 days earlier in LER females as compared to WR females, but all began testing on postnatal day 90, females and males, as well as females between the two rat strains, would have had different amounts of time to adjust to neurological changes as a result of THC administration prior to behavioural testing. This could have allowed for compensatory measures that could have taken more time to occur in one group and not another. This time differential was accounted for by limiting the analysis to within strain and sex groups. As well, despite the difference among females, there was no strain difference in puberty onset for males, therefore males would have been tested with relatively the same amount of time between the cessation of injection and the start of behavioural testing.

The final concern that is raised with these results is, again, changes in the volume of certain areas did not necessarily result in alterations in behavioural function. This may simply be an issue with using volumetric estimates to predict the function of these brain areas.

Conclusions

Post-pubertal administration of THC does not produce robust effects across strains or sexes or necessarily within a strain and sex group. Many of the effects were entirely dependent on the sex and strain of that rat, and many effects of THC were confounded as

they also occurred concomitantly with effects of VEH. This is contrary to many findings from other research groups who demonstrate long-term, stable effects of THC. However, this may be explained by multiple factors, including the age of THC administration as well as the age of behavioural testing. Ninety days is considered "adult" for rats, although other researchers, who did find long-term effects of THC, began behavioural testing at approximately 75 days. Perhaps, the extra two weeks of aging allowed these circuits to return to a baseline. If this is the case, then the effects of THC clearly are not stable enough to accommodate two more weeks of home cage exposure in order to produce behavioural and morphological effects.

This study clearly demonstrates that the genetic background of the individual is a large determinant of whether adolescent THC exposure will have any effects on brain and behaviour in adulthood. As argued in previous chapters, this may help identify individuals who are at risk. Outbred strains can help by identifying multiple behavioural differences between strains, both intrinsically and in response to treatment. If say, LER tend to be more stress-reactive than WR, perhaps THC administration would more easily perturb the system, shifting LER to becoming more disease prone. Although our data does not specifically address this issue, perhaps a reasonable screening procedure for identifying at-risk individuals could include examining both baseline and stress-induced CORT responses.

Despite these speculations, this study clearly demonstrates that THC does not produce robust behavioural deficits across all groups of rats. Care should be taken in interpreting results from other behavioural experiments, as an effect of THC is not consistently present or expressed in all genetic backgrounds.

CHAPTER 3.3: LONG-TERM EFFECTS OF THC ON SENSITIVITY TO AMPHETAMINE IN ADULT RATS

Introduction

Marijuana is one of the most commonly used drugs of abuse in Canada (Health Canada, 2013). Although marijuana contains over 60 different cannabinoids (Abood & Martin, 1992; Ashton, 2001), the main psychoactive properties of marijuana can be linked to the neurological actions of a single compound, Δ^9-tetrahydrocannabinol (THC; Mechoulam, 1970; Razdan, 1986). Acute effects of THC include pain relief, relaxation, slowing the perception of time and giddiness (Abood & Martin, 1992; Piomelli, 2003). However, the long-term consequences of marijuana use are not as consistently observed. There is mounting evidence that marijuana use is associated with an increased risk of psychosis and possibly depression (Moore et al., 2007), with particularly troubling outcomes when use occurs during key developmental epochs, specifically adolescence. For example, in an animal model, chronic cannabinoid exposure during adolescence resulted in more adverse consequences in comparison to chronic exposure during the perinatal period or adulthood (Cha et al., 2007b; Cha, White, Kuhn, Wilson, & Swartzwelder, 2006; O'Shea et al., 2004). In addition to the increased sensitivity of the adolescent period to the effects of marijuana, sex may also play a role in the consequences of both short- and long-term marijuana use. In a longitudinal Australian study, women were five times more likely to develop depression and anxiety symptoms if they had consumed marijuana as adolescents, even when accounting for symptoms of depression and anxiety as children (Patton et al., 2002). Therefore, sensitivity towards the development of negative consequences of marijuana consumption appears to be

dependent on a number of factors, including the time period when consumption began as well as individual differences, including sex.

Another possible consequence of marijuana use during adolescence beyond affective and psychotic disorders includes engaging in other, more physiologically and sociologically harmful drugs of abuse, the so-called "gateway" hypothesis. THC administration has been shown to potentiate the response to other drugs of abuse and to be correlated with increased risk of using other substances of abuse (Fiellin, Tetrault, Becker, Fiellin, & Hoff, 2013; Gardner, 2002; Kandel, 1975; Panlilio, Zanettini, Barnes, Solinas, & Goldberg, 2013) potentially through the facilitation of brain reward mechanisms (Gardner, 2002; Gardner et al., 1988). However, the interaction between the development of the consumption of one drug of abuse and another is a complex issue. There are many individuals who use drugs of abuse, never develop an addiction and engage in the consumption of few if any other drugs of abuse. Indeed, individual differences have been shown to account for and predict the sensitivity to certain drugs of abuse, such as methamphetamine (Anisman & Cygan, 1975; George et al., 1991; Klebaur et al., 2001; Piazza et al., 1989; Schenk et al., 1986). How individual differences, such as sex or genetic background, contribute to the sensitivity to drugs of abuse and their interaction with other supposed "gateway" drugs such as THC merits further investigation.

Beyond sex differences, another interesting question may be answered through the use of different rat strains in order to model the effects of individual differences, such as genetic background, on sensitivity to drugs of abuse. Rat strains have been shown to vary on a number of measures, including those related to learning and memory (Andrews et al., 1995; Hort et al., 2000; Mohn & Russell, 1983; Pare, 1996; van der Staay et al.,

2009), anxiety (van der Staay et al., 2009), development (Keeley et al., in submission) and the size and cytoarchitectural characteristics of multiple brain areas important for behaviour (Holahan et al., 2007; Holahan et al., 2006; Keeley et al., 2010). Not only do rat strains differ innately, but they can differ in their responses to multiple interventions, including stress (Pare, 1989; Tohei et al., 2003; Woolfolk & Holtzman, 1995; Wu & Wang, 2010), novelty (Camp et al., 1994), and certain drugs of abuse (Anisman & Cygan, 1975; Camp et al., 1994; Deiana et al., 2007; Fujimoto et al., 2007; George et al., 1991; Onaivi et al., 1992; Ortiz et al., 2004). Therefore, given that diverse rat strains are used in addiction research and the existence of innate differences between multiple rat strains, it can be argued that including various rat strains in any one study can help determine the strength of a specific experimental manipulation. Additionally, the use of rat strains can model individual differences and help illuminate how one individual might be predisposed to the development of addiction while another is not.

As discussed in previous chapters, two widely used rat strains are WR and LER rats. These two strains have been shown to differ on a number of metrics, including those mentioned above. Since these two strains are widely used to study both the effects of drugs but also the development of addiction, it is important to understand if and how these rat strains differ in their immediate response to specific drugs of abuse. In addition, we will also consider the interaction between strain and sex. For example, juvenile pre-training was found to facilitate learning in LER males but not in LER females or WR males or females (Keeley et al., 2010). For a discussion on the importance of considering sex in relation to stimulants, see (Gulley & Juraska, 2013) for review. Therefore, the interplay between strain and sex could contribute to individual differences in the sensitivity to drugs of abuse.

With these concepts in mind, this study set out to determine the long-term

consequences of THC administration during the post-pubertal period in LER and WR

males and females. To this end, following systemic administration of THC for 14 days

after puberty onset, rats were allowed to age to adulthood (90 days) at which point all rats

were trained in a conditioned place preference (CPP) task to a sub-threshold dose of d-

amphetamine (AMPH), i.e. a dose that in drug-naïve rats did not induce CPP (see the

results from rats originating from Charles River in Chap 2.3). It was hypothesized that if

a particular strain and sex group was more sensitive to the rewarding effects of THC and

if THC exposure increased the sensitivity to other drugs of abuse, then those rats would

develop CPP to the sub-threshold dose of AMPH. However, if THC itself does not

increase the sensitivity of rats to other drugs of abuse, then no strain or sex group should

show CPP.

Methods

Subjects

Subjects were acquired, bred and handled as previously described (Chap 2.1). All

rats handling and procedures were done in accordance to the University of Lethbridge's

Animal Welfare Committee and the Canadian Council on Animal Care guidelines.

Puberty onset & drug administration

Puberty onset and group assignment were conducted as previously described

(Chap 2.1 & 3.1). Briefly, on weaning day, rats were assigned to their experimental

groups: handled control (CON, N = 8/strain and sex group), vehicle (VEH; N = 8/strain and sex group) or 5mg/kg THC (THC; N = 8/strain and sex group).

Injection procedures and handling were conducted as previously described (Chap 3.1). Vaginal smears were taken on every injection day. Following injection, rats were returned to their home cages. All rats received treatment for 14 consecutive days following determination of puberty onset. For a pictorial representation of the injection sites, see Appendix 1.

Determination of Estrous Cycle & Vaginal Cytology

Vaginal cytology and determination of estrous cycle was done as previously described (Chap 2.2). Vaginal smears were collected during all handling days as well as on all days of behavioural testing.

Conditioned place preference (CPP) to a sub threshold dose of amphetamine:
Apparatus & training

Rats used for CPP were previously exposed to the EPM, as reported and discussed in Chap 3.2.

Apparatus and training were conducted as previously described (Chap 2.3). A sub-threshold dose of 0.7mg/kg of AMPH (Sigma Aldrich, UK) was determined from the experiments from a previous chapter (Chap 2.3) in rats purchased from Charles River (N = 8/strain and sex group).

Perfusion & fixation

One week after the final day of CPP, rats were injected with a single 1mg/kg dose of AMPH and allowed to sit for 1hr. AMPH will reach the brain within 5min in rats and remain stable for roughly 1hr (Kuhn & Schanberg, 1978). *Cfos* protein is present in neurons that were active 20-30min after a particular experience. Therefore, it was assumed that any *cfos* protein signal detected 1hr after AMPH injection would represent the population of neurons active 30min after AMPH injection, while AMPH was still present in the brain. Changes in sensitivity to AMPH as a result of THC administration during adolescence was assumed to cause changes in the activation of the immediate early gene *cfos* in brain areas associated with reward (NAc) and contextual memory (dorsal HP) brain areas. Rats were euthanized, as previously described (Chap 2.3), with a single i.p. injection of sodium pentobarbital (120mg/kg) and transcardially perfused with approximately 150mL of 1xPBS followed by 4% PFA in 1xPBS. Following decapitation, brains were removed from the skull and immersion fixed in 4% PFA in 1xPBS. PFA was replaced 24hr after the perfusion with 30% sucrose and 0.2% Na azide in 1xPBS until sectioning. Brains were sectioned in a series of 12 at 40µm using a cryostat (CM1900, Leica, Germany) and placed directly into Eppendorf tubes containing 0.2% Na azide in 1xPBS until immunohistochemical staining.

Cfos immunohistochemistry & quantification

Cfos staining procedures were conducted as previously described (Chap 2.3). Briefly, free-floating tissue was washed in 1xPBS. This was followed by a 30min quenching step in 0.3% H_2O_2 in 1xPBS to remove any endogenous peroxidises. After

washing in 1xPBS, the tissue was blocked in 1.5% goat serum in 0.3% triton-X 1xPBS

for 30min. Following this step, the tissue was incubated in 1˚ antibody (Santa Cruz,

California) at a concentration of 1:1000 in 0.33% triton-X in 1xPBS with 1.5% goat

serum for 24hr. The following day, the tissue was washed followed by a 24hr incubation

period in 2˚ antibody (1:1000, anti-rabbit; Vector Labs, Canada). On the third day, tissue

was washed then placed in AB Complex (Vector labs, Canada) for 45min. Tissue was

washed then bathed for 5min in a 0.5% 3,3'-diaminobenzidine (DAB) solution in 1xPBS

with $NiCl_2$-$6H_2O$ in order to turn the solution purple and 0.05% H_2O_2. Sections were

washed then mounted on 1% gelatin coated slides and let to dry for 24hr. Slides were

dehydrated and coverslipped with Permount.

Representative images from nucleus accumbens and dorsal hippocampus were

taken and quantified using particle analysis in Image J (NIH, US). Regions of interest

were traced and *cfos*-positive particles were counted per unit area of the region of interest.

Borders of the regions of interest were defined by using the Rat Brain Atlas (Paxinos &

Watson, 2007). Representative tracings of ROIs can be found in Appendix 4.

Statistical analysis

Dwell time in the paired and unpaired contexts on the pre-exposure and preference

days were compared within strain and sex groups using drug condition (group) as a

between subjects factor. *A priori* hypotheses were established such that within each

treatment group for each strain and sex group, comparisons between the paired and

unpaired contexts were always conducted. For *cfos* quantification, between subjects

comparisons within strain and sex groups were conducted in order to determine the

effects of treatment on a specific strain and sex group. No *a priori* comparisons between groups were conducted.

Results

CPP to a sub-threshold dose of AMPH

Weights during CPP task - In order to determine the appropriate dosages, weights were recorded for each rat throughout the CPP task. Also to verify that groups were not reacting to AMPH adversely or differentially, weight throughout the AMPH administration period of the task was compared within each strain and sex group.

LER males and females and WR males showed no significant effect of drug group on weights during the injection period, as well as did not display significant overall weight gain or loss through the weighing period (Fig 41A, B & D).

WR females showed no overall effect of day or drug group but did display a significant drug by day interaction on weight gain during the AMPH injection period ($F_{(10, 105)}$ = 1.985, p = 0.042; Fig 41C). However, comparisons between groups on each day revealed no significant differences between groups. Therefore, prior drug administration did not affect weight gain or loss over the course of AMPH administration in the CPP paradigm.

Pre-exposure - For all groups, there was no pre-existing bias to spend more time in the paired or unpaired context, regardless of strain, sex or drug administration (Fig 42A-D). No interaction between drug and contexts were observed in any strain and sex group.

Preference - The CPP preference day determined if animals formed a place preference to a sub-threshold dose of AMPH (here, 0.7mg/kg). Only LER females overall spent significantly more time in the paired context ($F_{(1, 21)}$ = 17.483, p < 0.001; Fig 43A). No overall effect of group was observed. Individual comparisons within groups revealed that CON (p = 0.04) and VEH (p = 0.028) LER females spent significantly more time in the context paired with AMPH. No such difference was observed within LER females exposed to THC, although this value did approach statistical significance (p = 0.065). LER males, WR females and WR males showed no significant effect of drug as well as did not show an overall preference for one context over the others (Fig 43B, C & D).

Cfos immunohistochemistry

No significant effects were observed for any strain and sex group for representative images of nucleus accumbens (Fig 44) or dorsal hippocampus (Fig 45). Therefore, drug condition had no effect on *cfos* protein content in either of these areas as a result of a high dose of AMPH (here, 1mg/kg).

Discussion

In none of the strain or sex groups did THC result in long-term effects on the sensitivity to amphetamines. For this study, we examined behavioural, using CPP (Fig 43), and activational, using immediate early gene *cfos* protein expression (Fig 44 & 45), differences and neither of these metrics demonstrated significant alterations as a result of exposure to THC following puberty onset. We did see some strain and sex-specific

effects in our control groups, however these results were discussed in a previous chapter (Chap 2.3) and will not be reiterated here.

One possible explanation of these null results may include the drug of choice included in this study. AMPH increases dopaminergic tone when applied systemically, and specifically blocks the actions of monoaminergic transporters, allowing increased and prolonged concentrations of dopamine to remain in the synaptic cleft (Melega, Williams, Schmitz, DiStefano, & Cho, 1995; Sulzer, Maidment, & Rayport, 1993; Sulzer, Sonders, Poulsen, & Galli, 2005; Taylor & Snyder, 1970). Dopamine has important regulatory roles for directed motor activity but also is critically involved in anticipatory and reward-related circuitry (Kalivas & Stewart, 1991; Salamone, Correa, Mingote, & Weber, 2003; Squire et al., 2008). Amphetamines are a highly rewarding drug of abuse (Pickens & Harris, 1968; Yokel & Wise, 1975), and tonic use of amphetamines can lead to incredibly harmful and deleterious addicted states (Nutt et al., 2007). It is estimated that roughly 1% of Canadians have engaged in lifetime amphetamine use (Health Canada, 2013). Given the premise that THC would potentiate reward circuitry to be more sensitive to other drugs of abuse, increasing the propensity to form associations between AMPH and a context, AMPH was considered a reasonable drug of choice.

Mixed results have been observed previously with regards to the specifics of the priming effects of THC to amphetamine responses. Some studies report no effect (Arnold, Topple, Hunt, & McGregor, 1998) while others have demonstrated priming effects (Gorriti, Rodriguez de Fonseca, Navarro, & Palomo, 1999; Lamarque, Taghzouti, & Simon, 2001; Pryor, Larsen, Husain, & Braude, 1978) of THC or other cannabinoid agonists to the physiological response to amphetamine. Part of the discrepancies observed between all these studies and ours might be due to the dose, the duration of

THC administration, the age of the subjects, etc. One factor that is of particular interest is the timing between exposure to THC and that of amphetamine. One study, using different time lags between the last injection day of THC and AMPH administration, demonstrated a priming effect of THC on locomotor activity only 3 days after the last THC injection (Lamarque et al., 2001). This effect was no longer present 55 days after the last THC injection. In addition to this, only a group of high responders to novelty demonstrated priming. Clearly, not only does the time frame affect the response to AMPH following THC exposure but individual differences may also contribute.

Previous studies have demonstrated priming effects of THC to other drugs of abuse, although the timing of THC administration varied from young adults to adults. Increased heroin or other opiate self-administration has been observed following pre-exposure to CB1R agonists (Cadoni, Pisanu, Solinas, Acquas, & Di Chiara, 2001; Ellgren et al., 2007; Pryor et al., 1978; Vela, Fuentes, Bonnin, Fernandez-Ruiz, & Ruiz-Gayo, 1995), and these effects were dependent on CB1Rs (Ledent et al., 1999). This may identify the endogenous opiod system as being particularly sensitive to the long-term consequences of THC. Therefore, the long-term consequences of THC administration may be more sensitive to drugs of abuse that target the endogenous opiod system, such as heroin or prescription opiates. Indeed, given the increased abuse of prescription opiates (for example, oxycodone) in Canada (roughly 1%; Health Canada, 2013), perhaps more research into the interplay between the endogenous cannabinoid and opiod systems will help prevent the transition of using marijuana to opiates. Although, this does not preclude the possibility of interactions between other drugs of abuse and THC pre-exposure.

One possible explanation for the achieved results may be the specifics of the implemented paradigm. Here, we used sub-threshold doses of AMPH in order to

examine differential sensitivity to AMPH resulting from THC pre-exposure. Although

for these measures, we saw significant effects of rearing environment (as discussed in

Chap 2.3), we did not observe any effects of THC. Most of the above-mentioned studies

using amphetamines or opiates implemented self-administration paradigms or examined

locomotor activity. Whether rats self-administer drugs or are given non-voluntary

administration (i.e., i.p. injections) can drastically alter the rewarding properties of those

drugs (Markou et al., 1999). Despite this fact, CPP is a standard metric for determining

the rewarding properties of drugs of abuse and has been observed for multiple doses and

types of amphetamines and cannabinoids (Braida, Iosue, Pegorini, & Sala, 2004;

Maldonado & Rodriguez de Fonseca, 2002; Tzschentke, 2007; Zangen, Solinas, Ikemoto,

Goldberg, & Wise, 2006). Therefore, although self-administration has been used

typically in THC pre-exposure paradigms, CPP was still a justifiable candidate for

determining if THC pre-exposure would behaviourally alter the sensitivity of an

individual to amphetamines. Future experiments using this schedule should consider

allowing animals to self-administer either THC or amphetamines, examining correlations

between self-administration of both drugs. THC has proven problematic in self-

administration paradigms, however. Although some studies have shown self-

administration of THC in rats and mice, others require pre-treatment with other

substances (Braida et al., 2004; Takahashi et al., 2002; Tanda, Munzar, & Goldberg,

2000). Therefore, the rewarding properties of THC alone are debatable, with many CPP

tasks showing bimodal responses to THC, with low doses (<0.3mg/kg) eliciting CPP

behaviour and high doses (>1mg/kg) inducing conditioned place aversion, depending on

the research group (as reviewed in, Tzschentke, 2007).

One other possibility is the exact timeframe of THC administration. Although the focus of this thesis has remained on post-pubescent exposure to THC, it is possible that given other time periods of exposure (for example, prenatally), individuals may become more susceptible to the effects of AMPH. However, other research has shown that the adolescent period is particularly sensitive to the effects of THC, as compared to the juvenile or adult period (for example, Cha et al., 2007b). Indeed, adolescence is marked by maturation of limbic structures to adult levels of signalling in the absence of adult levels of cortical control (Casey et al., 2008). Therefore, adolescents appear to have the emotional processes of an adult without the executive control of these emotional processes. This fits well with observations of increased novelty seeking and risk-prone behaviour observed in adolescents. Indeed, this shift in behaviour has evolutionary bases, such that upon reaching sexual maturation, it is beneficial for certain individuals to leave their familial surroundings to venture further away to find a partner to conceive children with (Spear, 2007). Increased novelty seeking could potentially help increase the diversity of the gene pool with individuals seeking out mates different from their own family (Spear, 2007). Although this is not necessarily observed, this could explain the evolutionary basis for the differential development of limbic and cortical areas observed in adolescence and the associated changes in behaviour in response to novelty.

Conclusions

These experiments investigated the relationship between adolescent THC and the propensity to engage in other drugs use. This study demonstrates no such link, where no strain and sex group was more sensitive to AMPH following the long-term exposure of THC. This is surprising, given the sensitivity of LER found in Chap 2.3 to developmental

perturbations (rearing environment) on CPP behaviour in response to AMPH. That may

simply identify LER as sensitive to early-life alterations and not to alterations in

adolescence/post-pubescence. In addition, WR were relatively stable in their behavioural

profiles, where rearing environment and/or THC administration did little to alter their

response to a sub-threshold dose of AMPH. Here, it is possible the chosen drug (AMPH)

may have contributed to the achieved null results, as more often, it is reported that THC

interacts with the opiod system. Additional research using our injection paradigm will

help elucidate if this is the case. Although our results do not demonstrate one group as

being more sensitive to the effects of THC in relation to sensitivity to AMPH, it does

identify groups that are immune or at least resilient to these effects. Further research into

discovering what determines resiliency in these groups may help identify mechanisms

that can be protective in at-risk groups for the development of addiction.

CHAPTER 4: PRINCIPAL COMPONENT ANALYSIS – BRIDGING THE GAP BETWEEN STRAIN, SEX AND DRUG EFFECTS

Introduction

Analysis of variance (ANOVA) is a statistical tool used for the identification of significant differences between group means. ANOVA is used extensively throughout the literature and is an appropriate measure when examining a small number of variables. However, as the number of variables increases, the interpretation of interaction effects between variables can become challenging. Indeed, with the advent of automation for the collection of multiple variables of interest in behavioural neuroscience, the number of variables has increased in size. With this in mind, it is important to simplify data and identify which variables best describe the variance in the system of interest.

As discussed in detail in previous chapters, multiple factors can contribute to the variance between individuals, including strain, sex, previous exposure to drugs of abuse, and the size of various brain areas. Each of these factors can act independently but can also have effects in one individual (for example, in females) and not another. Given the experimental design of the present experiments, as discussed in Chap 3.2, it was difficult to justify the use of ANOVAs in order to compare for the effects of drugs across strain and sex groups. In addition, the interaction effects of strain, sex and drug were not necessarily of interest and would have included some potentially superfluous and not necessarily informative interactions. Here, the numerical age of the animals differed for the injection period, also precipitating differences in time delay from drug exposure to behavioural testing. However, whether these factors interact and which factors are most important for each of these behavioural tasks are pertinent questions. The specific

analyses used in previous chapters did not address the cumulative effects of these factors and specifically, the proportion of variance that could be accounted for by each variable.

To this end, principal component analysis (PCA) was used. PCA is used to simplify data sets to the variables that drive observed group differences to enhance interpretability (Quinn & Keough, 2002; Shlens, 2005). PCA is a nonparametric statistical technique that generates vectors, based on the inputted variables (Wold, Esbensen, & Geladi, 1987) which can help identify the features and structure of multivariate data, including which variables account for high proportions of variance. In a theoretical data space, all variables, x, can be represented in an x-dimensional space. PCA attempts to simplify this data space to a k number of eigenvectors, which consist of combinations of the x variables, into a k dimensional space, allowing for ease in interpretation as well as graphical representation. To this end, you can determine which variables are most valuable to your question of interest. For example, PCA has been applied to identify the primary features in the EPM (Ramos, Berton, Mormede, & Chaouloff, 1997) and the MWT (Wolfer & Lipp, 2000). Both analyses revealed that specific behavioural strategies were best able to explain the variance in the data set, above that which was explained by genetic differences.

In this case, we analyzed the data set for each behavioural task to determine which variables accounted for the largest proportion of the variance. Therefore, we ran separate PCAs for each behavioural task discussed in earlier chapters and included strain, sex and drug effects. Brain volumetrics and *cfos* measurements were also included to determine the contribution of each of these values to the variance in the data. Additionally, the contribution of litter effects and parity status (i.e. nulliparous versus multiparous dams)

were also included given their influence on physiology (Fleming, O'Day, & Kraemer, 1999).

Methods

Subjects, behavioural tests and volumetrics.

All subjects were used as described from previous chapters (Chap 2 and 3). Data from the skilled reaching task (SRT), the Morris water task (MWT) and discriminative fear-conditioning to context (DFCTC) with corresponding brain volumetrics for hippocampus (HP), dentate gyrus (DG), CA1, CA3, orbital frontal cortex (OFC), medial prefrontal cortex (mPFC) and amygdala (AMYG), were assessed. *Cfos* activation in dorsal HP and nucleus accumbens (NAc) were used in lieu of brain volumetrics for rats run in elevated plus maze (EPM) and conditioned place preference (CPP) because these tasks and measurements were conducted in a separate group of rats.

Data pre-processing

PCA requires that all values exist on the same scale and are normalized relative to their mean and standard deviation. Given this requirement, all raw values were converted to Z-scores and normalized relative to the maximum absolute Z-score for that variable. Following pre-processing, all values occupied a range between -1 and 1 and had a mean of 0.

Statistical analysis

All statistical methods were conducted in SPSS (IBM ver21) unless otherwise mentioned. Separate PCAs were conducted for each behavioural test in order to determine the contribution of each factor and to keep the number of variables low. Variables of interest were identified for each behavioural test based on experimental experience, the results achieved in previous chapters' ANOVAs and *a priori* assumptions.

All chosen variables in each behavioural task were subjected to a PCA with direct oblimin rotation, an oblique rotation method that assumes correlations between variables and aids in the simplification of the data space generated by the eigenvectors (Gorsuch, 2013; Vogt, 2005). The Kaiser-Meyer-Olkin measure of sampling adequacy was used, and all PCAs demonstrated a value of > 0.6, which is within acceptable parameters. Bartlett's test of sphericity was also conducted, and for all PCAs, this test was significant ($p < 0.05$), which is within acceptable parameters. The number of factors (k) was determined through multiple tests. Only factors with eigenvalues larger than 1 were kept. Additionally, Monte Carlo PCA for parallel analysis was implemented (software developed by M.W. Watkinson, 2000) which generated random sets of eigenvalues. If the randomly generated eigenvalues were larger than the eigenvalues present in our results, they were not included in the final model. Therefore, once k was determined, PCA was rerun with k values.

Factor loadings > 0.5 were considered to contribute to the factor and were included in the interpretation.

Results

SRT

Four factors that met our inclusion criteria emerged from the analysis of brain volumetrics and SRT behaviour representing 63% of the total variability. Factor 1 correlated with all measures of brain volumetrics for HP, and associated areas (DG, CA1 and CA3), and AMYG and the sex of the individual. Factor 2 correlated only with the strain and litter effects. Factor 3 correlated with performance in SRT, and Factor 4 received the most contributions from the size of prefrontal cortex areas (mPFC and OFC). The results can be seen in Table 8.

MWT

For the MWT, 3 factors met our inclusion criteria, which represented 47% of the variance in the data set. Factor 1 received the most contributions from latency across all days of training to a submerged platform, as well as strain and litter effects. Factor 2 comprised the size of hippocampal areas (DG, CA1 and CA3), total HP and AMYG. Factor 3 was formed entirely of the ability to navigate to a visible platform and the sex of the individual. The results for the PCA can be found in Table 9.

DFCTC

Five factors emerged for the PCA with the DFCTC data that accounted for 63% of the variance in the data set. Sex and the size of the AMYG, HP and hippocampal subregions (DG, CA1 and CA3) loaded on Factor 1. Factor 2 was correlated exclusively with the dwell time in contexts that had or had not yet been paired with aversive stimuli. Sex and litter loaded on Factor 3. Factor 4 was correlated with the size of prefrontal cortex subregions (mPFC and OFC), previous exposure to drug and the amount of

baseline freezing behaviour. Finally, the effects of being born to a nulliparous or multiparous dam and the amount of freezing in the paired context loaded on Factor 5. The results for the PCA for DFCTC can be found in Table 10.

EPM

EPM, followed by CPP, was conducted in a separate group of rats from that were exposed to SRT, MWT and DFCTC. All rats in these two experiments were born of nulliparous dams, therefore litter order was not included.

Three factors accounted for 78% of the variance in the data set for EPM. Factor 1 was correlated with the absolute and relative time spent in either the open and closed arms as well as, the less anxious behaviour, number of arm entries. The absolute and relative number of arm entries and the number of closed arm entries, an indicator of increased locomotor activity were loaded on Factor 2. Factor 3 was comprised of the strain and the litter of the individual. These results are summarized in Table 11.

CPP

A PCA with 3 factors accounted for 66% of the variance in the data for the CPP task. Factor 1 was comprised primarily of dwell time in either context for both pre-exposure days and preference days. Strain and litter loaded heavily on Factor 2. The amount of *cfos* activation in the dorsal hippocampus and the sex of the individual correlated highly with Factor 3.   These results are summarized in Table 12.

Discussion

PCA can help identify which variables contribute to the variance in the data set. Given the large number of variables considered in the present study, it was of interest to determine which of the variables were the most influential as well as aid in the interpretation of the extensive and often confusing results reported in previous chapters. Five factors appeared throughout the PCAs for each task: early-life experience, the volumes of the HP, DG, CA1, CA3 and the AMYG, sex, performance and motor skill. Each of these factors will be discussed in turn in relation to its contribution to the variance in the data for each behavioural task. Task-specific factors, which were only observed in a small fraction of the observed behaviours, will also be discussed. Finally, the benefits and shortcomings of PCA will be discussed.

Early-life experience

One factor that loaded heavily on all the behavioural tasks was early life experience. This factor was primarily formed from the variance associated with strain and litter. Strain has been included here as early-life experience for one main reason: although the genetic background of strain may be contributing to the variance in the data set, it is also possible than any effects of strain were products of the strain of the mother, not of the individual. Indeed, strain-dependent maternal behaviours have been observed previously (Moore, Wong, Daum, & Leclair, 1997), and maternal behavioural can have large effects on behavioural (Liu, Diorio, Day, Francis, & Meaney, 2000), neuroanatomical (Bredy, Zhang, Grant, Diorio, & Meaney, 2004; Caldji, Diorio, & Meaney, 2003) and neuroendocrine (Francis, Diorio, Liu, & Meaney, 1999; Liu et al., 1997) functions in rats. Since no cross-fostering across strains was included in this

analysis, it is not possible to account for the possibility that part, or all, of the variance in the data may in fact be due to the effects of being reared by either a WR or LER mother.

Given this possibility, the factor that included the variance between strain and litter was named, "early-life experience," and this factor accounted for 23% of the variance in the MWT, 19% of the variance in the CPP task, 16% of the variance in the SRT task, 12% of the variance in the EPM and 11% of the variance in DFCTC. Although this factor was not always the first factor in each PCA, it was the only factor to be included in the analysis across all data from behavioural tasks. In light of these results, caution must be taken in interpretation across rat strains, and both strain and litter effects must be considered. Litter effects are a well-known phenomenon in ontogeny studies (Festing, 2006; Holson & Pearce, 1992), and the present experiments attempted to minimize the effects of litter as much as possible. This was done by including subjects from one litter to multiple behavioural groups, which was suggested previously (Zorrilla, 1997). In our study, in a litter of 6, two subjects were assigned to the handled control group, two subjects to the vehicle group and two subjects to the THC injected group. This methodology is recommended to account for litter effects. Despite this attempt, clearly litter does play a significant effect in the behaviour of individuals.

Litter effects can be the result of multiple factors. Not only do litter effects include the influence of prenatal environment, which if altered, can alter brain and behaviour, it also includes the perinatal and preweening environment. Multiple interventions during the perinatal period can have long-term consequences on brain and behaviour (Kolb, Forgie, Gibb, Gorny, & Rowntree, 1998), including stress (Lupien, McEwen, Gunnar, & Heim, 2009) or exposure to drugs (Fernandez-Ruiz, Berrendero, Hernandez, & Ramos, 2000).

Clearly, care must be taken when interpreting the results that do not consider litter effects as well as preweening environment.

Despite the possibility of the strain differences being reliant on maternal behaviour, it is clear that strain differences can account for differences in behaviour (for example, van der Staay et al., 2009). This has been discussed extensively in earlier chapters. Despite this discussion, PCA offers a compelling argument as the amount of variance accounted for by strain can help account for differences observed across all of these widely used behavioural tasks. If readers were not convinced earlier, it is clear that strain is a vastly important factor and should always be considered when examining the literature and comparing across research groups.

Performance

The next factor that was commonly seen across behavioural tasks was dubbed, "performance." This factor was typically composed of variables assessing learning and retention in each behavioural task. For example, in the MWT, performance was composed of the latency to reach the platform across multiple training days. Performance accounted for 34% of the variance in the CPP task, 23% of the variance in the MWT, 15% of the variance in the DFCTC task and 12% of the variance in the SRT task. Performance accounted for a high amount of variance across most of these metrics demonstrating that, if a rat is showing high performance in one metric, they are likely to show high performance on another within a behavioural task. This is not surprising, as many of these measures assess learning across multiple days and are not independent. For example, latency to reach the platform on the second day of MWT training is related to the performance on the first day.

However, not all variables from each behavioural task were included in the PCA. Typically, only values that are found to be either significantly different in earlier analyses or, more importantly, variables that are integral to assessing performance in the task of interest, were included in the PCAs. Although it would have been beneficial to run a PCA including all metrics in our behavioural tasks, the number of subjects did not permit this analysis. It is recommended that you have 5-10 subjects per variable of interest when using PCA (Field, 2013), and with 96 subjects included in this experiment, only 19 variables, at most, were advisable to be included to maintain enough statistical power for the PCA. Additional research with more subjects may be able to determine the interrelatedness of these tasks. It would be interesting to determine whether performance in the MWT would load on the same factor as performance in the DFCTC, although an analysis of that nature requires a higher number of subjects.

HP & AMYG volumes

One factor that accounted for some of the variance in the behavioural tasks was the volume of the HP, its subregions (DG, CA1 and CA3) and the AMYG. If these variables loaded heavily (i.e. > 0.5) on a factor, they always loaded together. In the SRT, the volumes of the HP, its subregions and the AMYG accounted for 25% of the variance. This is a surprising feature for the SRT task, as it is not reliant on hippocampal and amygdalar function. This may be a reflection of brain size and not necessarily volumetrics, as the volumes of these brain areas will increase with increasing brain size, and brain volume was not normalized for these measures. However, given that OFC and mPFC did not load heavily with HP and AMYG, this is likely not the case. Alternatively, even though the HP and AMYG are not necessary for the accurate learning and

expression of SRT behaviour, they are still acquiring representations and participating in some aspects of the behavioural expression of this task (Driscoll, Howard, Prusky, Rudy, & Sutherland, 2005; Sutherland, Lehmann, Spanswick, Sparks, & Melvin, 2006). This has been seen in other learning and memory tasks (Holahan, Hong, Chan, & McDonald, 2005; McDonald, Lo, King, Wasiak, & Hong, 2007; White, Packard, & McDonald, 2013). Instead, given the relationship of these two areas to learning and memory as well as emotional control (Ferbinteanu & McDonald, 2000), perhaps their relationship in this task is much more about performance in general and not necessarily in relation to motor skill. Additionally, given that only two measures of performance were considered in this task, it simply could be that differences between these individuals, on all the metrics, was more highly accounted for by the size of these brain areas. In PCA, all of these factors were included in the same analysis, therefore, it could be that the largest proportion of the variance between individuals was due only to the size of the hippocampus, its subregions and the amygdala, completely independent of performance in the SRT. This is likely because no significant differences were observed between any groups in SRT (Chap 2.2) regardless of drug exposure (Chap 3.2).

For the DFCTC tasks, the volume of HP, its subregions and AMYG accounted for 19% of the variance in a factor that also included sex. This is not surprising given the dependence on the integrity of hippocampus and amygdala for performance in the DFCTC task (Antoniadis & McDonald, 2000). Additionally, sex differences were observed in hippocampal volume and amygdalar volume in these same rats (see Chap 2.2 and 3.2), therefore it is not surprising that these variables all contribute in a significant way to the same factor which accounts for a proportion of the variance. Clearly, the size of the hippocampus, its subregions and the amygdala are important contributing factors to

performance in the DFCTC, and these volumetric estimates are closely linked with the sex of the individual.

Sex

One variable that accounted for a large proportion of the variance was sex, although it often loaded on a factor with other variables. For the MWT, the sex of the individual loaded uniquely on the third factor and accounted for 9% of the variance in the data. This is not surprising given the effects of sex (as discussed in Chap 2.2) on both performance in the MWT (Jonasson, 2005) but also sex differences in the HP (McEwen & Milner, 2007), which is essential for acquisition and recall in the MWT (Moser, Moser, Forrest, Andersen, & Morris, 1995). Clearly, sex plays a large role in the variability in this task, which has been well discussed and described elsewhere (for example, Jonasson, 2005). Research using this task should consider not only the inclusion but also the discussion of sex differences, as this phenomenon is not isolated to rodents and has been observed in humans (for example, Astur et al., 1998; Astur et al., 2004; Sandstrom, Kaufman, & Huettel, 1998). This has clear implications for human health and disease progression, as the hippocampus is a target for many disease of aging, including Alzheimer's disease (West, Coleman, Flood, & Troncoso, 1994), and some diseases of aging show sexual dimorphisms (Gao, Hendrie, Hall, & Hui, 1998), although the longer lifespan of women (Mascitelli, Pezzetta, & Sullivan, 2006; Waldron, 1976) could confound these results.

In addition to the sex effects mentioned above for the MWT and DFCTC task (as discussed in the HP and AMYG volume section), sex also contributed to the third factor in conjunction with hippocampal activation following exposure to 1mg/kg AMPH for

variables measured in the CPP task. Here, these two variables contributed heavily to

factor 3, which accounted for 13% of the variance in the data. Here, like with MWT,

hippocampal measurements were paired with sex, given the hippocampus' role in both

the establishment of contextual memories and specifically the CPP task (Ferbinteanu &

McDonald, 2001) and the effect of sex on hippocampal gross and fine morphology

(McEwen & Milner, 2007).

Motor Skill

All of the tasks discussed here rely on motor skill and locomotor activity, where

issues with sensory processing and motor skill would have consequences on performance,

which could be interpreted as memory or learning deficits. In the MWT, motor skill, as

assessed by latency to reach the platform on two days of visible platform training,

contributed highly to the second factor, which accounted for 15% of the variability in the

data. Although no significant differences between groups were observed on the visible

platform day, clearly, the actual motor performance of an individual contributes highly to

the variance in the data set. This is a prime example of the information that can be

gleaned by PCA in comparison to standard parametric analyses.

Locomotor activity or motor performance also contributed highly to the fourth

factor in the DFCTC analysis. Here, in conjunction with the volume of prefrontal cortex

areas and group effects, motor skill accounted for 10% of the variance in the data.

Although this data is difficult to interpret, there is a relationship between DFCTC freezing

behaviour and the OFC, where lesions of OFC result in non-discriminative and elevated

freezing in the unpaired context (Zelinski, Hong, Tyndall, Halsall, & McDonald, 2010).

Further investigation into the relationship of OFC and motor activity in this and other behavioural tasks may help clarify this result.

Task-specific weightings

Certain weightings of variables were observed exclusive to a behavioural task. In the EPM, measures that indicated decreased anxiety loaded heavily on the first factors and accounted for 45% of the variability in the data. The second factor consisted of measures that would indicate increased anxiety and accounted for 21% of the variability. These task-specific weightings demonstrate that primarily, measures that are associated with decreased anxiety account for the majority of the variance in this particular data set. This is contrary to earlier work assessing EPM using PCA in both mice (Rodgers & Johnson, 1995) and rats (Ramos et al., 1997) which both discovered that measures of anxiety loaded primarily on the first factor and accounted for the highest proportion of variance in the data. However, Rodgers and Johnson (1995) acknowledged that this could partially be mediated through differences in procedures, including handling, time of testing and species used. Additionally, Ramos et al. (1997) concluded that the loadings of these factors differed by strain. Here EPM appeared to be a better metric for locomotor activity than anxiety, although this may be a function of our training procedure as well as specific to the rat strains chosen in our study.

Little to no effect of drug exposure

One interesting fact that should be discussed is the absence of high loadings that the variable "group" had on any factor. Group, which represented whether or not a rat had been exposed to THC, vehicle or control conditions, only loaded highly on the fourth

factor for DFCTC, and that was also in combination with other factors. Despite the multiple studies indicating long-term consequences of THC during adolescence (for example, Rubino, Guidali, et al., 2008; Rubino, Realini, Braida, Alberio, et al., 2009; Rubino, Vigano, et al., 2008; Rubino et al., 2012), in this sampling of 96 rats, the variability in the data set is barely accounted for by the exposure of these rats to THC in adolescence. These results, in combination with the results achieved in Chap 2.2, 2.3, examining strain and sex differences and Chap 3.2 and 3.3, examining the long-term consequences of THC, demonstrate that the effects of THC on rat brain and behaviour are not as harmful or effective at altering the brain or behaviour as previous literature or public opinion would predict, and strain and sex accounted for and produced more differences in behaviour. It is in this author's opinion that although it is not recommended for adolescents to engage in drug use of any kind, due to the changing and maturing brain during this time period (Casey et al., 2008), clearly the harmful effects of THC from adolescence to adulthood may only have negative consequences in certain subgroups within a population. Here, when using a standard model that should be representative of the norm, no long-term effects were observed. Rearing environment and genetic background accounted for more individual variability than whether or not an individual was exposed to THC as an adolescent. It is of this author's opinion that this demonstrates that the long-term consequences of THC on adult brain and behaviour are negligible overall, but certain subgroups are more sensitive than others. Research should instead attempt to determine which factors confer altered sensitivity or resilience to a drug of abuse. For example, alterations in the HPA axis in the prenatal environment could potentially reset HPA set points which could differentially alter the sensitivity to this drug of abuse, which has a defined relationship with the HPA axis (for example, Hill et al.,

2010). THC during adolescence should perhaps be examined in terms of a factor, like the

AD co-factor model (McDonald, 2002; McDonald, Craig, & Hong, 2010), which

contributes to a mental health state, but that, in a population with no genetic risk, will not

likely result in a disease state unless presented concurrently with other factors. Only time

and additional research will be able to shed light on this matter.

PCA

PCA is an invaluable tool that can help determine the dynamics of a system.

Although it does not address exact hypothesis testing, as can be done using ANOVA, it

can help identify which variables account for large proportions of variance in data sets

which can then inform the choice of statistical tests among variables for ANOVA testing.

It is not a "straightforward" tool, like ANOVA, as it does not have clear rules about

significance levels. Instead, it can act as a qualitative analysis, shedding light on which

variables contribute to the differences between individuals. It can help shed light on

relationships between variables as well as save time when collecting data for analysis, so

researchers can focus on variables that clearly contribute and are informative of other

variables. PCA is a tool in a statistical toolbox that can help measure and describe some

aspects of the data set.

For this particular study, it has helped demonstrate the critical variables that

contribute to the variance in specific data sets. As discussed above, it identifies the effect

of early life experience, including the effects of litter and strain, as critical to the variance

in these behavioural tasks. Additionally, it demonstrates the lack of effect of drug

exposure, despite discussions in the scientific literature and media that state otherwise.

PCA demonstrated here, that across multiple behavioural tasks, THC experience

following puberty onset contributed very little to the variance overall. The impact of this

finding will be discussed in conjunction with the results of earlier chapters in the final

general discussion.

CHAPTER 5: GENERAL DISCUSSION

This study examined strain and sex differences in brain and behaviour between laboratory rats. First, strain differences during in development were examined in a myriad of behavioural tasks and in response to low doses of amphetamine. In addition to strain differences, males and females were examined in this same set of experiments to identify sex differences amongst these measurements. Finally, separate analyses were conducted within strain and sex groups to examine the effects of adolescent THC exposure. This aspect of the study sought to determine if a specific strain or sex group was more susceptible or resilient to the effects of THC as compared to the others. Finally, all of these factors were combined in principal component analyses to determine the contribution of these factors to the variance in the data. Each of these factors (strain, sex and drug), and their contribution in the PCA, will be discussed in turn as well as the contributions of this study to our current understanding of the mammalian brain and behaviour. Alternative explanations and interaction effects, as possible caveats of this study, will then be discussed. Finally, future directions and implications will be discussed as well as how this study might impact policy and decision making in a broader, health care perspective.

Effects of strain

One of the most widely observed effects in this series of studies on brain size and behavioural performance was the effect of strain. Strain differences were observed during the juvenile period, such that LER females reached puberty sooner and gained more weight following puberty onset as compared to their WR counterparts. Differences

between LER and WR were observed across all behavioural tasks. In the EPM, WR were more active overall, and individual comparisons revealed that this effect was due to differences between females of either strain. Additionally, female WR were less anxious than female LER as measured by one metric of EPM. LER outperformed WR on MWT, including measures of acquisition, probe and mass training, with no differences observed in visible platform training. LER also outperformed WR in discriminative freezing and active avoidance in the DFCTC paradigm. Female LER outperformed their WR counterparts in discriminative freezing. WR had larger hippocampal and amygdalar volumes despite the overall superior performance of LER to that of WR. In response to a low dose of amphetamine, CPP behaviour in LERs was more sensitive to rearing environment, such that whether they were raised at the CCBN or at Charles River significantly changed their CPP behaviour, more so in male LER. Differences on the CPP task occurred in conjunction with increased activation of the immediate-early gene *cfos* in the dorsal hippocampus of WR females in comparison to their LER counterparts. Finally, PCA analysis revealed that strain, always in conjunction with litter and once in conjunction with performance, accounted for 12 to 23% of the variance of the data observed in rats through all behavioural tasks. Strain differences were consistent, although these differences occasionally were dependent on sex.

Strain differences have been observed across a variety of tasks, including variants of those discussed here (for example, van der Staay et al., 2009). There are robust, latent genetic differences between these two strains, as a result of years of breeding within a particular strain group, with little to no outside genetic influences. Because both of the strains here are outbred (Krinke, 2000), meaning that there are no brother-sister pairings to maintain the strain, within the strains, there is genetic diversity. Despite the influence

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of genetics, strain differences can originate from more than just the genetic diversity between individuals (e.g. maternal effects; Liu et al., 2000; Liu et al., 1997; Moore et al., 1997). Using a cross-fostering study could have helped to determine whether these strain differences were a function of maternal strain. Given our results in the CPP task, the LER strain appears to be particularly sensitive to the effects of rearing environment, which may identify them as more susceptible to the effects of cross fostering. Only additional research will help determine if this is the case. Despite these possibilities, LER drastically outperform WR in a myriad of behavioural tasks.

LER and WR are not the only two strains of rats used in behavioural neuroscience. Indeed, multiple strains persist, some of which are offshoots of outbred or inbred lines that have become inbred or outbred, respectively. Some rats have been bred to be seizure-prone or -resistant (Racine et al., 1999), drug sensitive or insensitive (Deiana et al., 2007), and some closely resemble wild-caught rats due to multiple outbreedings with wild rats (Krinke, 2000). Despite access to many possible strains, LER and WR have been directly, or in combination with other strains, compared for a multitude of tasks and behaviours (Fujimoto et al., 2007; Holahan et al., 2007; Holahan et al., 2006; Hort et al., 2000; Keeley et al., 2010; Nakajima, 2014). However, data on SRT, DFCTC and our variant of the MWT in conjunction with volumetric estimates have never been conducted. Furthermore, this is the first report of a difference in puberty onset between these two strains. Our results are unique but fit well in the literature, which appears to agree that WR are not as proficient in cognitive tasks as LER (Holahan et al., 2006; Keeley et al., 2010). This identifies LER as more easily trained to perform these tasks. Researchers should consider which strain of rat to use and choose wisely, as it can be difficult to detect cognitive differences in a strain that does not perform, or takes

longer to perform, in a specific task. Further, the duration of training may alter the underlying neurophysiology of task performance. This does not mean that WR should not be included in any behavioural neuroscience research *per se*, but that care should be taken to interpreting results achieved in the subset of behavioural neuroscience studies that use WR in learning and memory based tasks. On a brighter note, determining the exact origins of these strain differences, i.e. how exactly the different genetic backgrounds interact with environment, may help in the understanding of independent mechanisms of behavioural diversity. Indeed, comparing genetically distinct populations could be a useful endeavour in the pursuit of that knowledge.

What do these results mean for animal research? Although it has been argued that the use of any albino outbred strain is an irresponsible and non-defendable choice (as discussed in Lockard, 1968), there are others who come to their defence (Clause, 1993). Reports of the use of albino strains, including WR, in research began in the early 1900s. Using inbred strains of rats may ease the identification of genes of interest for particular behavioural styles or differences in volumetrics, but outbred strains may be more representative of the diverse human population. It must be acknowledged that strain differences do exist, and depending on the strain used, they may either amplify or mask the effects of experimental manipulations. This can also help determine which effects are robust (i.e. those that occur across rat strains). This point will be discussed further in relation to the effects of THC.

It is also of interest to note that some of these strain differences occurred only in one sex and not another. Indeed, for some measurements and some behavioural tasks, many differences were exclusively viewed in females and not males. The bulk of studies exploring strain differences are done almost exclusively in males therefore it is of interest

to determine why these behavioural differences of strain would occur in one sex and not another.

Effects of sex

Sex differences were less ubiquitous than strain differences but were still present in most of the measures discussed here. In juveniles, there was an obvious, and expected, sex difference in puberty onset (Engelbregt et al., 2000), which was accompanied by larger volumes of the hippocampus and its subregions in males. In adults, behavioural differences were observed in all the tasks save for CPP. In the EPM, females were more active than males overall. Sex differences in the MWT were restricted to the high-performing LER strain, were LER males outperformed females early in acquisition as well as early in the acquisition of a new spatial location on the mass training day. In DFCTC, females overall performed better in discriminative freezing, although this difference was accounted for largely by results achieved within the LER strain. The sex differences in brain volumes observed in juveniles did not persist to adulthood, as females were found to have larger CA1 and amygdalar volumes. This amygdalar volume difference was accounted for by sex differences within the WR strain. Finally, using PCA to group all of these factors together, sex was found to load with hippocampal and amygdalar measurements as well as motor skill. However, sex did not contribute to any main factors for EPM. Despite this, sex contributed to factors that accounted for between 9 and 25% of the variance in the data.

Sex differences are believed to originate from the activational and organizational effects of sex hormones. Early in development, exposure to sex hormones determines the sex of the individual (Carrer & Cambiasso, 2002). However, additional periods of sex

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hormone exposure determine secondary sex characteristics, and exposure to sex hormones during the adolescent period can mediate some of these effects. Organizational effects of sex hormones may account for behavioural differences in a multitude of tasks. However, given the well-conserved observation of spatial learning and memory differences between the sexes, the organizational effects of sex hormones on spatial learning and memory have been extensively discussed (Jonasson, 2005). Dependent on the developmental time of the manipulation, genetically female rats can develop male-typical hippocampi (Stewart & Kolb, 1994; Zuloaga et al., 2008), neurotransmitter levels (Mitsushima, Takase, Funabashi, et al., 2009) and strategies in spatial tasks (Williams et al., 1990).

In addition to the organizational effects, transient exposure to sex hormones during non-critical time periods can result in local, small-scale and plastic changes in the brain. Again, this has been studied extensively in the hippocampus, where local or systemic application of sex hormones can alter hippocampal cytoarchitecture (Roof & Havens, 1992; Woolley, 1998; Woolley & McEwen, 1992) as well as behavioural strategies in hippocampal-dependent tasks (Frye, Duffy, & Walf, 2007; Fugger et al., 1998; Luine et al., 2003; Roof & Havens, 1992; Vanhaaren et al., 1990). The activational effects of sex hormones can be studied through artificial elevations or applications of sex hormones using systemically or local application to brain regions of interest. However, naturally fluctuating levels of sex hormones observed in females offers an opportunity to study the activational effects of these hormones using endogenous concentrations and pulsatile changes in these hormones on brain and behaviour. Differences in brain and behaviour across the estrous cycle in rats (Conrad et al., 2004; Stackman et al., 1997; Woolley & McEwen, 1992) and the menstrual cycle in humans (Hampson, 1990; Hausmann et al.,

2000; Kimura, 1996; Protopopescu et al., 2008; Rosenberg & Park, 2002; Schoening et al., 2007) are apparent, although the results are not entirely clear. In rats, at least, changes in hormone levels over the course of the naturally fluctuating estrous cycle does appear to alter chosen behavioural strategies between spatial and non-spatial strategies (Korol, 2004; Korol & Kolo, 2002; Korol et al., 2004).

Here, estrous cycle infrequently altered behavioural differences within females. This may be due to the features of the experimental design itself and not indicative of a conflict with the literature. Here, we did not cycle synchronize females to induce the same hormones levels at the same time in each behavioural task. Even if this had been conducted, the results could have been problematic to interpret, as effects on one day of behavioural testing could then be amplified or masked by the effects of estrous cycle. Another option would have included, following determination of estrous cycle, subsets of rats in different phases of the estrous cycle could have been run and counterbalanced across days, such that 2 out of 8 LER females on each day would be in estrous, 2 would be in metestrous, 2 in diestrous and 2 in proestrous. The same could have been conducted for WR. However, implementing this strategy would have resulted in small group sizes (N = 4/estrous cycle group, if no strain differences were observed), which would have decreased the power of any analyses. Instead, we chose to run all rats at the same numerical age, leaving phase of estrous cycle as a random variable. This method did result in small group sizes for each estrous cycle phase across certain days and not others. However, because we did conduct most behavioural tasks, except EPM, across multiple testing days, it was assumed that estrous cycle just increased the variance in the data set. Indeed, using estrous cycle as a covariate, in most cases, did not alter the results of the achieved statistical model. In addition, estrous cycle was not considered in our PCA, as it

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was of great importance to keep the number of variables low. Future research should, however, implement estrous cycle, perhaps in one-day variants of the MWT, to determine how performance is altered by estrous cycle. Only additional research will help elucidate and clarify the specific contributions of estrous cycle to each of these behavioural tasks.

Despite the minimally controlled effects of estrous cycle, differences were observed in cognitive performance, such that males had greater proficiency in the spatial MWT, and females showed higher levels of performance in the DFCTC task. This may identify males as having superior spatial learning and memory skills, which has been observed previously (Jonasson, 2005), whereas females more easily demonstrate fear-conditioning behaviour (Markus & Zecevic, 1997; Toufexis, 2007). Although the mechanisms behind male-biased performance in spatial learning and memory tasks has been discussed extensively, in relation to evolutionary origins (Gaulin & Fitzgerald, 1989; Jones et al., 2003) among others, differences in fear-conditioning behaviour has not been as consistently discussed. There are multiple potential causes of these differences, including differential stress-responses (Critchlow et al., 1963; Kajantie & Phillips, 2006), but the exact mechanisms require further research.

This leads to the question of what are the broader implications for these sex differences for the rest of the behavioural neuroscience community. This identifies males and females as functionally and formatively different animals. Indeed, not only are behavioural differences between the sexes identified, but the biochemical mechanisms behind memory formation appear to be unique to each sex (as discussed in Mizuno & Giese, 2010). Therefore, like strain, the sex of the individual could mask or amplify certain experimental manipulations.

Effects of THC

Unlike the effects of strain and sex, exposure to THC during the post-pubertal period did not consistently produce effects. The only consistent effect was observed in the juveniles such that during the injection period, all strain and sex groups had lower relative weight gain with THC administration. Fourteen days of THC administration also resulted in a sex-specific increase in hippocampal volume in LER and WR females. Behavioural effects were specific and unique to each strain and sex group and often occurred only in females. In the EPM, THC exposure following puberty onset decreased one metric of anxiety in WR females. In the DFCTC task, THC exposure decreased discriminative freezing in LER females. However in WR males, THC exposure resulted in the expression of active avoidance, which wasn't observed in the CON or VEH groups. In adults, THC decreased the volume of the HP, DG and CA1 as compared to the VEH group in LER females. THC administration increased the volume of the mPFC in WR females as compared to the VEH group and increased the volume of the amygdala in LER males compared to the VEH and CON group. PCA analysis very clearly indicated that whether rats had been exposed to THC following puberty onset contributed very little to the variance in most behavioural tasks. Only for DFCTC, group contributed to one factor of the PCA, in combination with the size of prefrontal cortex areas and baseline freezing, and this factor only accounted for 10% of the variance in that data set. Here, THC had minimal effects, and they were specific and unique to each strain and sex group.

The mechanism behind the strain- and sex-specific effects are wide and varied, and, from the information in this thesis, only speculative. One possibility is that given the varied genetic background of these individuals, including the sex chromosomes, THC can have differential effects, some causing deficits in behaviour and some causing

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improvements. Most effects were observed in females, regardless of strain. In previous

chapters, we discussed the increased sensitivity of females to the effects of adolescent

THC exposure, specifically on measures of affect (Patton et al., 2002; Rubino, Realini,

Braida, Alberio, et al., 2009). Here, since any effects of THC were observed almost

exclusively in females across both strains, females may be particularly at risk for the

effects of THC during adolescence. Mechanisms for these effects have been discussed in

earlier chapters, including the sex-specific metabolism of THC (Narimatsu et al., 1991),

but it could also be a function of the particular sensitivity of females to more easily

develop depressive-like symptoms as compared to males. Although this has been

discussed extensively in humans, perhaps this is more than just a sociologically

determined phenomenon, is a biological reality and may be a function of sex-specific

circuitry and behavioural predispositions (Parker & Brotchie, 2010). Only further

research into this sex difference will help elucidate this matter.

Another possibility of the strain- and sex-specific effects of THC includes the

possibility of differential dose-response curves dependent on strain and sex. This may be

due to differences in both the initial response to THC, which is dependent on its binding

to CB1Rs, as well as differential metabolism of THC. Further research examining dose-

response relationships to THC using the adolescent period in both male and female LER

and WR will help to determine if this is the case. However, given the acute effects

observed here on weight gain as well as other studies that used much smaller doses and

the dosage used here in both of these strains of rats, among others as well (discussed

below), a dose-response relationship between strain and sex was not deemed necessary.

Indeed, our study was mostly interested in examining the long-term effects of THC on

brain and behaviour as a model of the long-term effect of marijuana use in adolescent humans.

One unavoidable point includes the lack of cohesiveness of the current results and previous research in the literature. This research project was based on the assumption that THC would have long-term consequences on brain and behaviour as well as potentially alter sensitivity to other drugs of abuse, as has been discussed extensively (for example, Kandel, 1975; Kandel et al., 2006; Pryor et al., 1978). Furthermore, the dose of THC implemented has been shown to have both immediate (Braida et al., 2004; Cha et al., 2006; Craft & Leitl, 2008; McGregor, Arnold, Weber, Topple, & Hunt, 1998b; Onaivi, Green, & Martin, 1990) and long-term effects (Cha et al., 2007b; Navarro et al., 1994; Pryor et al., 1978; Vela et al., 1995) in other research paradigms using the similar routes of administration. Indeed even lower doses of this drug have been used previously and have had observed short- and long-term effects (Cadoni et al., 2001; Chen et al., 1991; Ellgren et al., 2007; Gardner et al., 1988; Harte & Dow-Edwards, 2010; Lamarque et al., 2001; Lepore, Liu, Savage, Matalon, & Gardner, 1996; Lichtman et al., 1995; Mallet & Beninger, 1998; Onaivi et al., 1990; Parker & Gillies, 1995; Robinson, Hinder, Pertwee, & Riedel, 2003; Wenger et al., 1988). However, very few effects were observed in our series of studies, and clearly their impact is dependent on the background of the individual. As discussed in earlier chapters, the differences between the injection schedules of our and other's research may help account for these discrepancies. However, if post-pubertal administration of THC was a robust phenomenon, these minor methodological issues should not be of concern.

Indeed, the inclusion of PCA highlighted the different conclusions that could be achieved depending on the implemented statistical tool. Using standard ANOVA testing,

we observed strain- and sex-specific effects of THC whereas using PCA, we demonstrated that very little variance in the data set was accounted for by the treatment group. This demonstrates the different levels of information that can be learned from each statistical tool. In addition, it demonstrates that although the long-term consequences of THC may be specific to strain and sex groups, when looking across the entire population, it appears to have little effect. This highlights the importance of considering large population phenomena as well as the choice of groups in human studies looking at the long-term consequences of THC.

The alternative explanation, which is more likely representative of real-world situations, is that certain individuals are particularly at risk for the long-term negative consequences of THC. Here, we see that, for the most part, females seem to be at particular risk. However, the effects in females were often restricted to one strain and not the other, and in one case, improved anxiety behaviour and increased the size of an associated brain area. Therefore, it is of interest to determine the mechanism behind these sensitivities, as well as the resilience of the males, to be able to identify which factors might place an individual at particular risk. It could be a behavioural element, like novelty, which could predict the sensitivity to drug reward (for example, Klebaur et al., 2001), specifically to THC. Additionally, there could be multiple factors, including sex (McGregor & Arnold, 2007), genetic background (for example, Ortiz et al., 2004) or behavioural predispositions (Coria et al., 2014; Hamilton et al., 2014) that could account for an increased sensitivity or resilience to the effects of THC. Characterizing and identifying the factors that determine the response to THC could help identify at-risk groups for the long-term consequences of THC use during the adolescent period. This

concept, in relation to future directions and implications will be discussed following a discussion of the major caveats to this research procedure.

Caveats and limitations

Here, the main caveats and alternative explanations to the achieved results will be discussed. Only a subset of the possible limitations of this study will be discussed.

Effects of injection

One very large caveat in our study was an effect of injection. Not all research paradigms include a handled control group. Often, only a vehicle group is included as a control, in order to affirm that any effects were exclusive to the drug administered and not due to secondary effects of receiving an injection or the solution in which the injection was dissolved. Here, a handled control group was included in order to examine which effects could be due to an injection alone and which were not. Here, injection effects were observed in the EPM, MWT and DFCTC, with each effect unique to a strain and sex group. In the EPM, receiving an injection decreased anxiety metrics in WR males. In the MWT, receiving an injection during the adolescent period decreased performance in the probe trial for WR males and females and increased performance in LER females. Finally, in the DFCTC task, LER males who had received an injection did not show the same levels of active avoidance. In addition to these effects, other differences were observed only between VEH and THC, and not between THC and CON groups.

The effect of injection could have originated from multiple sources. One of them includes the actual contents of the vehicle injection. THC, received from Sigma Aldrich, was dissolved in ethanol and diluted into a saline solution. Therefore, the vehicle

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solution, as well as the THC solution, contained very low concentrations of ethanol. Any effects of injection could have been the result of chronic low doses of ethanol during adolescence, and any effects of THC could have been due to a synergistic interaction between ethanol and THC. Other research has used other vehicle injections that do not include ethanol, and the use of this as a vehicle was a caveat to the behavioural and brain metrics observed here. However, the effects of such a low dose of ethanol seem very unlikely simply due to the very low ethanol content.

This effect of injection is worrisome as it may identify certain metrics as sensitive to the effects of an injection during the adolescent period. However, when the effects of treatment group are examined via PCA, treatment group accounted for very little of the variance in the data set. Although this does not completely discount the effects of an injection on subsequent measures of brain and behaviour, it does help indicate that apparent effects of injection do not alter the data in a meaningful way. Despite this, the effect of injection is an important result, and highlights the importance of including a handled control group in any research paradigm involving drug administration, specifically THC.

Interactions with stress

Injection effects could be the result of the physiological response to a chronic unpredictable stressor. This was discussed in detail earlier (Chap 2.2) and will not be reiterated in detail. Briefly, chronic elevations of CORT could account for the differences observed between handled control and injected groups.

In addition to the effects of injection, endocannabinoids provide a strong regulatory role for the hypothalamic-pituitary-adrenal (HPA) axis (Hill et al., 2010),

which regulates the physiological response to stress. Given this role, any effects of THC may be simply a result of disruptions to the compensatory and regulatory role of the endocannabinoid system to the stress response. The inclusion of measurements of stress hormones into this study would have helped to elucidate this role, and future research using this paradigm should include measurements of CORT before and after the handling period in order to answer this question.

Interaction with social development

One very interesting interaction effect was not considered in the initial design and is rarely, if ever, discussed in the animal literature examining the adolescent administration of THC. This caveat only came as a result of a collaboration, which examined the effects of THC administration on play behaviour during the injection period. All of our metrics, except for puberty onset and weight gain, were taken following the administration of THC.   Therefore, as a side project, we investigated the effects of THC on play behaviour to examine some immediate effects of THC. This was included in Appendix 3, only for LER, and there are differential effects of THC and injections on the type and frequency of play behaviour. Briefly, if THC alters play behaviour, it is possible that any effects of THC during adolescence are not the product of the drug itself, but a product of altering the social interactions of rats during a critical period of development. Deprivation of play, but not social interactions, can have large effects on later sociality (Pellis & Pellis, 2007) and development of cortical circuits (Bell, Pellis, & Kolb, 2010). Injections with THC could alter how the rats interact with their cagemates, and changes in social interactions could cause modifications in the development of brain and behaviour that persists to adulthood. Although this is a difficult phenomenon to study, it can easily be

extended to human research. The adolescent period in humans is considered a critical

time for the development of sociality with authority figures, peers and potential sex

partners. Additionally, differential development of limbic and prefrontal brain areas is

thought to mediate the behavioural changes and responses to novelty and risk observed in

adolescence (Casey et al., 2008; Galvan et al., 2006). Simply put, during adolescence,

emotional circuitry reaches adult levels in the absence of an adult prefrontal cortex.

Therefore, this period of development is critical for the maturation of emotional reactivity

and subsequent cortical control. This makes the adolescent period particularly sensitive

to any manipulations that alter emotional circuitry. From a recent Canadian survey, as

reiterated throughout this thesis, the first exposure to marijuana occurs on average during

the adolescent period (Health Canada, 2013). Perhaps any adverse consequences are not

due to marijuana itself, but to the alterations in social interactions and emotional

regulation when "high." Modifying social and emotional development in at risk

individuals, such as those already predisposed to schizophrenia, could be the proverbial

straw that breaks the camel's back. This concept is rather speculative, but raises

important concerns as to the origin of the long-term consequences of marijuana use in

adolescence.

Future directions and implications

Throughout this thesis, the pattern of results obtained here and in relation to many

additional research streams and areas of focus have been discussed. This includes

examining the effects of THC on cytoarchitectural changes, stress hormones and the

stress response as well as sociality. The most important point on this matter is that the

background of the individual, as determined by multiple factors including genetics and experience, is a large determinant to the sensitivity or resilience to the effects of THC.

The implications of this finding are interesting. Although this may appear to be a shortcoming of the present thesis, it is also its strength. These results could help determine the largest factors that contribute to the development of aversive consequences of THC on brain and behaviour as well as in response to other drugs of abuse. Once these factors have been identified, prevention strategies could be implemented in order to target high-risk youth preferentially. This could change policy and decision-making in light of the increasing popularity and identified utility of medicinal marijuana. There may be some individuals for whom the side effects of THC use outweigh the medicinal benefits, and identifying these factors before prescription are of great interest to the medical community.

Like medicinal marijuana, studies have identified the endocannabinoid system for drug development in anti-depressant and anti-obesity mediation (for example, CB1R antagonists and fatty-acid amide hydroxylase inhibitors; Bortolato et al., 2007; Christensen, Kristensen, Bartels, Blidda, & Astrup, 2007; Gaetani et al., 2009). Identifying individuals who may be more sensitive or more resilient to alterations in the endocannabinoid system may help develop personalized medication strategies, saving time and money as well as improving quality of life for the individuals requiring treatment.

This study is the first of its kind to examine, across multiple strains and between the sexes, the differential effects of the psychoactive component of marijuana on development, brain and behaviour. It offers a unique set of behavioural results that have broad implications for the development of intervention strategies towards marijuana

abuse prevention in adolescents as well as the development of drug-treatments that target the endocannabinoid system. This thesis reveals that individual variability related to genetics and experience are important mediators of subsequent experiences and can alter the effects of pharmacological agents across the lifespan, even in a relatively genetically homogenous animal. Further, the different analyses included in this thesis, highlight the importance of mindfulness in regards to whether phenomena are clinically significant, rather than just statistically so.

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TABLES

Table 1: Mean volume (mm^3) and maximum coefficient of error (CE) for each area of interest for juveniles LER and WR male and females.

Strain	Sex	Hippocampus ($mm3$)		DG (mm^3)		CA1 (mm^3)		CA3 (mm^3)		mPFC (mm^3)		OFC (mm^3)		Amygdala (mm^3)	
		Mean	CE	Mean	CE	Mean	CE	Mean	CE	Mean	CE	Mean	CE	Mean	CE
LER	female	26.04	<0.03	1.27	<0.03	1.04	<0.04	1.39	<0.03	5.63	<0.03	8.70	<0.03	8.59	<0.02
	male	34.51	<0.02	1.77	<0.02	1.53	<0.03	1.92	<0.03	4.92	<0.04	8.87	<0.02	10.96	<0.02
WR	female	26.05	<0.02	1.05	<0.04	0.88	<0.05	1.36	<0.05	4.81	<0.03	7.48	<0.03	8.43	<0.03
	male	32.41	<0.02	1.64	<0.03	1.38	<0.03	1.74	<0.03	5.39	<0.02	8.87	<0.03	10.87	<0.02

Table 2: Mean volume (mm^3) and maximum coefficient of error (CE) for each area of interest for adult LER and WR males and females.

Strain	Sex	Hippocampus (mm^3)		DG (mm^3)		CA1 (mm^3)		CA3 (mm^3)		mPFC (mm^3)		OFC (mm^3)		Amygdala (mm^3)	
		Mean	CE	Mean	CE	Mean	CE	Mean	CE	Mean	CE	Mean	CE	Mean	CE
LER	female	41.86	<0.02	1.78	<0.03	1.677	<0.02	2.10	<0.03	5.96	<0.04	8.31	<0.02	14.23	<0.07
	male	40.15	<0.04	1.83	<0.03	1.57	<0.04	2.05	<0.03	6.95	<0.03	7.45	<0.03	14.40	<0.08
WR	female	43.01	<0.02	1.88	<0.03	1.80	<0.03	2.06	<0.03	6.26	<0.03	6.83	<0.03	16.03	<0.04
	male	45.39	<0.02	1.88	<0.03	1.60	<0.05	2.04	<0.03	6.32	<0.03	7.35	<0.03	15.30	<0.04

Table 3: Summary of effects of strain and sex on measures of interest. </> symbols indicate comparative superior performance.

Task	Measure	Effect of Strain	Effect of Sex
SRT	Attempts	-	-
	Trials	-	-
MWT	Acquisition	LER >> WR	LER♂ > LER♀
	Probe	LER > WR	-
	Mass training	LER >> WR	LER♂ > LER♀ during early training
	Motor/Visual performance	-	-
DFCTC	Pre-exposure	-	-
	Test	LER > WR LER♀ > WR♀	♀ > ♂ LER♀ > LER♂
	Preference	LER > WR	-
EPM	Dwell time	-	-
	# Arm entries	WR more active LER♀ more anxious that WR♀ WR♀ more active than LER♀	♀ more active than ♂
	Open: Closed	-	-
Volumetric estimates	HP	WR > LER	-
	DG	-	-
	CA1	-	♀ > ♂
	CA3	-	-
	OFC	-	-
	mPFC	-	-
	AMYG	WR > LER	♀ > ♂ WR♀ > WR♂

Table 4: Mean volume (mm^3) and maximum coefficient of error (CE) for each area of interest for juveniles LER and WR males and females exposed to THC following puberty onset.

Strain & sex	Group	Hippocampus (mm^3)		DG (mm^3)		CA1 (mm^3)		CA3 (mm^3)		mPFC (mm^3)		OFC (mm^3)		Amygdala (mm^3)	
		Mean	CE	Mean	CE	Mean	CE	Mean	CE	Mean	CE	Mean	CE	Mean	CE
LER ♀	CON	26.04	<0.03	1.27	<0.03	1.04	<0.04	1.39	<0.03	5.46	<0.03	9.09	<0.03	8.60	<0.02
	VEH	33.16	<0.02	1.51	<0.03	1.46	<0.03	1.83	<0.03	4.38	<0.03	8.06	<0.03	9.40	<0.02
	THC	32.85	<0.02	1.52	<0.03	1.43	<0.03	1.72	<0.03	4.52	<0.03	8.63	<0.02	10.64	<0.02
LER ♂	CON	34.58	<0.02	1.77	<0.02	1.53	<0.03	1.92	<0.03	4.92	<0.04	8.87	<0.02	10.96	<0.02
	VEH	36.88	<0.02	1.45	<0.03	1.22	<0.03	1.65	<0.03	5.40	<0.03	8.58	<0.03	11.03	<0.02
	THC	37.26	<0.02	1.70	<0.03	1.45	<0.03	1.86	<0.04	4.75	<0.03	10.17	<0.02	9.79	<0.03
WR ♀	CON	22.40	<0.02	1.05	<0.04	0.88	<0.05	1.37	<0.05	4.81	<0.03	7.48	<0.03	8.51	<0.03
	VEH	26.00	<0.02	1.12	<0.03	0.96	<0.04	1.41	<0.03	3.83	<0.04	7.05	<0.02	7.66	<0.02
	THC	29.87	<0.02	1.22	<0.03	1.06	<0.03	1.52	<0.03	3.74	<0.03	8.14	<0.03	6.98	<0.02
WR ♂	CON	32.41	<0.02	1.64	<0.03	1.38	<0.03	1.74	<0.03	5.39	<0.02	8.87	<0.03	10.87	<0.02
	VEH	30.76	<0.02	1.45	<0.03	1.18	<0.04	1.69	<0.03	4.02	<0.03	8.33	<0.02	9.16	<0.02
	THC	27.81	<0.02	1.41	<0.03	1.21	<0.03	1.49	<0.03	3.70	<0.03	8.36	<0.02	10.40	<0.02

Table 5: Mean volume (mm^3) and maximum coefficient of error (CE) for each area of interest for adult LER and WR males and females exposed to THC following puberty onset.

Strain & sex	Group	Hippocampus (mm^3)		DG (mm^3)		CA1 (mm^3)		CA3 (mm^3)		mPFC (mm^3)		OFC (mm^3)		Amygdala (mm^3)	
		Mean	CE	Mean	CE	Mean	CE	Mean	CE	Mean	CE	Mean	CE	Mean	CE
LER ♀	CON	41.86	<0.03	1.78	<0.03	1.68	<0.03	2.10	<0.03	5.96	<0.04	8.31	<0.02	14.23	<0.07
	VEH	43.22	<0.02	1.97	<0.03	1.82	<0.03	2.07	<0.03	7.32	<0.03	7.80	<0.03	14.75	<0.03
	THC	38.17	<0.03	1.70	<0.03	1.54	<0.03	1.98	<0.03	5.72	<0.03	7.93	<0.02	14.90	<0.03
LER ♂	CON	40.15	<0.04	1.83	<0.03	1.57	<0.04	2.05	<0.03	6.95	<0.03	7.45	<0.03	14.41	<0.08
	VEH	44.21	<0.02	1.89	<0.03	1.69	<0.02	2.27	<0.03	6.99	<0.03	7.99	<0.03	15.16	<0.03
	THC	44.62	<0.02	2.07	<0.02	1.75	<0.02	2.22	<0.03	6.74	<0.04	7.38	<0.03	16.53	<0.03
WR ♀	CON	43.01	<0.02	1.88	<0.03	1.81	<0.03	2.06	<0.03	6.26	<0.03	6.83	<0.03	16.04	<0.04
	VEH	41.67	<0.03	1.77	<0.03	1.56	<0.03	1.93	<0.03	6.14	<0.03	7.27	<0.04	14.89	<0.05
	THC	42.40	<0.02	1.82	<0.03	1.60	<0.03	1.96	<0.03	8.27	<0.03	8.05	<0.02	15.26	<0.03
WR ♂	CON	45.40	<0.02	1.88	<0.03	1.60	<0.05	1.04	<0.03	6.32	<0.03	7.35	<0.03	15.30	<0.04
	VEH	46.23	<0.03	2.01	<0.03	1.74	<0.03	2.09	<0.02	6.99	<0.04	8.19	<0.02	16.10	<0.03
	THC	45.47	<0.02	2.13	<0.03	1.87	<0.03	2.17	<0.03	6.63	<0.03	7.03	<0.03	16.20	<0.03

Table 6: Number of subjects (N) included in volumetric estimate analysis for all strain and sex groups for all brain areas of interest.

Strain & sex	Group	Hippocampus N	DG N	CA1 N	CA3 N	mPFC N	OFC N	Amygdala N
LER ♀	CON	14	13	14	14	14	14	14
	VEH	15	15	15	15	15	15	15
	THC	15	15	14	14	14	14	14
LER ♂	CON	16	15	15	15	15	15	15
	VEH	15	14	14	14	14	14	14
	THC	12	12	12	12	12	12	12
WR ♀	CON	12	11	11	11	11	11	11
	VEH	16	15	14	14	14	14	15
	THC	14	13	12	12	13	13	13
WR ♂	CON	12	12	12	12	12	12	11
	VEH	14	14	14	14	14	14	14
	THC	14	14	14	14	13	13	14

Table 7: Summary of the effect of injection or THC on rat behaviour and brain volumes. > Denotes improved performance. < Denotes worse performance for behavioural tasks. For volumetric differences, </> indicates comparatively smaller or larger volumes. Injection rats were those who received either vehicle or THC treatments.

Task	Measurement	Treatment group			
		LER females	LER males	WR females	WR males
SRT	Attempts/Trials	-	-	-	-
MWT	Acquisition	-	-	-	-
	Probe path length	> VEH	-	-	< with injection
	Probe dwell time	> with injection	-	< with injection	< with injection
	Mass training	-	-	-	-
	Motor/visual performance	-	-	-	-
DFCTC	Pre-exposure	-	-	-	-
	Test	< THC	> VEH	-	-
	Preference	< VEH	< injection	-	> THC
EPM	% dwell time	-	-	-	-
	# arm entries	-	-	THC ↓ anxiety	Injection ↑ anxiety
	Open:Closed arm	-	-	-	-
Volumetric estimates	Hippocampus	THC < VEH	-	-	-
	DG	THC < VEH	-	-	-
	CA1	THC < VEH	-	-	-
	CA3	-	-	-	-
	OFC	-	-	-	-
	mPFC	-	-	THC > VEH	-
	Amygdala	-	THC > VEH/CON		

Table 8: Factor loadings >0.5 (in bold) with oblimin rotation for SRT

	Factor 1 **HP and AMYG volumes & sex**	Factor 2 **Early life experience**	Factor 3 **Performance**	Factor 4 **PFC volumes**
DG	**.874**	.011	.029	.020
HP	**.776**	.119	.053	.048
CA1	**.733**	-.044	-.041	-.297
CA3	**.670**	-.198	-.056	-.339
AMYG	**.614**	.187	.227	-.001
Sex	**.523**	-.122	-.138	.359
Litter	-.090	**.935**	-.053	.037
Strain	.100	**.897**	-.142	.118
Attempts	-.080	-.056	**.929**	-.033
Success	-.003	-.338	**.868**	.188
Litter Order	-.053	-.235	-.380	.346
Group	.123	.096	.237	-.050
OFC	.033	-.193	-.080	**-.806**
mPFC	.070	.023	.030	**-.732**
Variance explained	25%	16%	12%	10%

Table 9: Factor loadings >0.5 (in bold) with oblimin rotation for MWT

	Factor 1 Early life experience & performance	Factor 2 HP & AMYG volumes	Factor 3 Motor skill & sex
Strain	**.882**	.008	-.162
Litter	**.785**	-.093	.030
D4 Latency	**.777**	-.096	.053
D2 Latency	**.767**	-.071	-.003
D3 Latency	**.764**	-.035	.039
D6 Bin 3 Latency	**.741**	.040	-.013
D6 Bin 2 Latency	**.693**	-.091	-.104
D6 Bin 4 Latency	**.660**	.129	-.012
D1 Latency	.439	.134	.109
Probe – Target Dwell Time	-.425	-.062	-.125
DG	.082	**.810**	-.265
CA1	-.032	**.805**	-.076
CA3	-.128	**.771**	.074
HP	.086	**.708**	-.299
AMYG	.153	**.638**	-.192
mPFC	-.076	.434	.273
OFC	-.243	.388	.307
Group	.044	.238	.132
D7 Latency	-.182	-.163	**.670**
D8 Latency	.195	-.125	**.670**
Sex	-.094	.278	**-.609**
Litter Order	-.010	-.336	-.498
D6 Bin 1 Latency	.128	.106	.213
Variance explained	23%	15%	9%

Table 10: Factor loadings >0.5 (in bold) with oblimin rotation for DFCTC

	Factor 1 HP & AMYG volumes & Sex	Factor 2 Dwell time in unpaired and not-yet paired contexts	Factor 3 Early life experience	Factor 4 PFC, Baseline freezing and Drug	Factor 5 Litter order & Active avoidance
DG	**.885**	-.059	.031	.046	.024
HP	**.810**	.085	.135	.154	-.092
CA1	**.793**	-.043	-.062	-.053	-.154
CA3	**.633**	-.226	-.224	-.236	-.194
AMYG	**.594**	-.083	.198	-.318	.072
Sex	**.419**	.099	-.076	.084	.324
PreExp Unpaired	.051	**.926**	.164	-.084	.216
PreExp Paired	.000	**-.870**	.050	-.008	-.124
Pref Unpaired	-.181	**.557**	.010	-.090	-.242
Litter	-.053	.060	**.939**	-.017	-.199
Strain	.116	.033	**.920**	.021	-.044
Test Unpaired	.289	.122	-.055	**.700**	-.096
mPFC	.151	.181	-.075	**-.590**	-.311
OFC	.093	.021	-.301	**-.565**	-.277
Group	.088	.119	.065	**-.505**	.118
Test Paired	-.004	.301	-.254	.470	-.451
Litter Order	-.138	.141	-.180	-.001	**.817**
Pref Paired	-.051	-.488	.153	-.069	**.573**
Variance explained	19%	15%	11%	10%	8%

Table 11: Factor loadings < 0.5 (in bold) with oblimin rotation for EPM

	Factor 1 **Time spent in arms & less anxious behaviour**	Factor 2 **Relative number of arm entries & more anxious behaviour**	Factor 3 **Early life experience**
Time Closed	**-.947**	.104	.009
% Time Closed	**-.944**	.110	.000
Time Open	**.925**	-.194	.016
% Time Open	**.919**	-.198	.025
# Open Entries	**.768**	-.037	.357
Sex	-.351	-.020	.165
% Closed Entries	-.227	**.926**	-.059
% Open Entries	.227	**-.926**	.059
Open: Closed Entries	.250	**-.916**	.053
# Closed Entries	.602	**.676**	.303
Strain	.151	.102	**.905**
Litter	.053	.075	**.900**
Group	.033	.032	-.070
Variance explained	45%	21%	12%

Table 12: Factor loadings < 0.5 (in bold) with oblimin rotation for CPP

	Factor 1 **Dwell time**	Factor 2 **Early life experience**	Factor 3 **HP activation and Sex**
PreExp Unpaired	**.924**	.019	-.017
Pref Paired	**-.921**	-.020	.018
Pref Unpaired	**.888**	.165	.129
PreExp Paired	**-.888**	.106	.108
Strain	.043	**.922**	.044
Litter	.058	**.908**	.022
Cfos Dorsal HP	-.202	.17	**.768**
Cfos NAc	.105	-.349	.59
Sex	.095	.044	**.532**
Group	-.024	.014	.286
Variance explained	34%	19%	13%

A

B
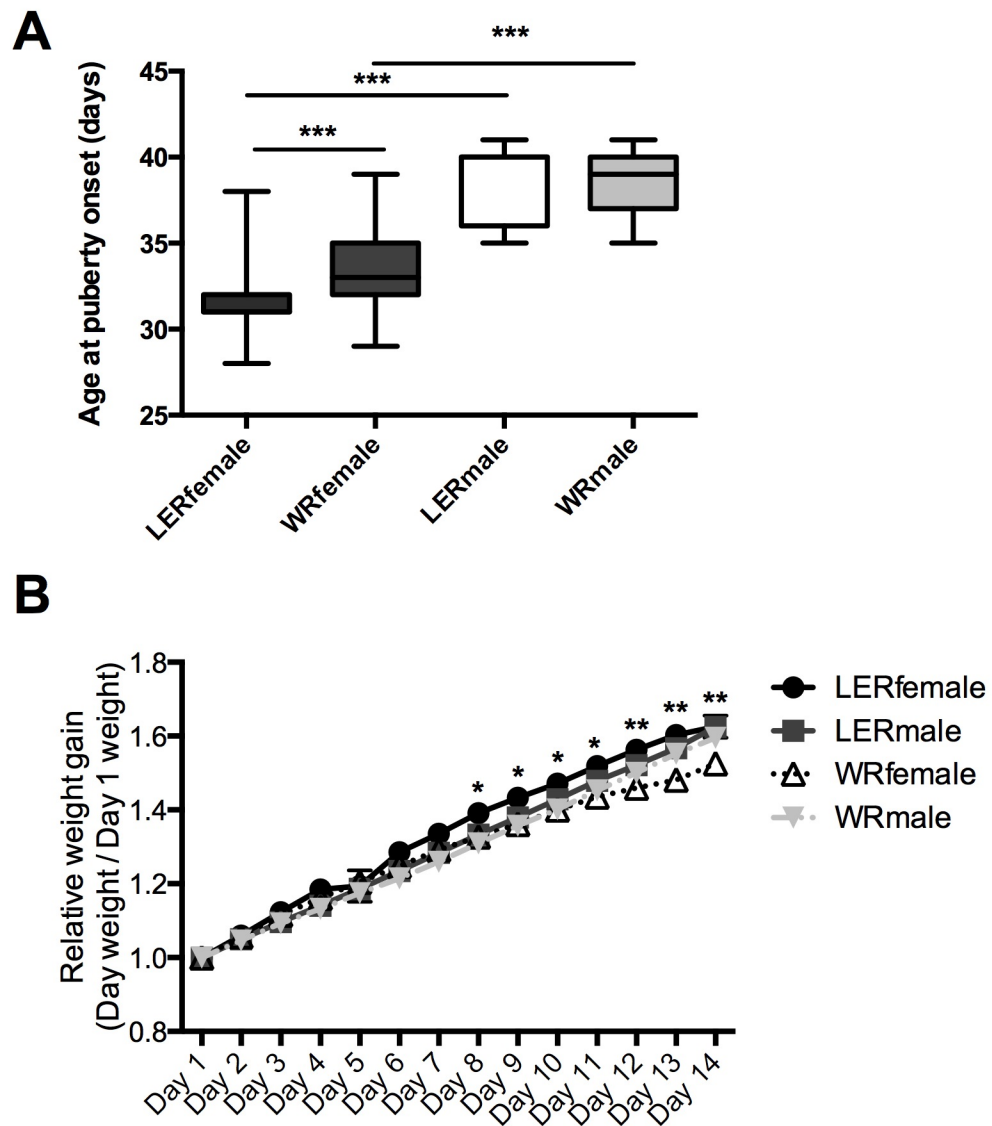
Figure 1: A. Age of onset at puberty. B. Weight gain during pubertal period. Weight gain is reflected as a ratio of the weight of the rats on that day relative to that measured on the day of puberty onset (day 1). * indicated a significant difference between LER and WR females. * p < 0.05. ** p < 0.01. *** p < 0.001.
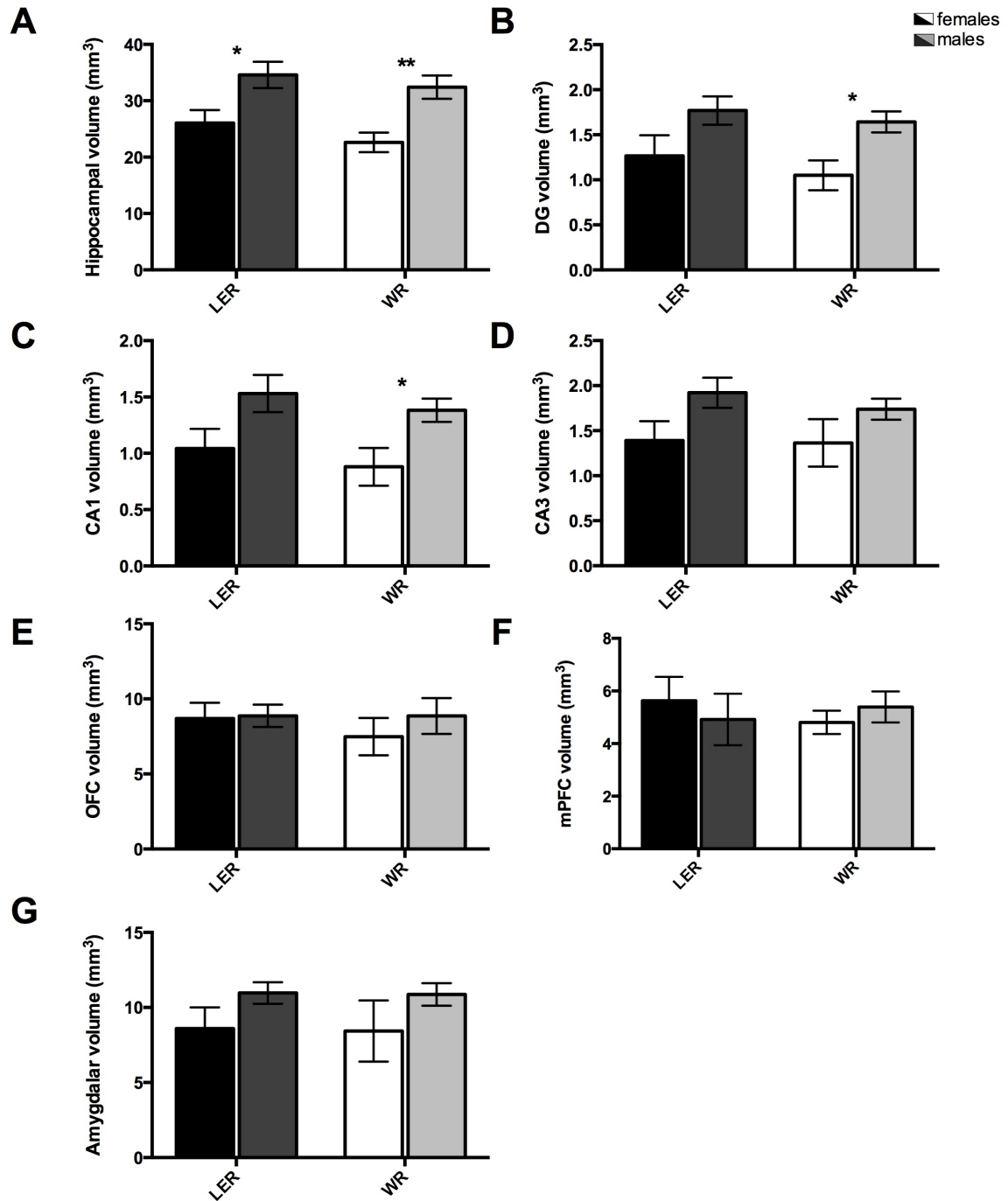
Figure 2: Volumetric estimates of right hemispheres measurements for A. HP, B. DG, C. CA1, D. CA3, E. mPFC, F. OFC and G. AMYG for LER and WR males and females. * p < 0.05. ** p < 0.01.
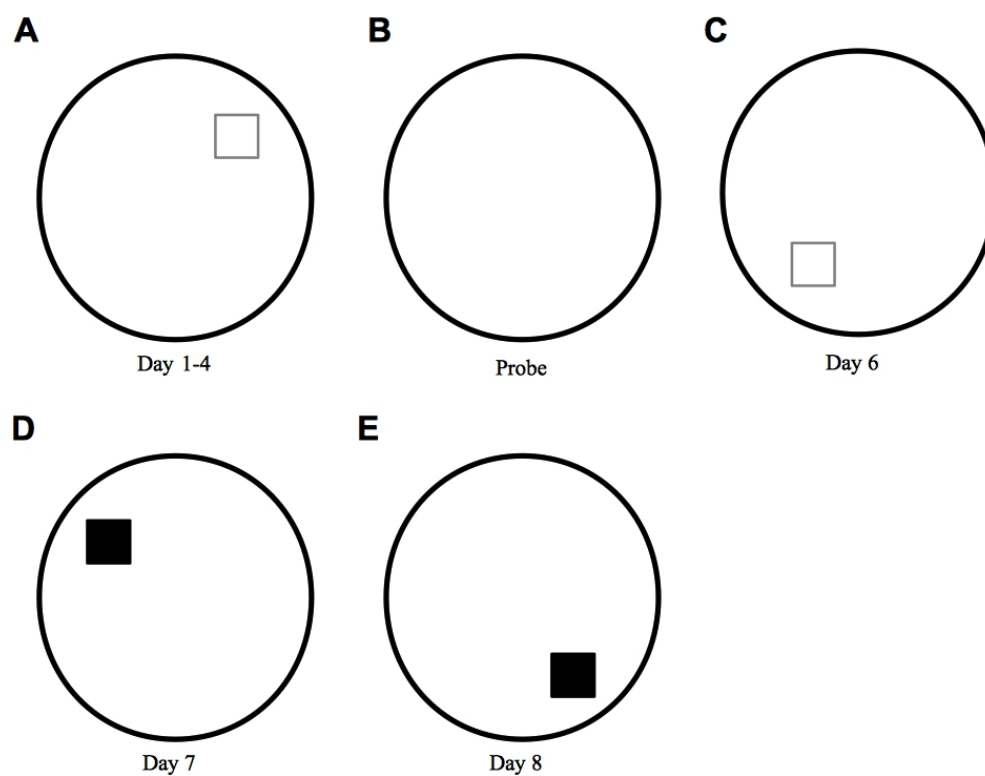
Figure 3: Pictorial representation of a bird's eye view of the MWT. A. Submerged platform location (grey outlined square) for days 1-4. B. Probe trial with no platform present. C. Submerged platform location for the mass training day to a new spatial location. D. Visible platform location (black square) for day 7. E. Visible platform location for day 8.
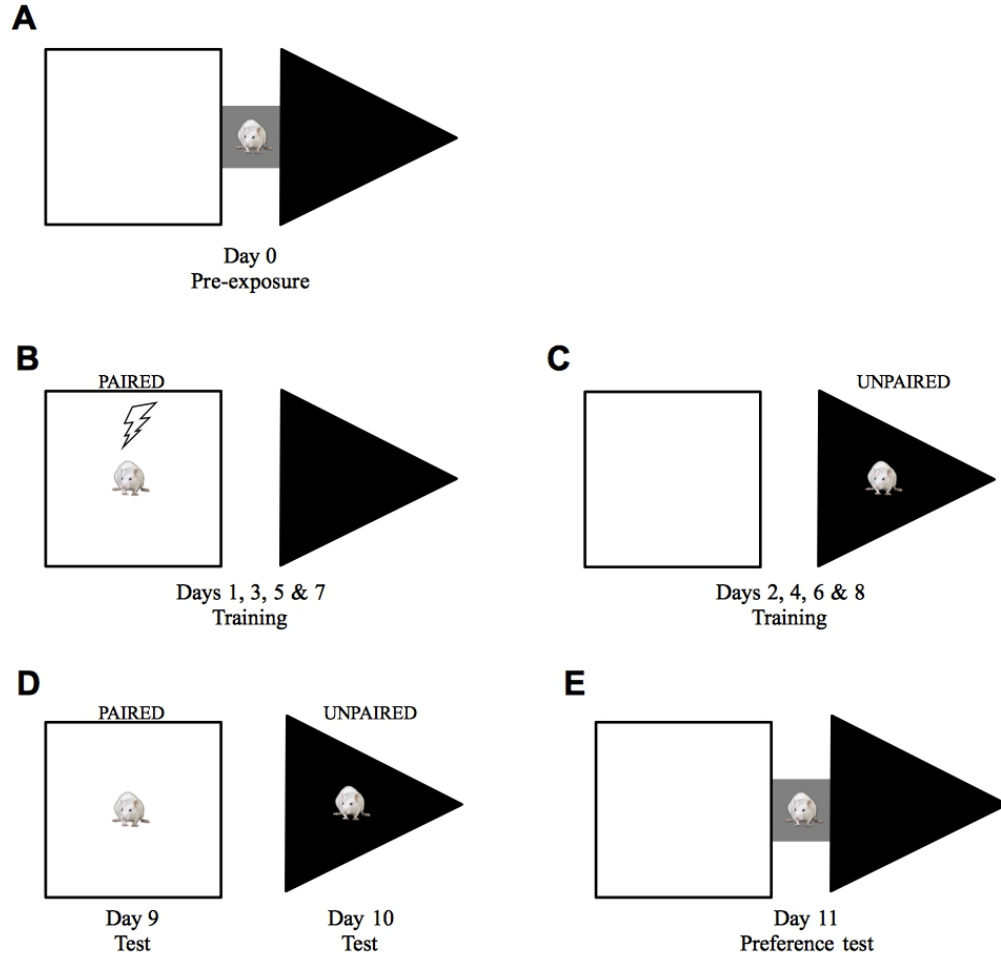
Figure 4: Pictorial representation of a bird's eye view of the DFCTC. A. Day 0: Pre-exposure day. B. C. Day 1-8: Training. This graphic represents the training for one rat. Rats were counterbalanced such that half received a shock stimulus in the white square (as shown here) and the other half received a shock stimulus in the black triangle. Rats were also counterbalanced such that half were exposed to their paired context on the first day of training (as shown here) and the other half were exposed to their unpaired context on the first day of training. D. Day 9 & 10: Testing. Rats were counterbalanced such that half were exposed to their paired context on the first test day and the other half to their unpaired context on the first test day. E. Day 11: Preference test.
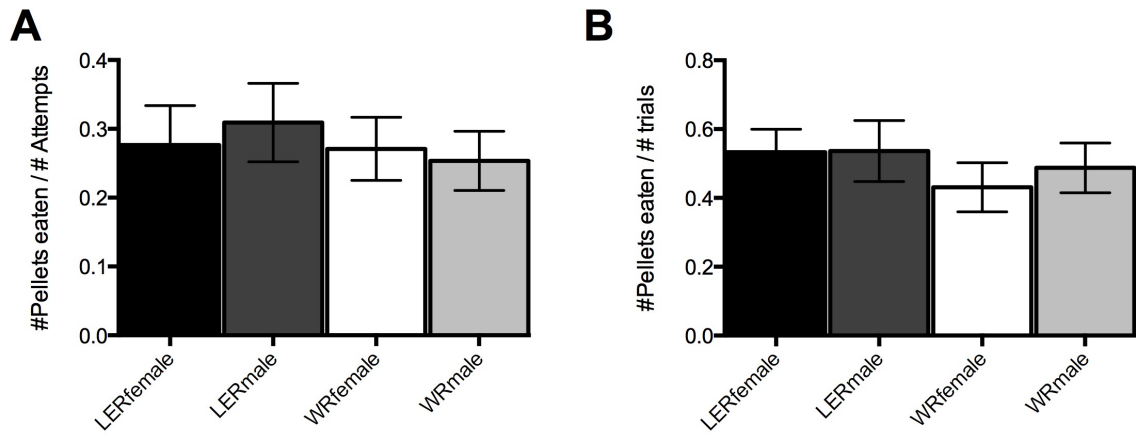
Figure 5: SRT. A. Success as measured by a ratio of number of pellets eaten and number of attempts. B. Success as measured by the number of pellets eaten over the trials.
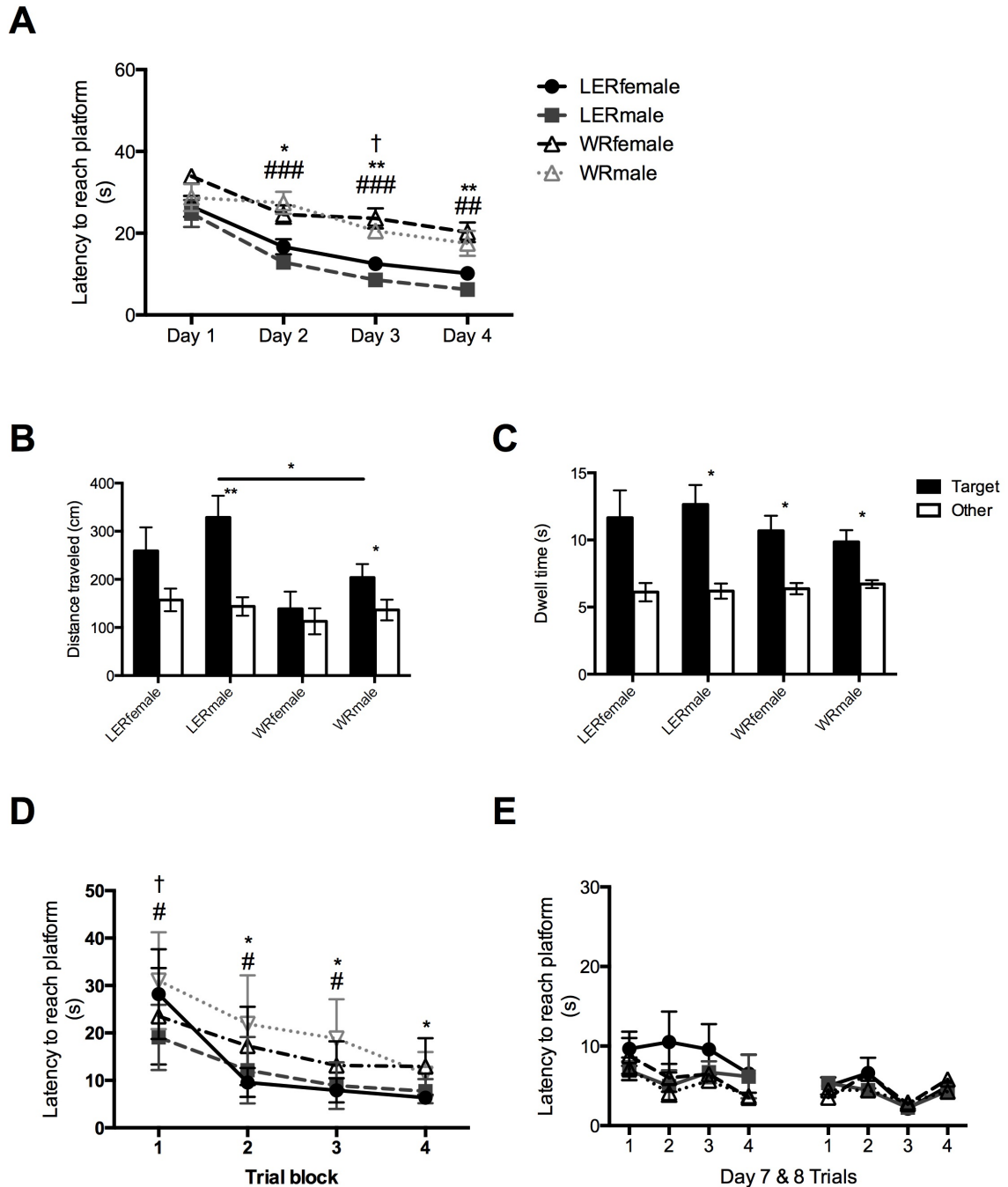
Figure 6: MWT. A. Acquisition. Day 1-4 of training. B. Retention. Day 5 probe trial displaying distance travelled during the first 30s of the probe trial. C. Day 5 probe trial displaying dwell time during the first 30s. D. Mass training to a new platform location. Data is separated into 4 time bins of 4 trials each. E. Visible platform. Day 7 & 8. * indicates a significant differences between LER and WR females. # indicates a significant difference between LER and WR males. † indicates a significant difference between LER males and females @ indicates a significant differences between WR females and males. * p < 0.05. ** p < 0.01. *** p < 0.001.
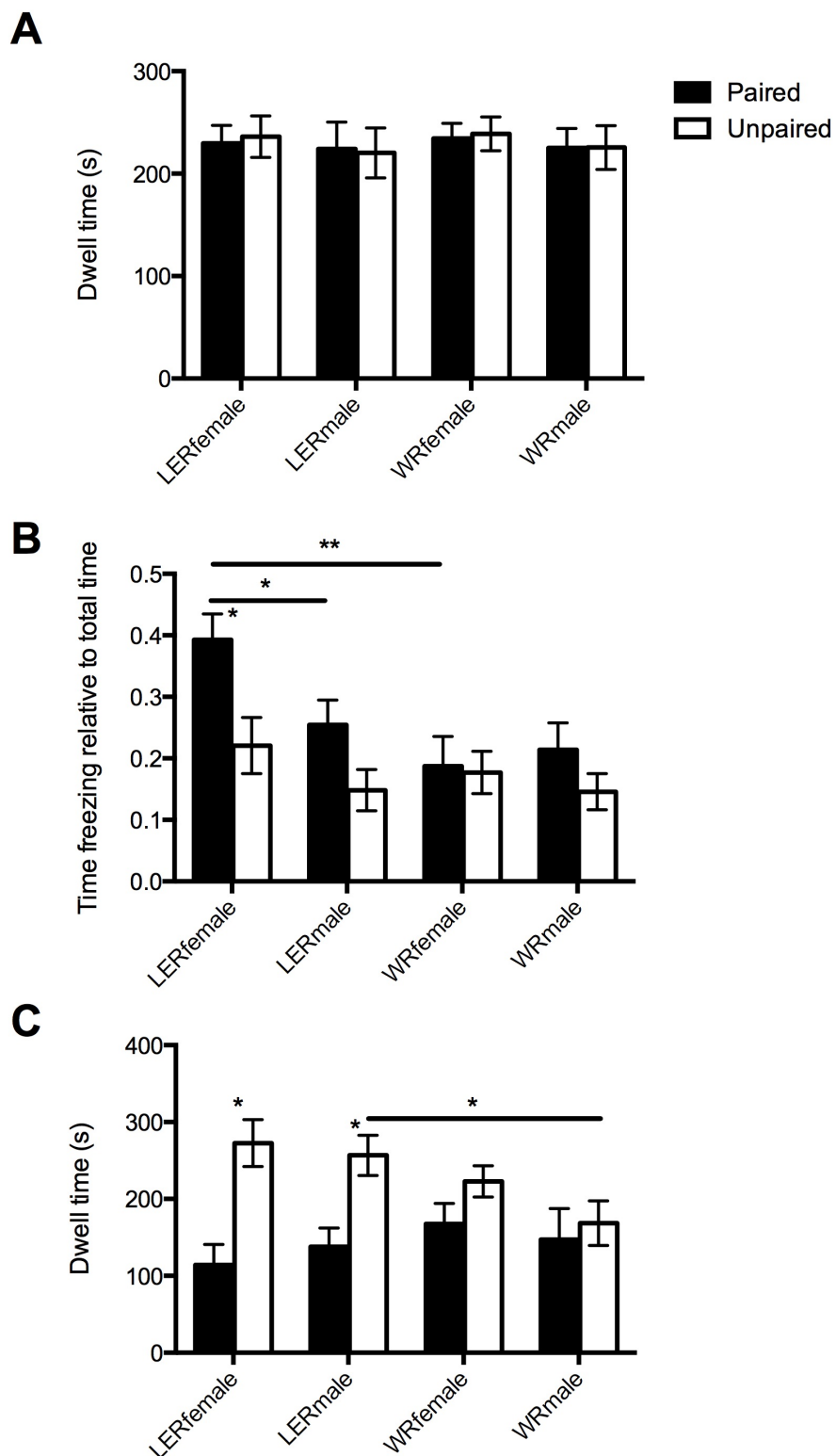
Figure 7: DFCTC. A. Pre-exposure. B. Test day. C. Preference day. *p<0.05. **p<0.01.
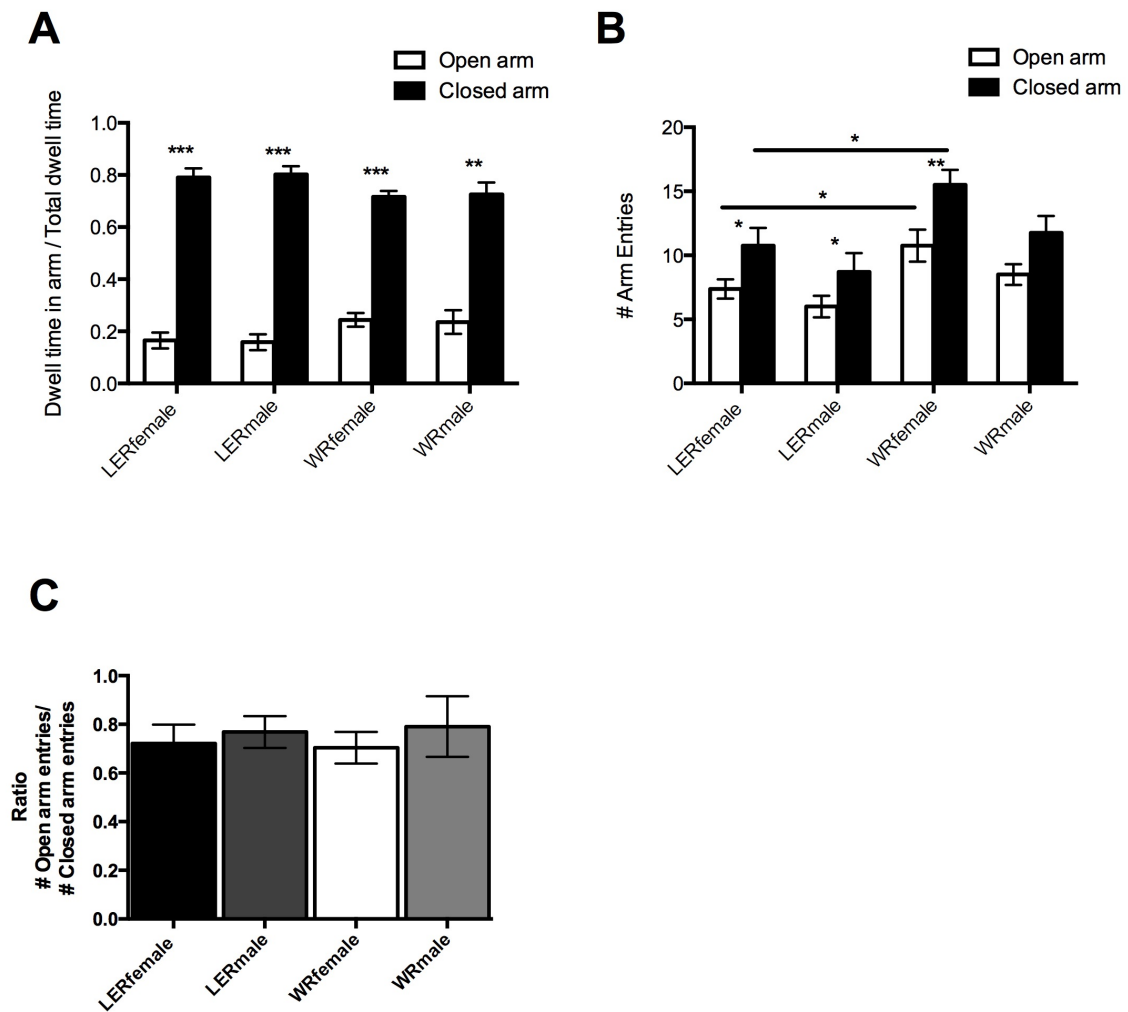
Figure 8: EPM. A. Percentage dwell time in open and close arm. B. Number of open and closed arm entries. C. The ratio of open to closed arm entries. * p < 0.05. ** p < 0.01. *** p < 0.001.
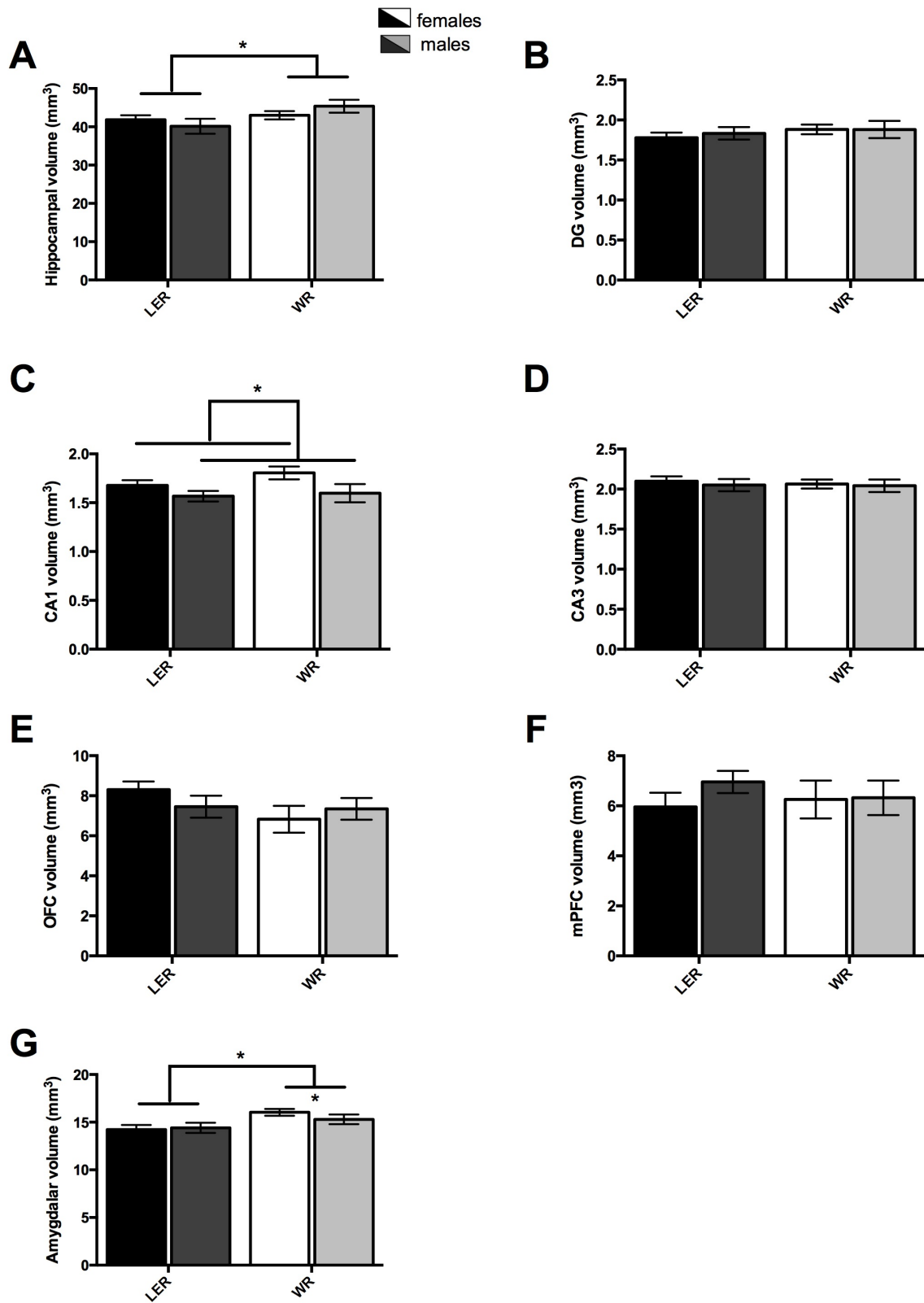
Figure 9: Volumetric estimates of A. Hippocampus, B. DG, C. CA1, D. CA3, E. OFC, F. mPFC, and G. Amygdala. *p<0.05.
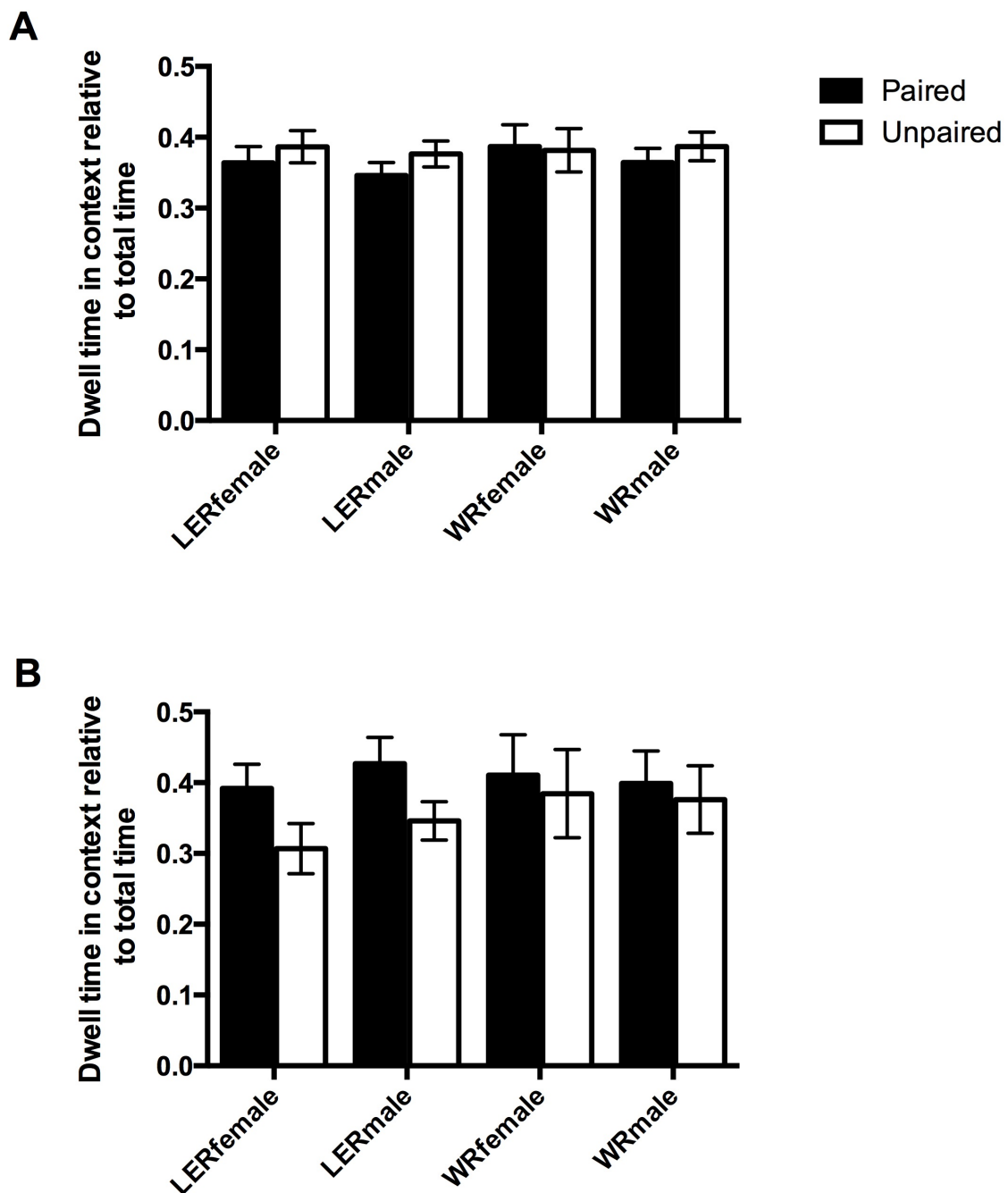
Figure 10: CPP using a 0.5mg/kg dose of amphetamine from rats purchased from Charles River. A. Pre-exposure. B. Preference.
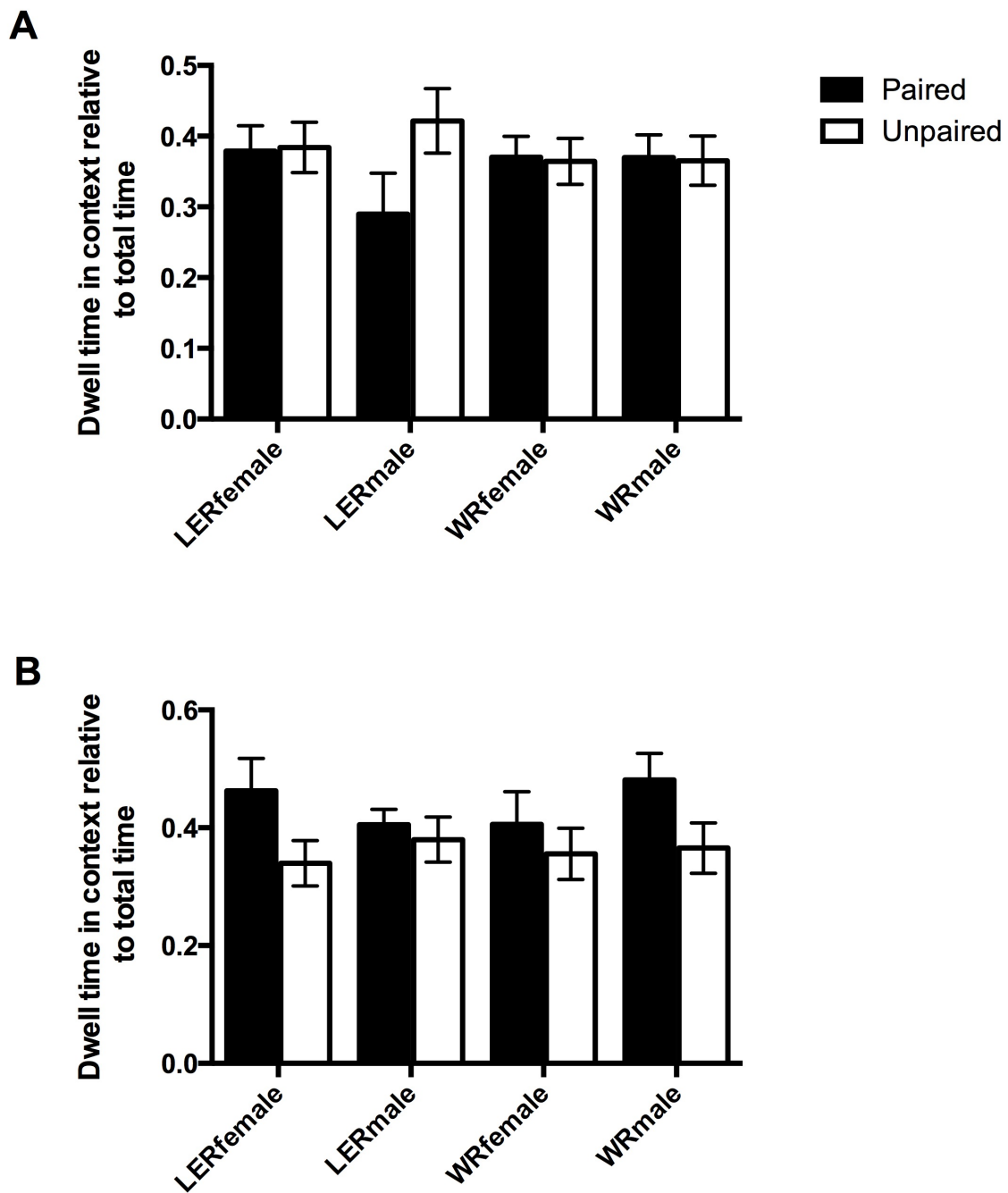
Figure 11: CPP using a 0.7mg/kg dose of amphetamine from rats purchased from Charles River. A. Pre-exposure. B. Preference.
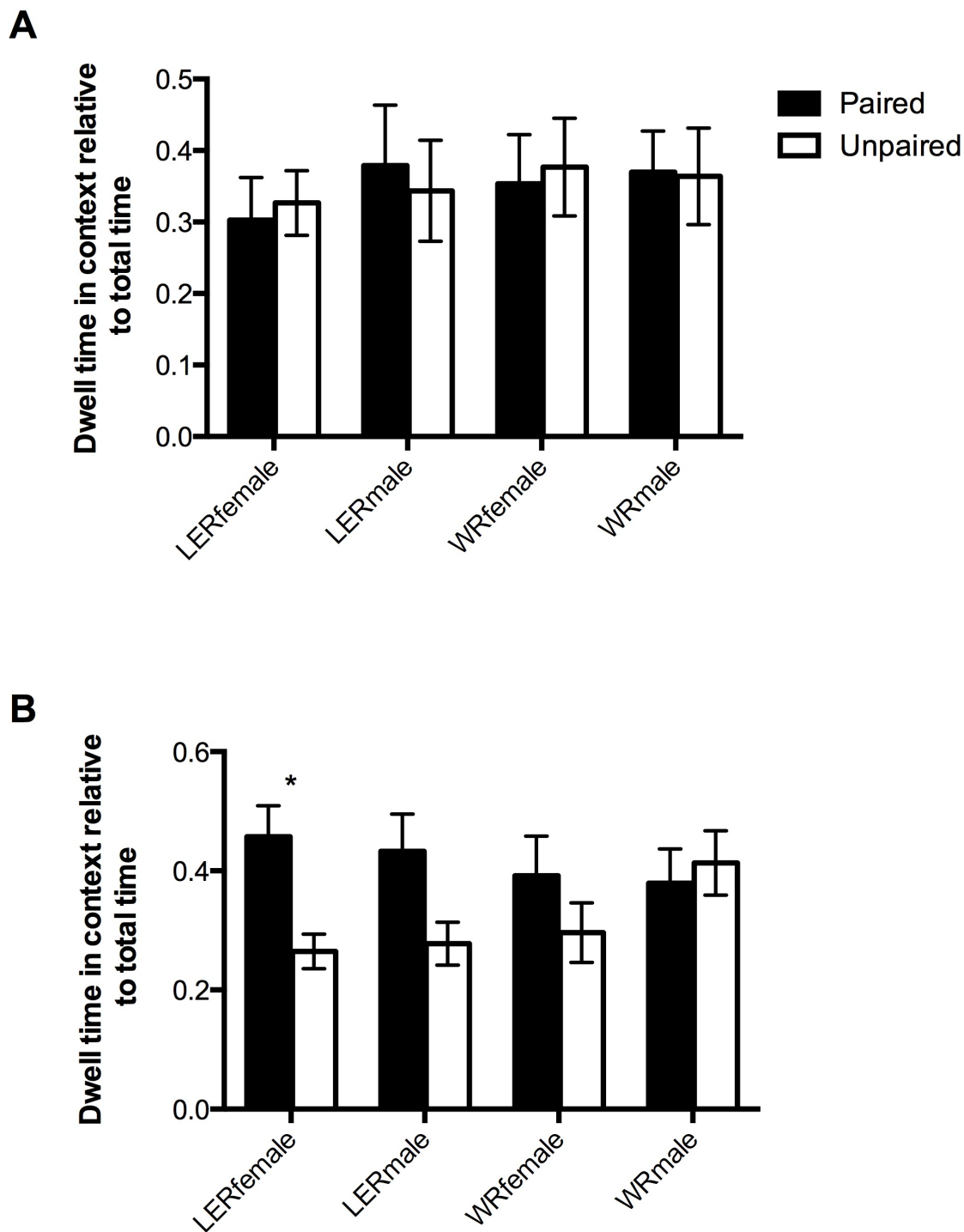
Figure 12: CPP using a 0.7mg/kg dose of amphetamine from rats bred in house at the University of Lethbridge CCBN. A. Pre-exposure. B. Preference. * p < 0.05.

A

B
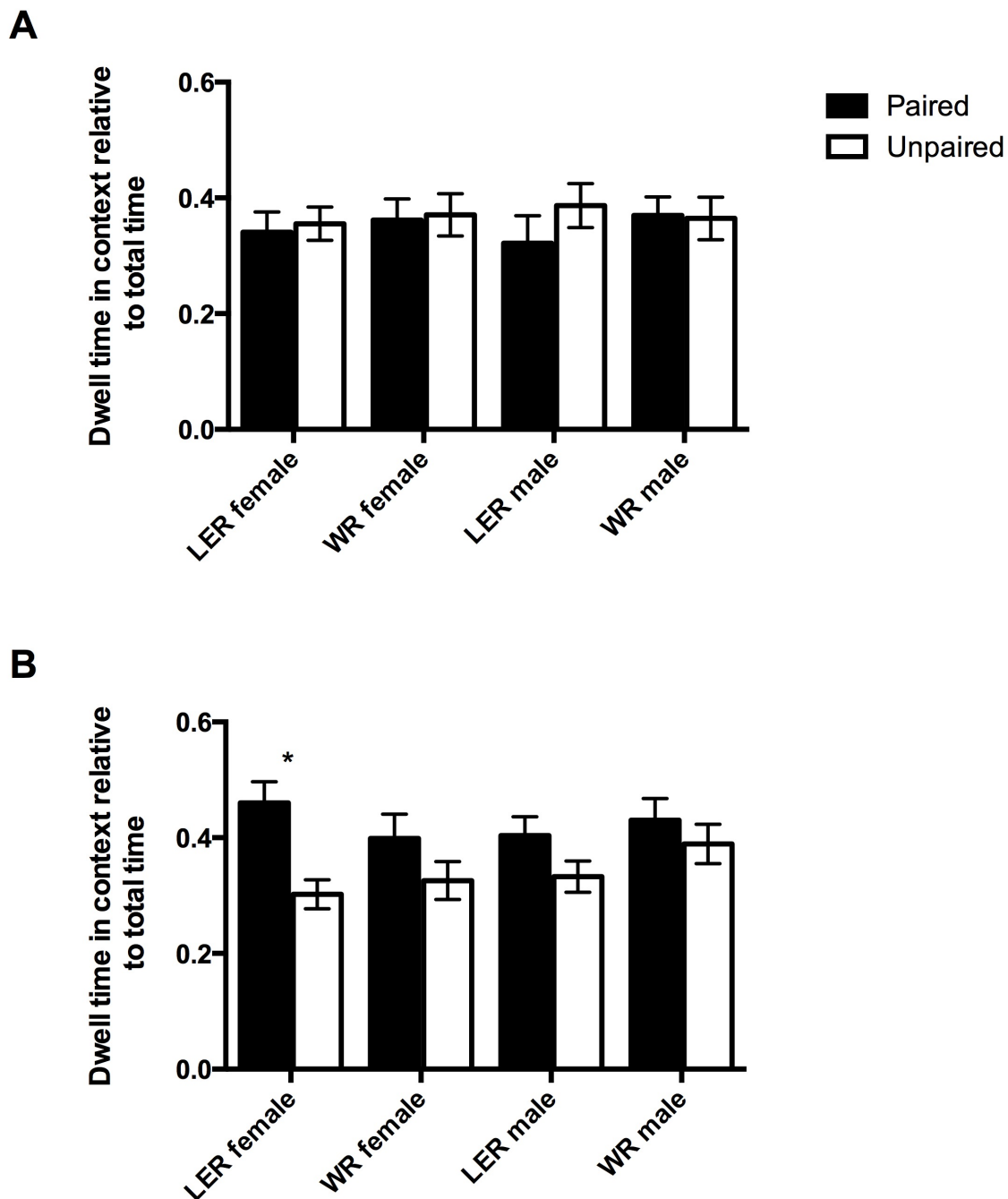
Figure 13: CPP using a 0.7mg/kg dose of amphetamine from rats pooled from both Charles River and the CCBN. A. Pre-exposure. B. Preference. * p < 0.05.
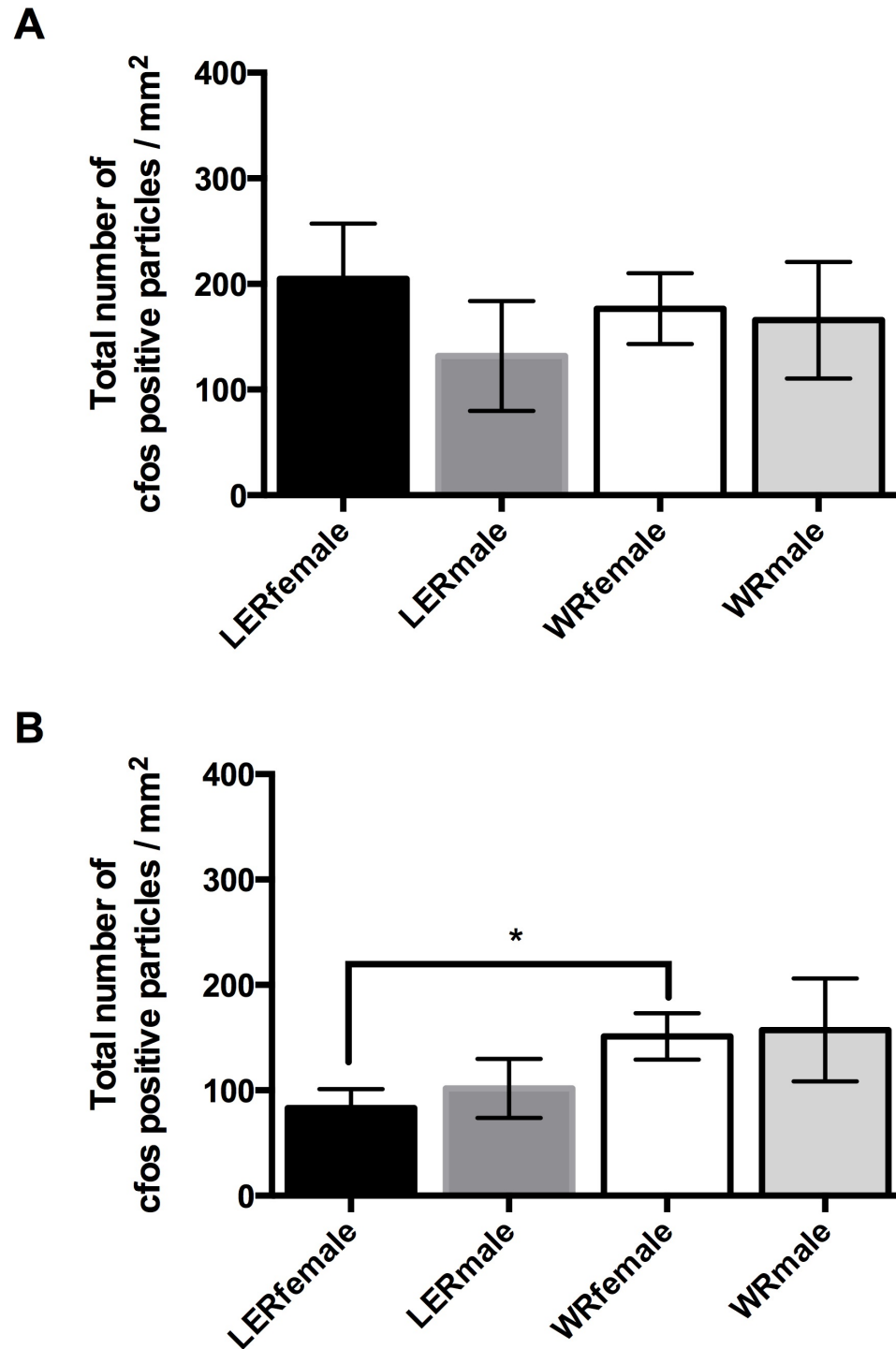
Figure 14: Total number of cfos particles per mm^2 of tissue from representative images of A. nucleus accumbens and B. dorsal hippocampus. * p < 0.05.
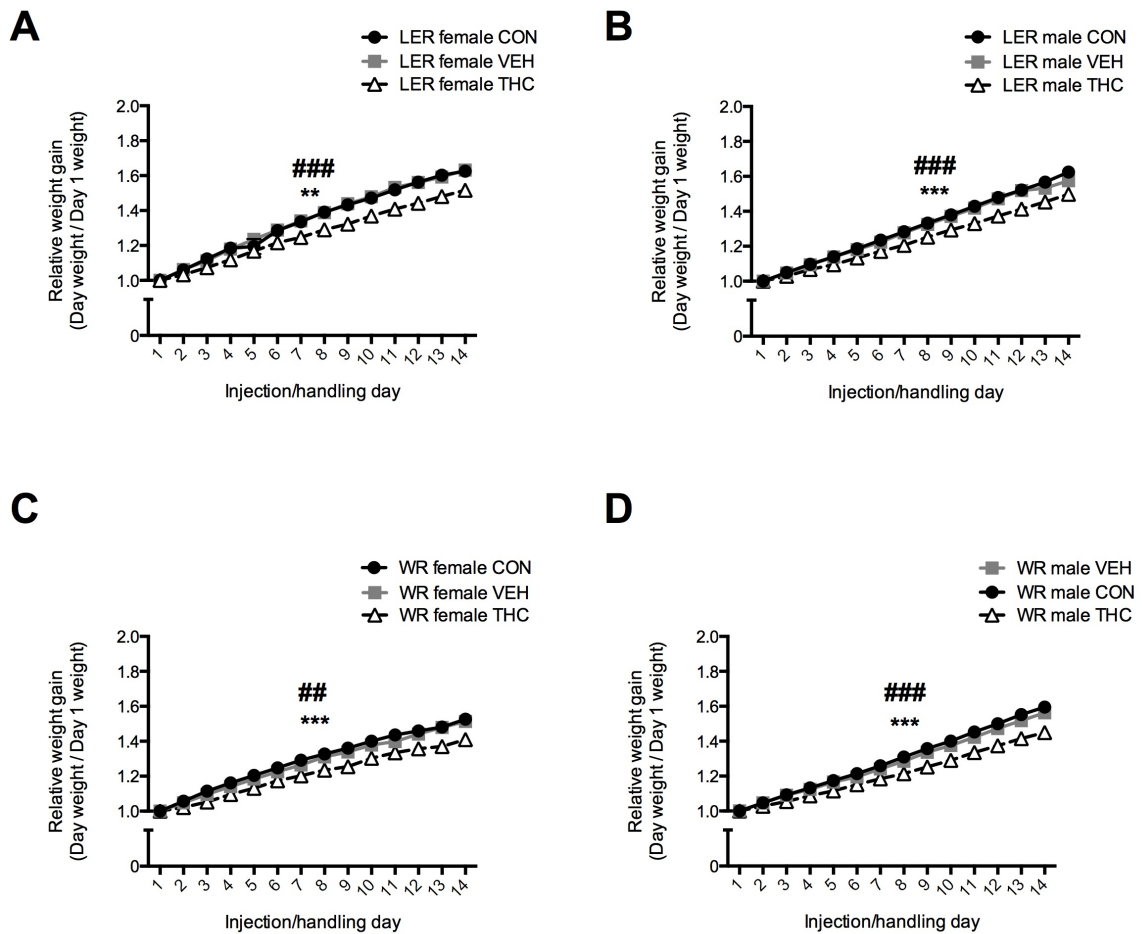
Figure 15: Weight gain during the injection period. A. LER females. B. LER males. C. WR females. D. WR males. Note * indicates a significant differences between controls and THC and # indicates a significant difference between vehicle and THC for all strain and sex groups. */# indicates p<0.05. **/## indicates p<0.01. ***/### indicates p< 0.001.
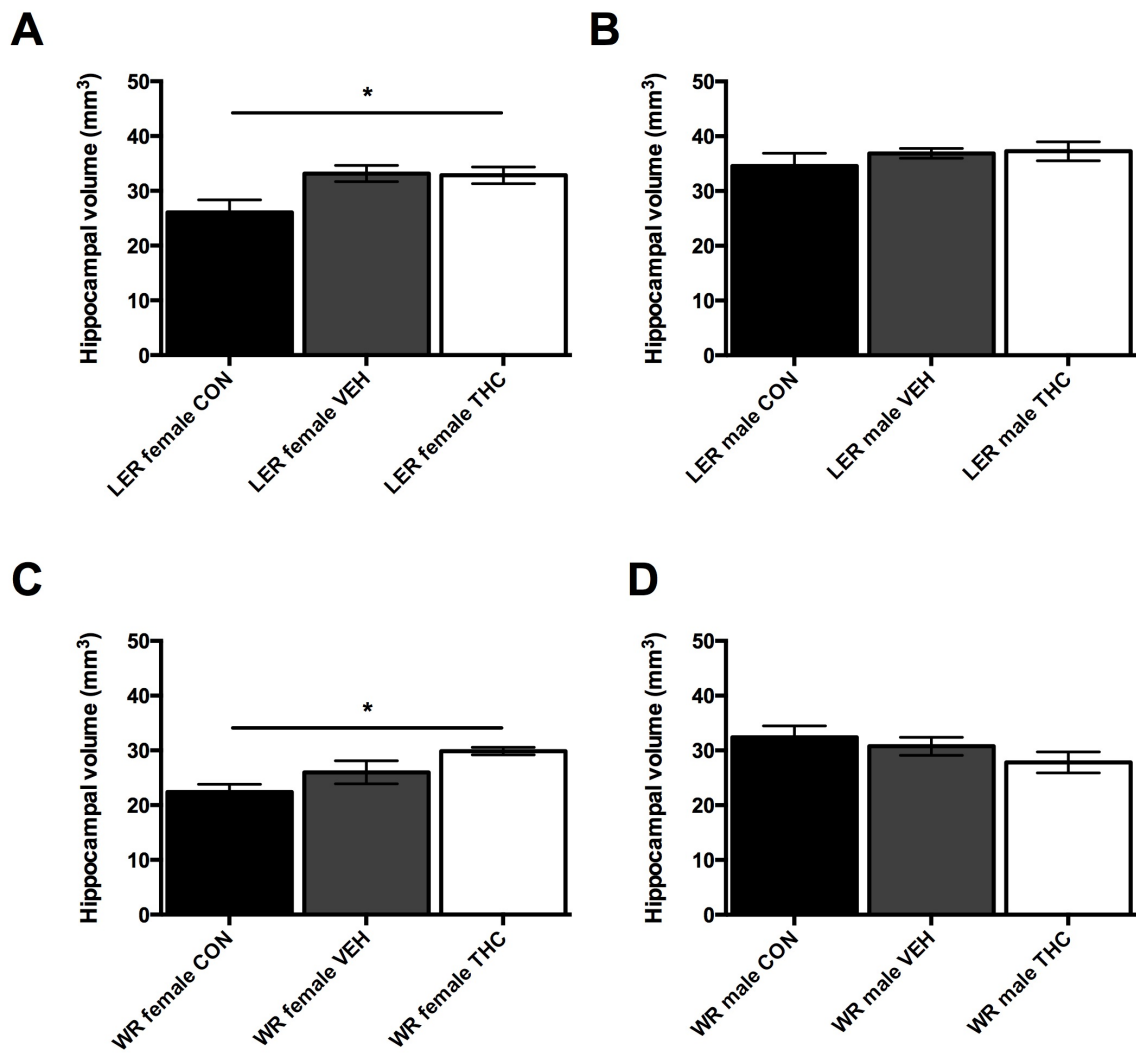
Figure 16: Total HP volume. A. LER females. B. LER males. C. WR females. D. WR males. * indicates a significant difference between CON and THC (p < 0.05).
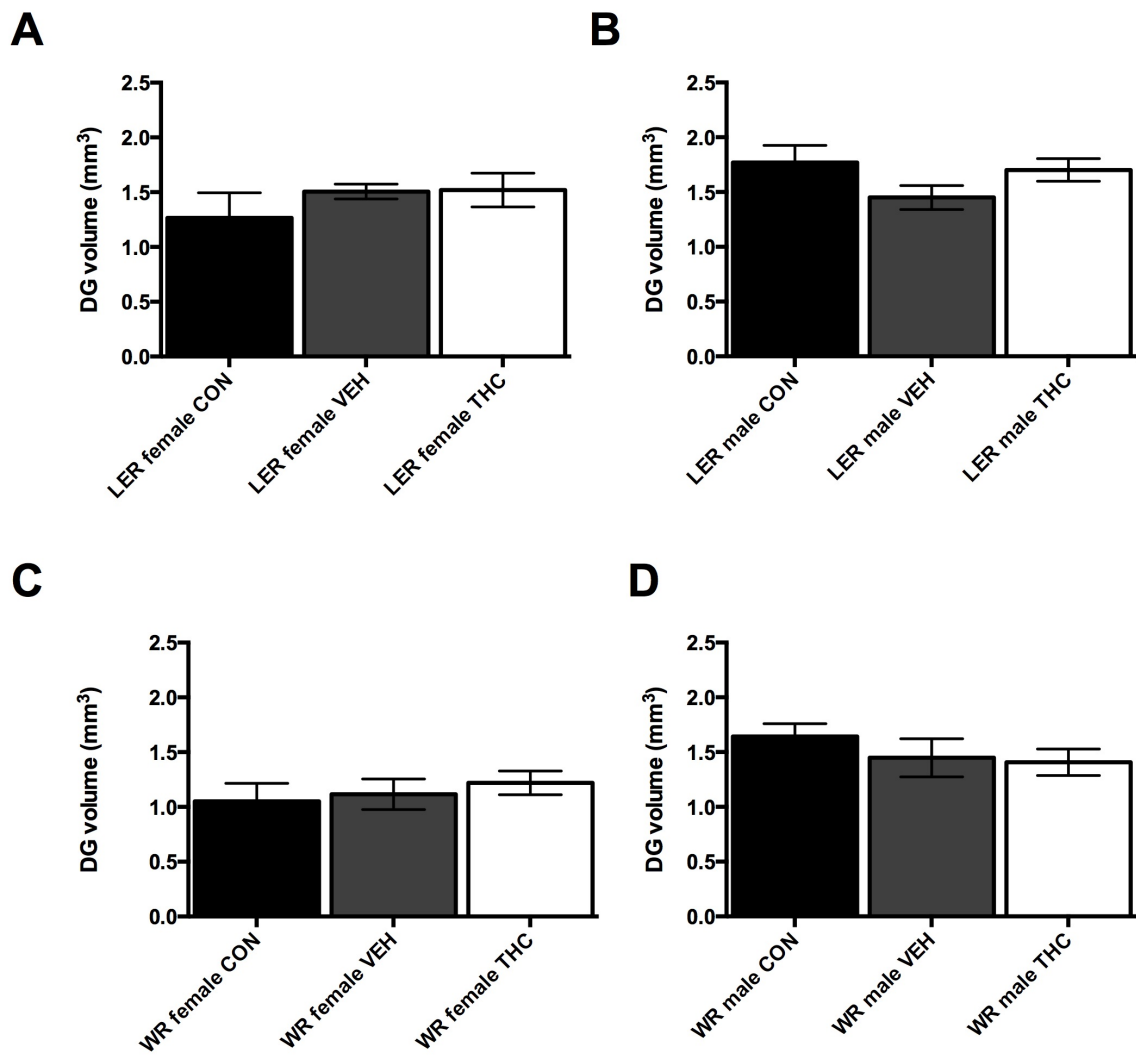
Figure 17: Dentate gyrus (DG) volume. A. LER females. B. LER males. C. WR females. D. WR males.
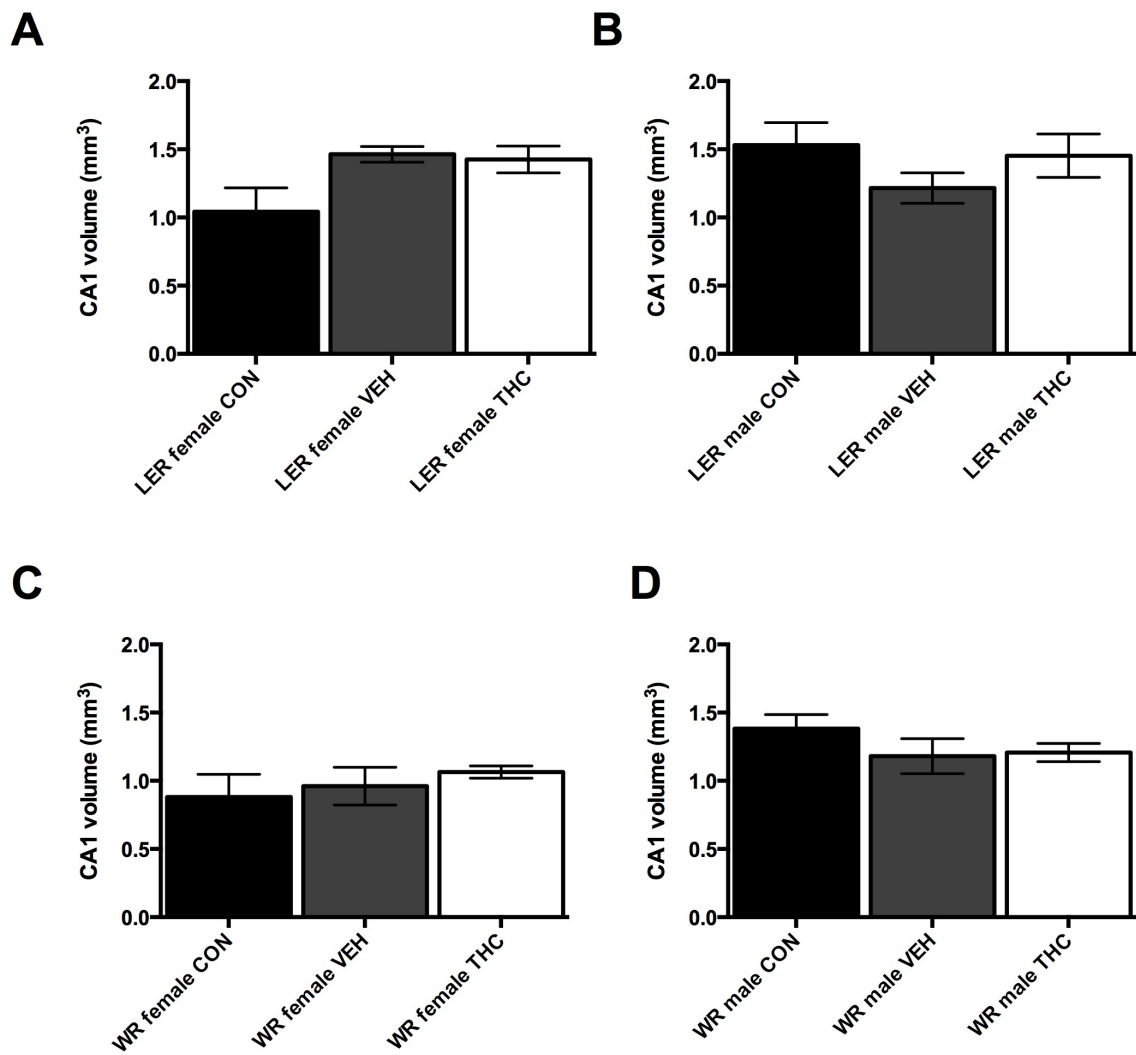
Figure 18: CA1 volume. A. LER females. B. LER males. C. WR females. D. WR males.
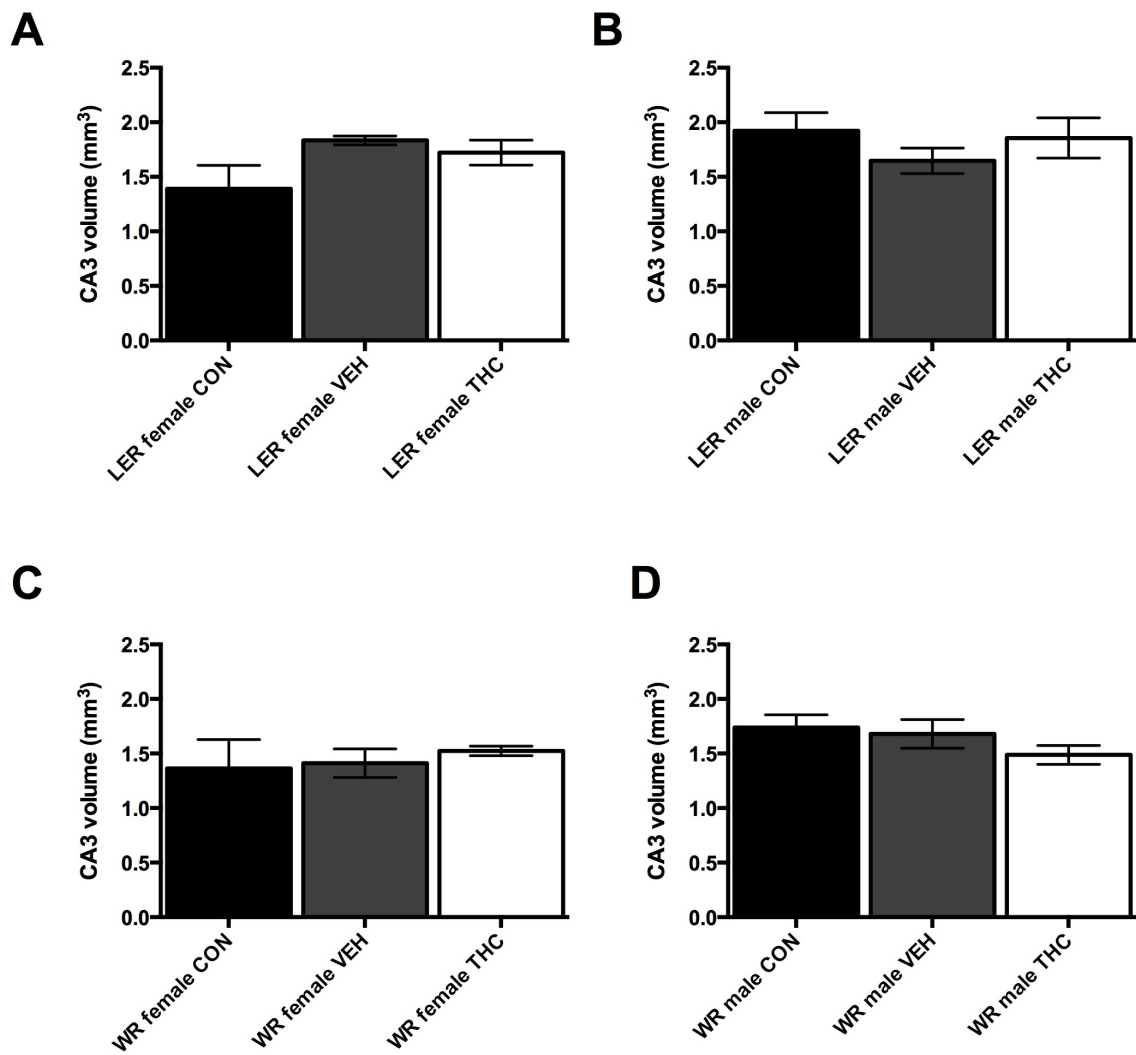
Figure 19: CA3 volume. A. LER females. B. LER males. C. WR females. D. WR males.
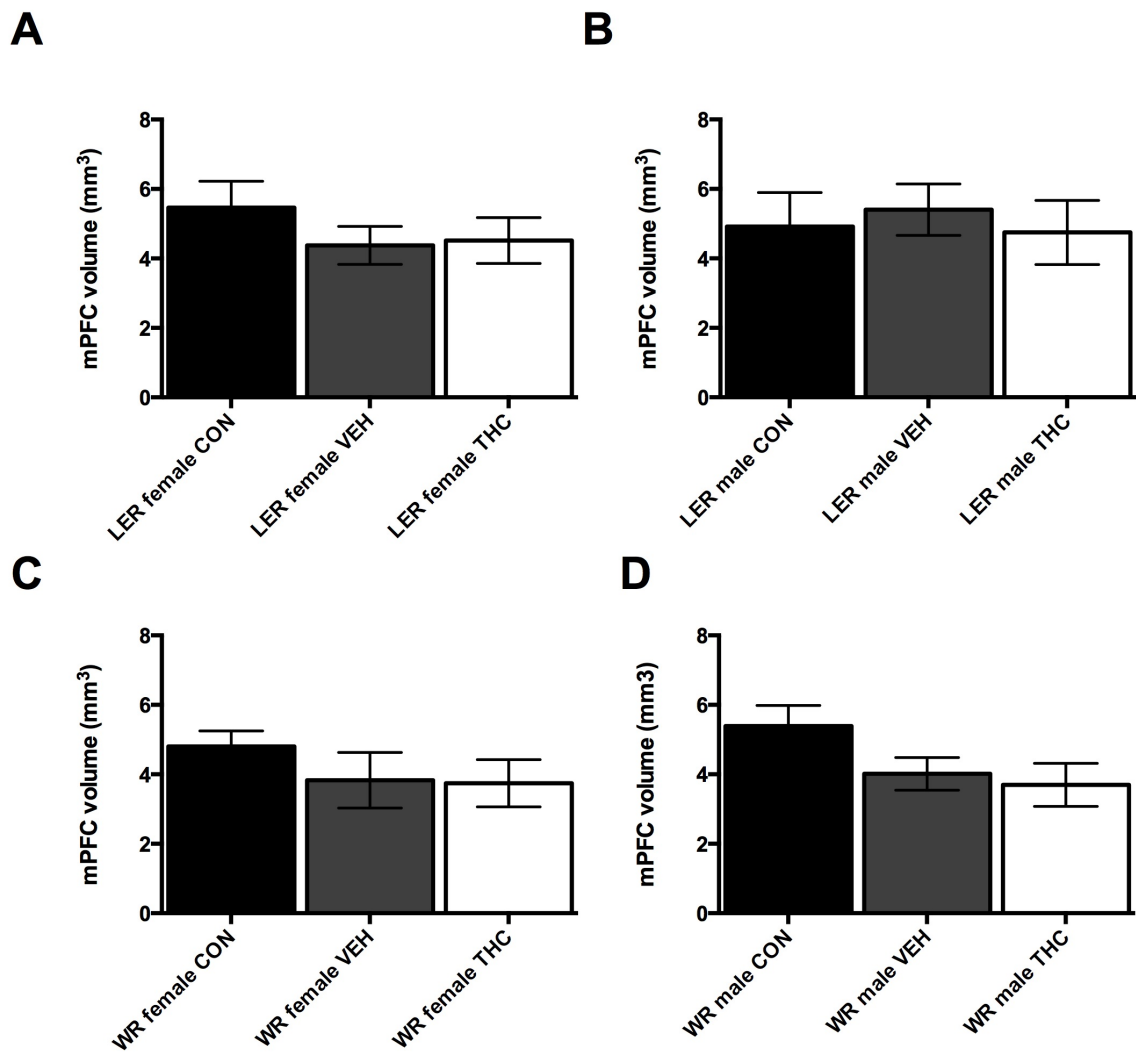
Figure 20: mPFC volume. A. LER females. B. LER males. C. WR females. D. WR males.
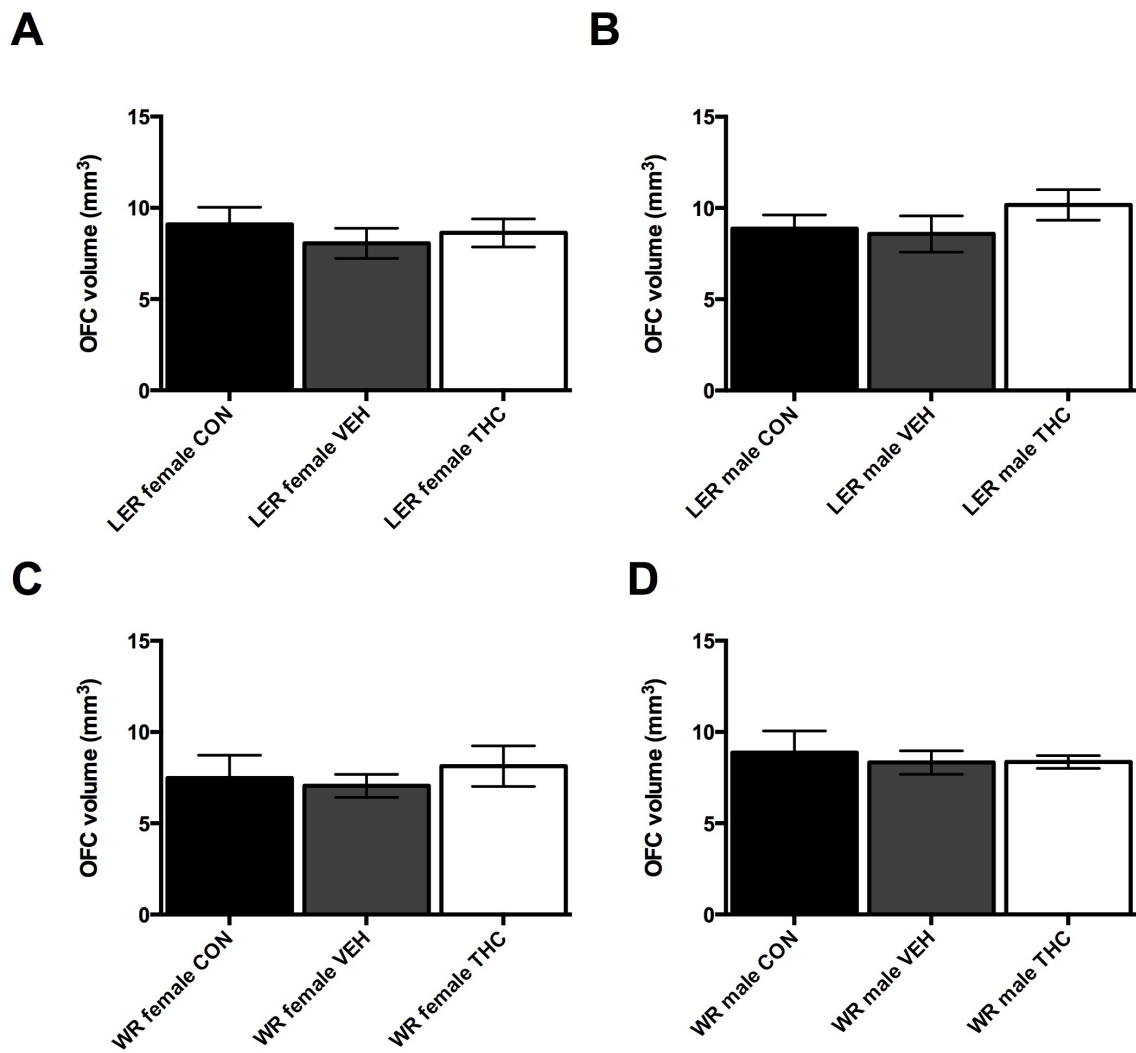
Figure 21: OFC volume. A. LER females. B. LER males. C. WR females. D. WR males.
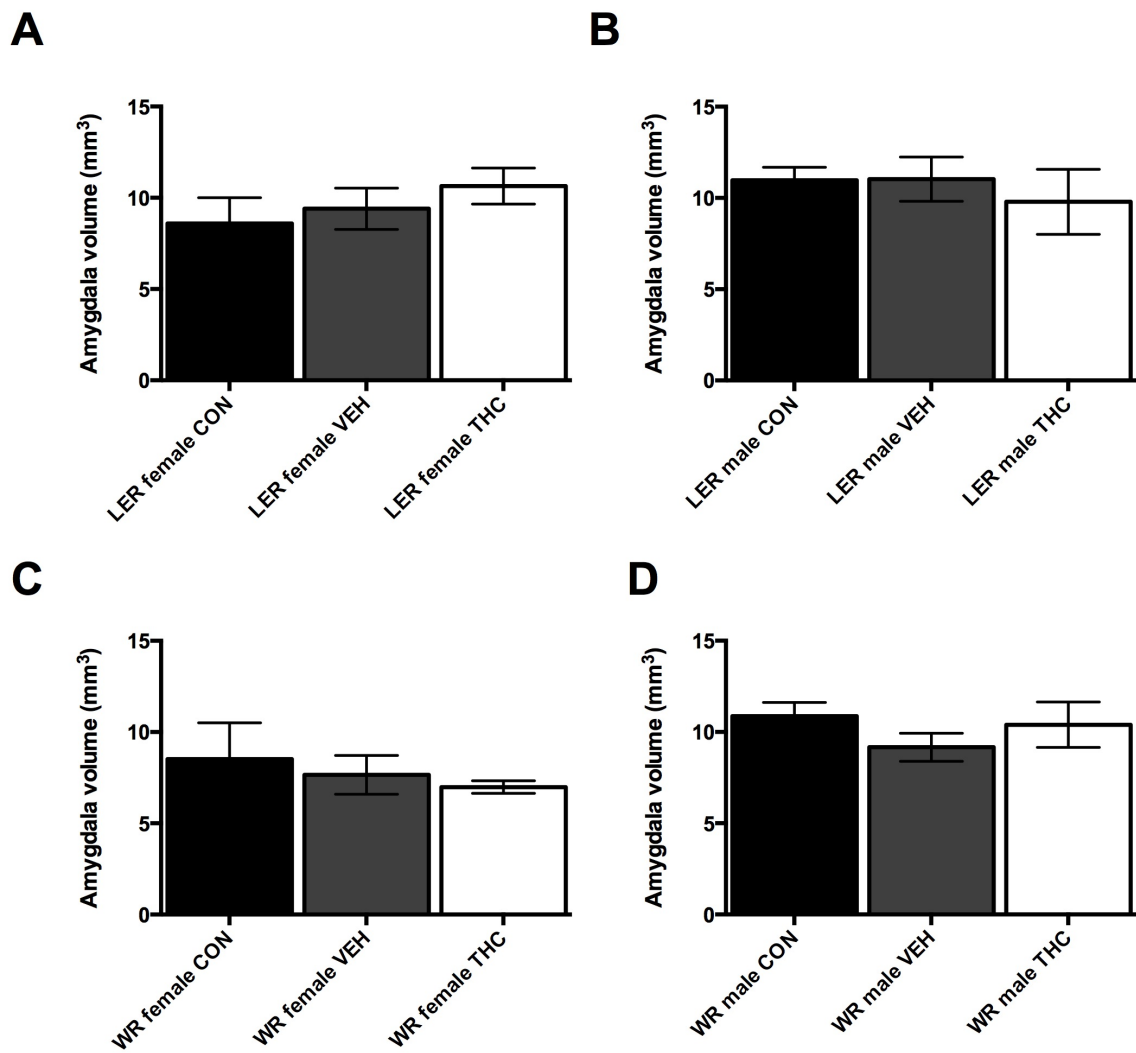
Figure 22: AMYG volume. A. LER females. B. LER males. C. WR females. D. WR males.
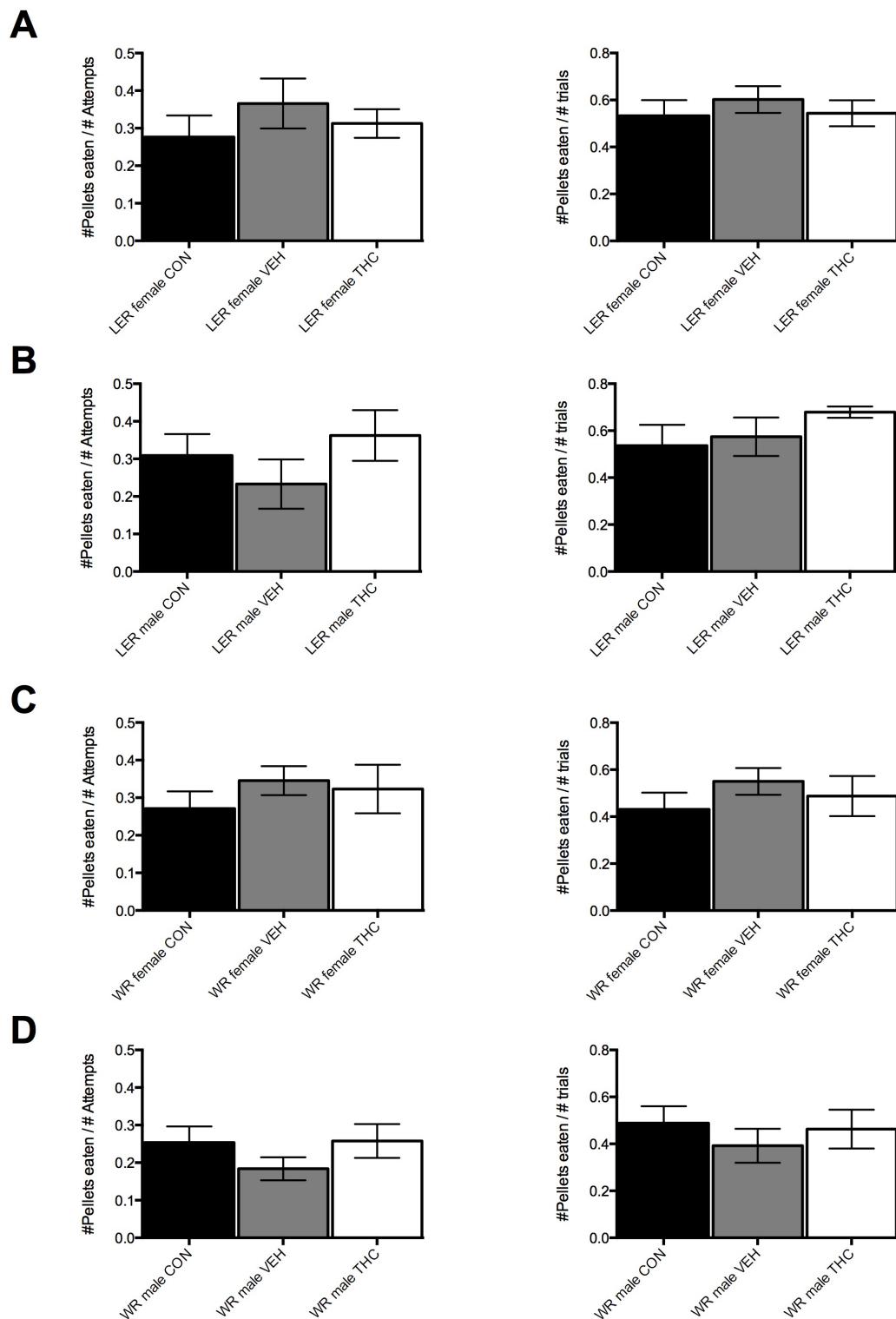
Figure 23: Effects of THC on motor learning in the SRT. Both successful attempts and successful trials are presented. A. LER females. B. LER males. C. WR females. D. WR males.
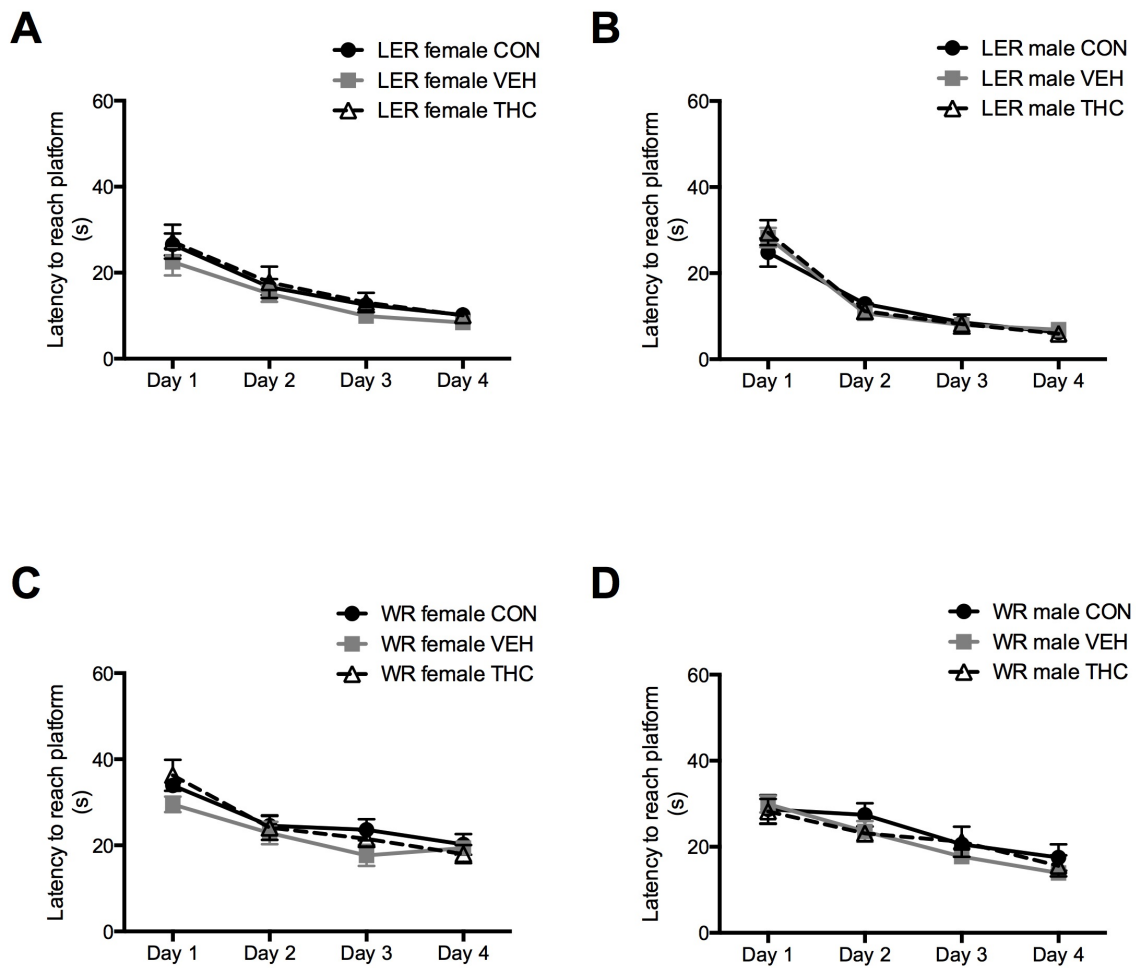
Figure 24: Effects of THC on MWT acquisition. A. LER females. B. LER males. C. WR females. D. WR males.
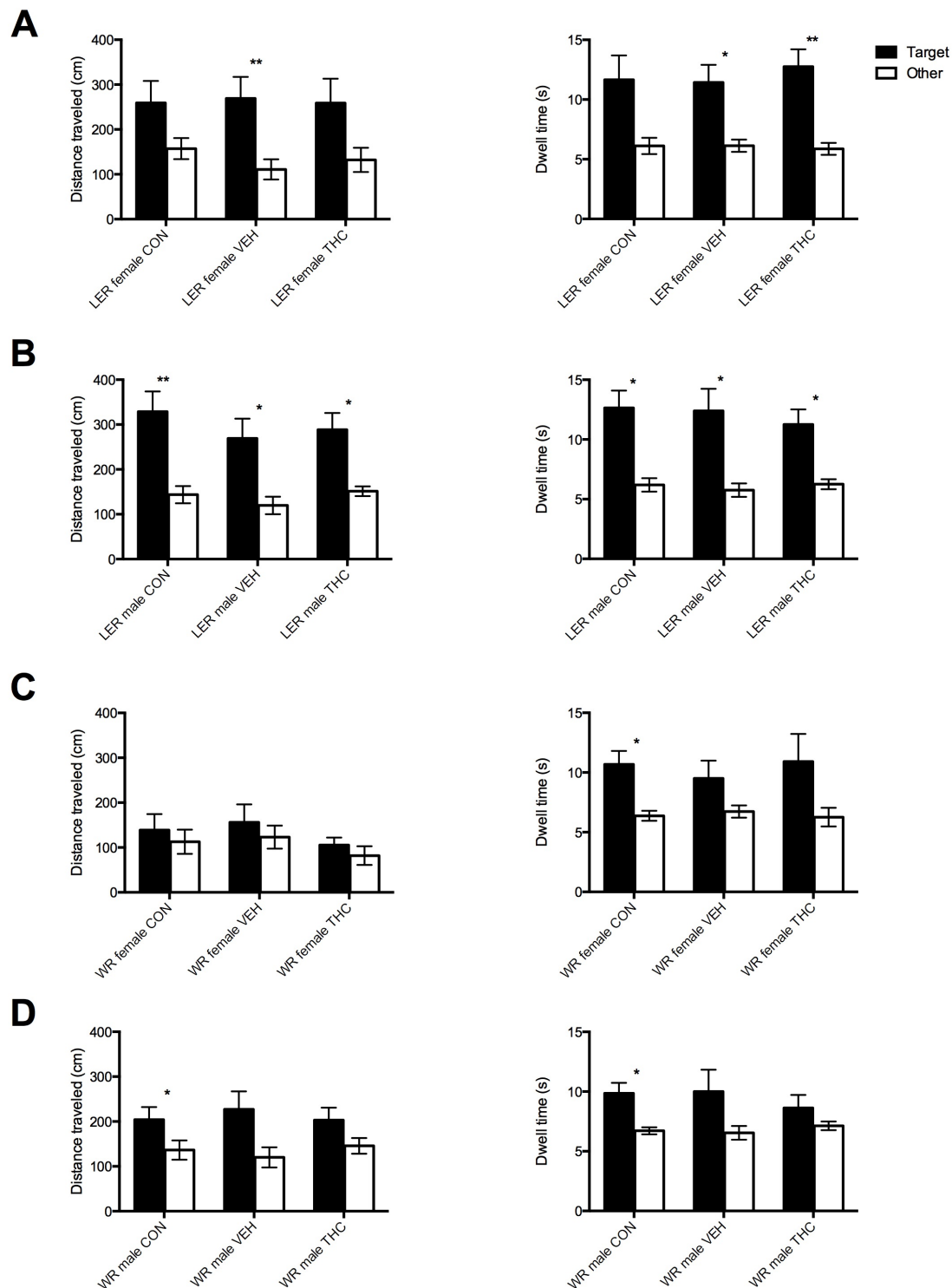
Figure 25: Effect of THC on retention. Probe trial performance for the first 30s. A. Distance travelled and dwell time for LER females. B. Distance travelled and dwell time for LER males. C. Distance travelled and dwell time for WR females. D. Distance travelled and dwell time for WR males. * indicates a significant difference < 0.05. ** indicated a significant difference < 0.01.
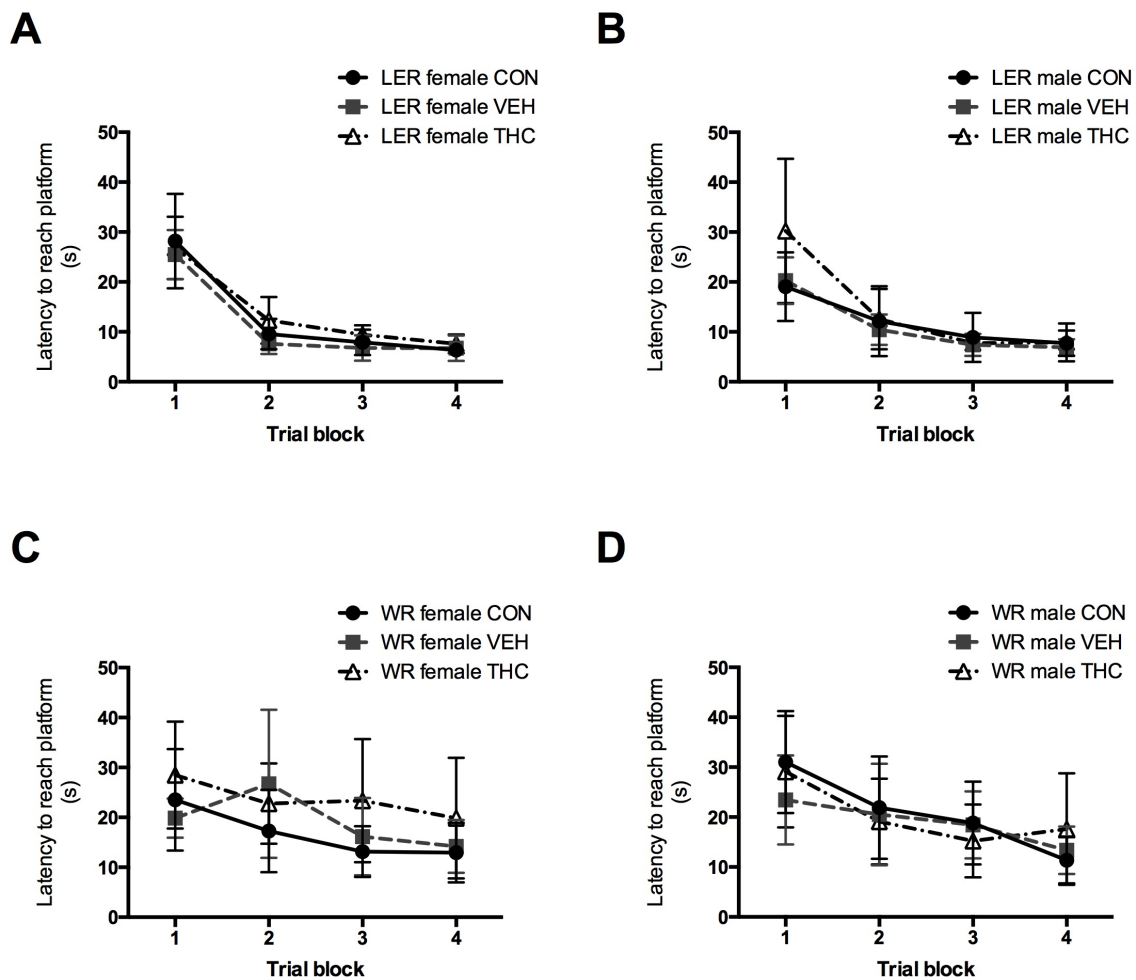
Figure 26: Effect of THC on Mass training to a new location. Trials were binned into blocks of 4. A. LER females. B. LER males. C. WR females. D. WR males.
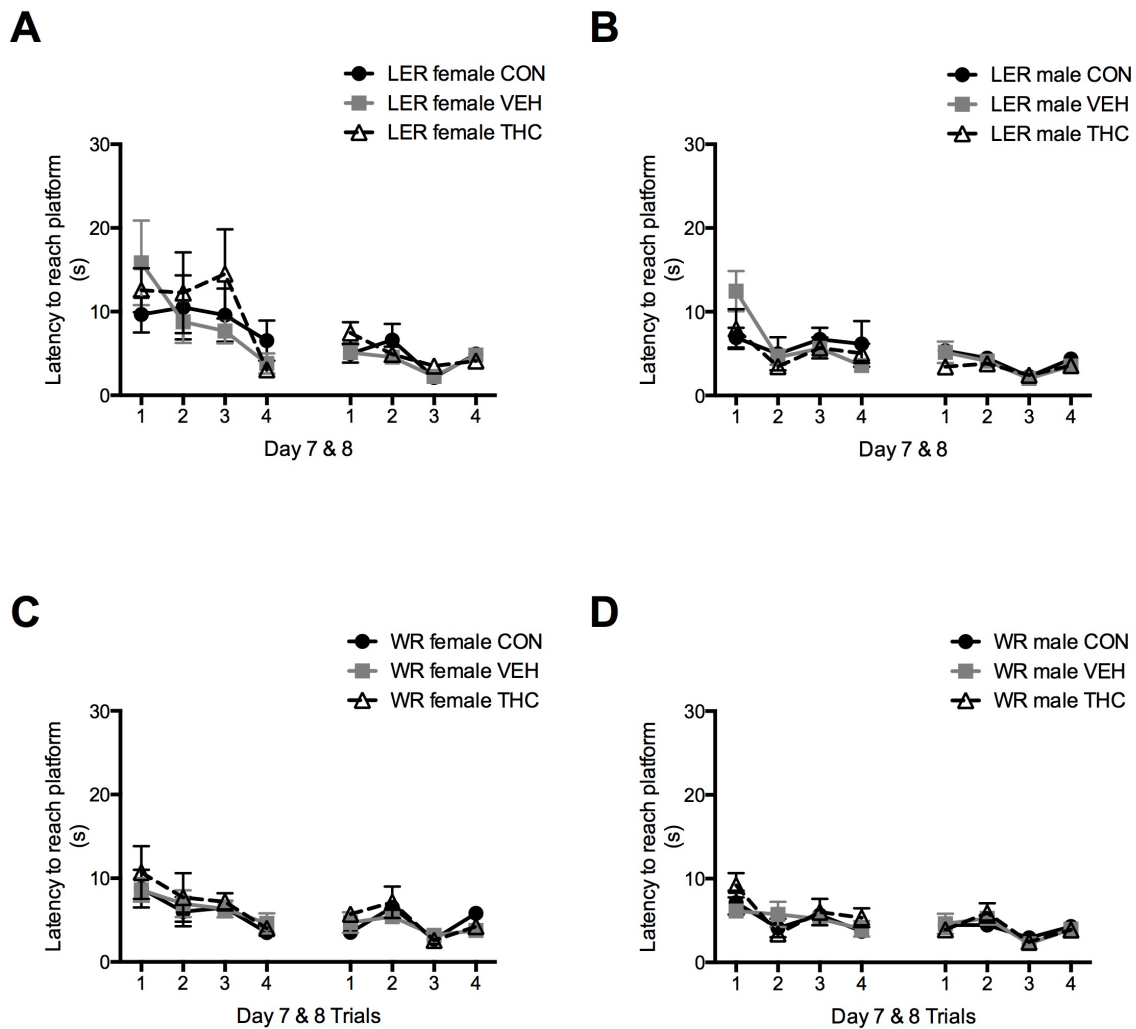
Figure 27: Effects of THC on visual and motor performance in the MWT. A. LER females. B. LER males. C. WR females. D. WR males.
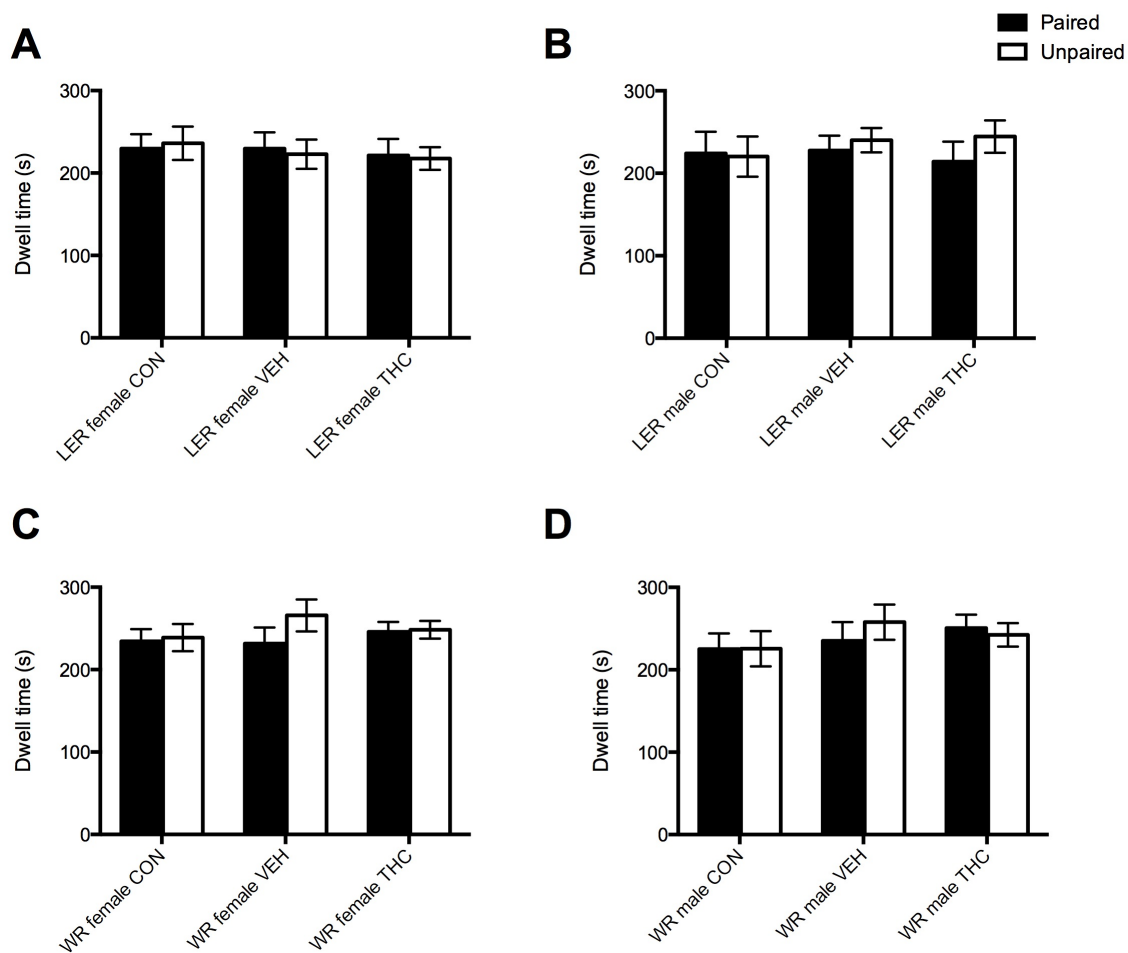
Figure 28: Effect of THC on pre-exposure of the DFCTC. A. LER females. B. LER males. C. WR females. D. WR males.
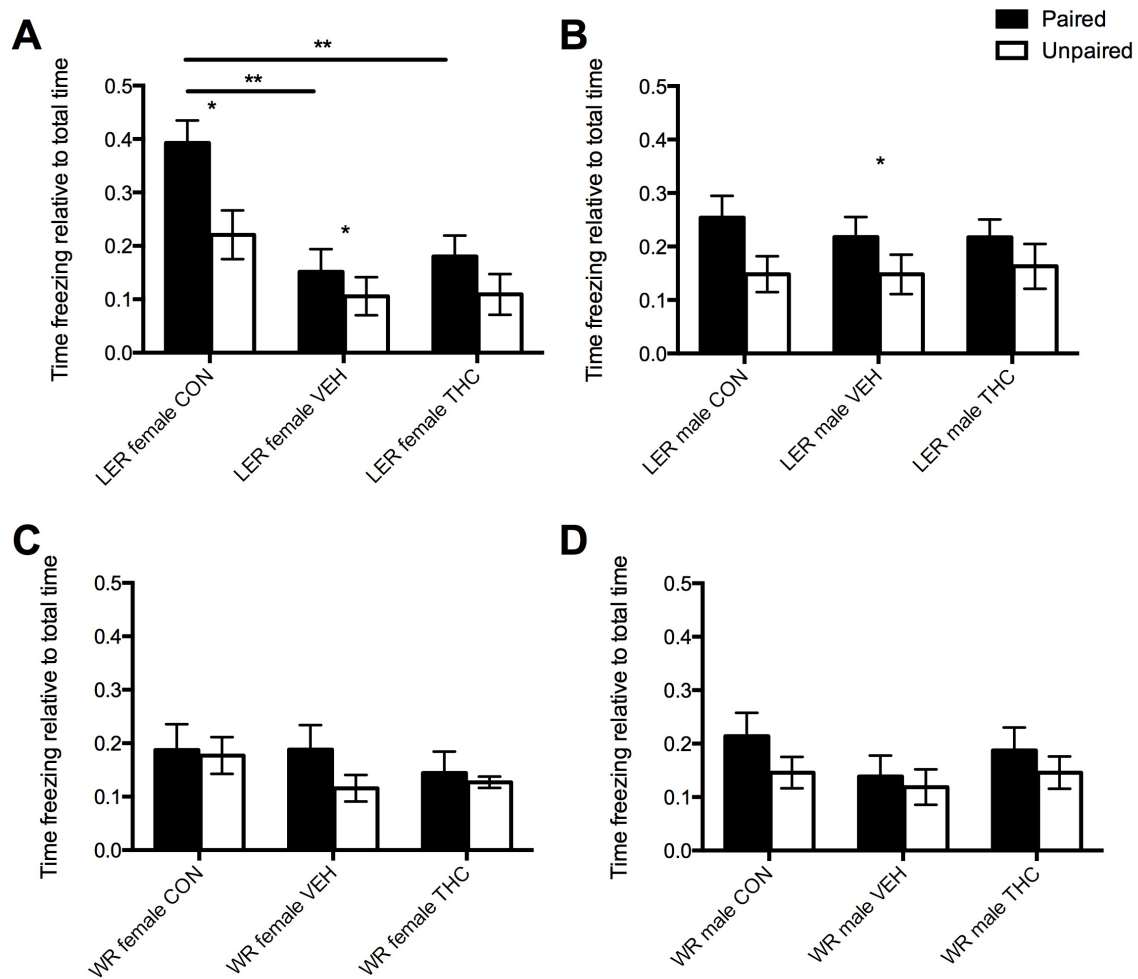
Figure 29: Effect of THC on discriminative freezing behaviour in the DFCTC paradigm. A. LER females. B. LER males. C. WR females. WR males. * indicates a significant difference < 0.05. ** indicated a significant difference < 0.01.
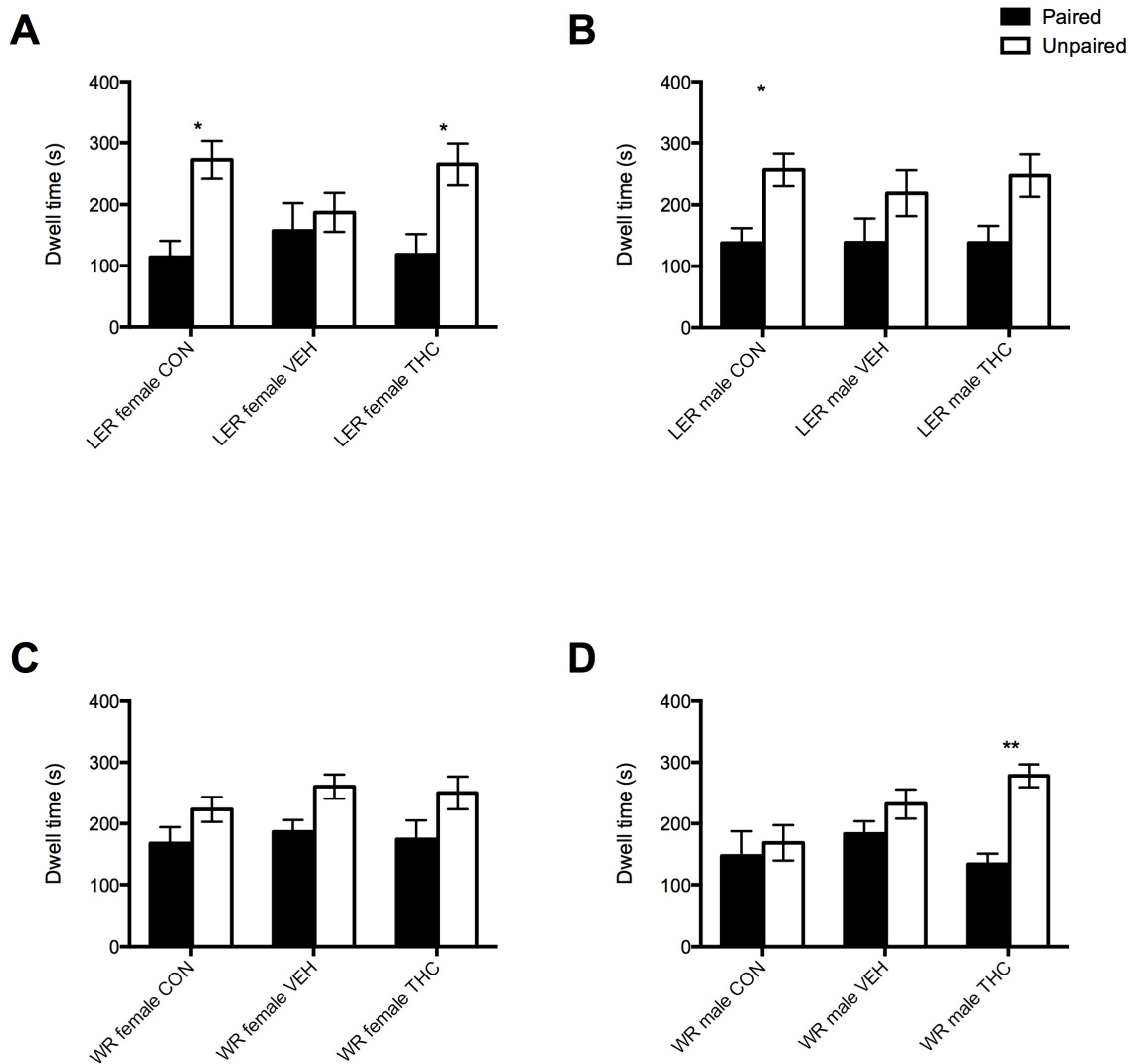
Figure 30: Effect of THC on DFCTC preference behaviour. A. LER females. B. LER males. C. WR females. D. WR males. * indicates p<0.05. ** indicates p<0.01.
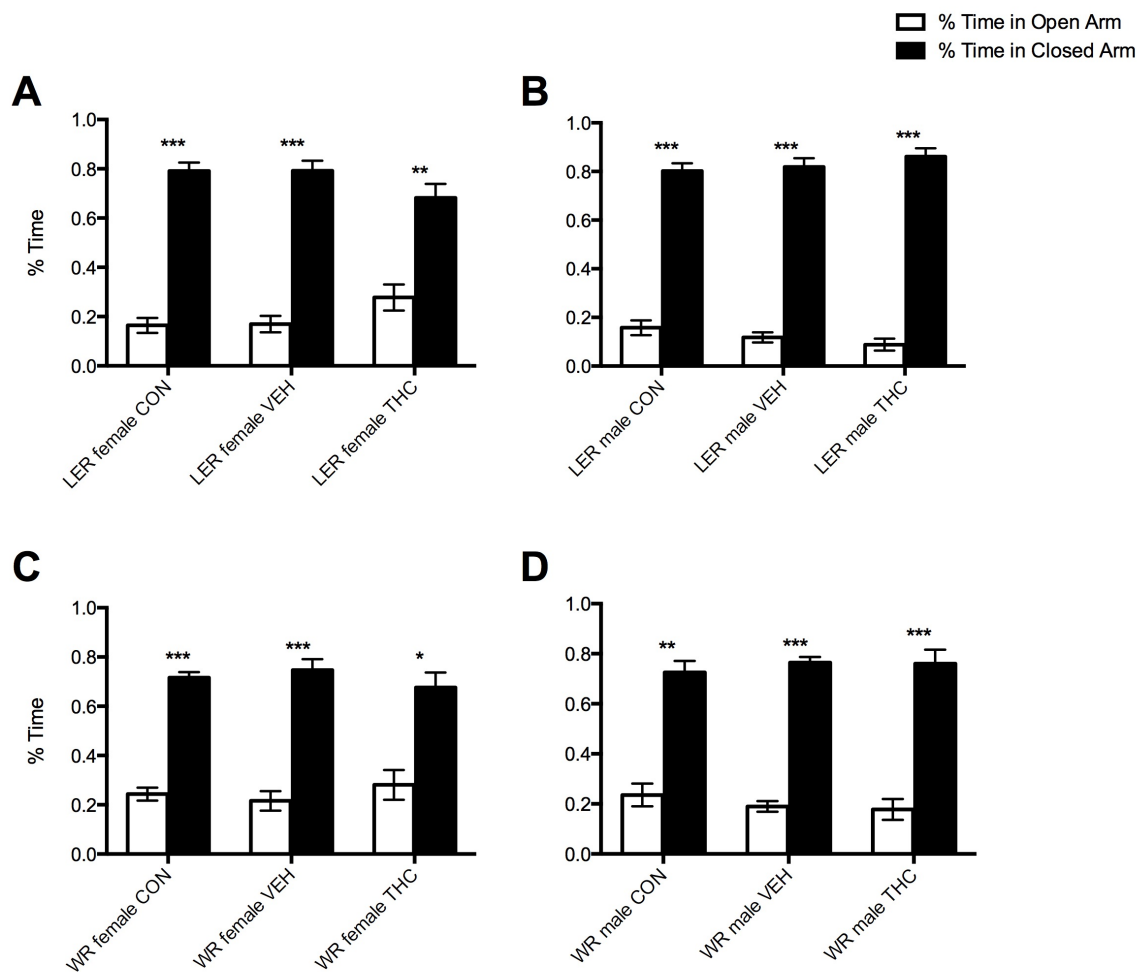
Figure 31: The effect of THC on dwell time in the open and closed arms in elevated plus maze. A. LER females. B. LER males. C. WR females. D. WR males. * indicates $p<0.05$. ** indicates $p<0.01$. ***indicates $p<0.001$.
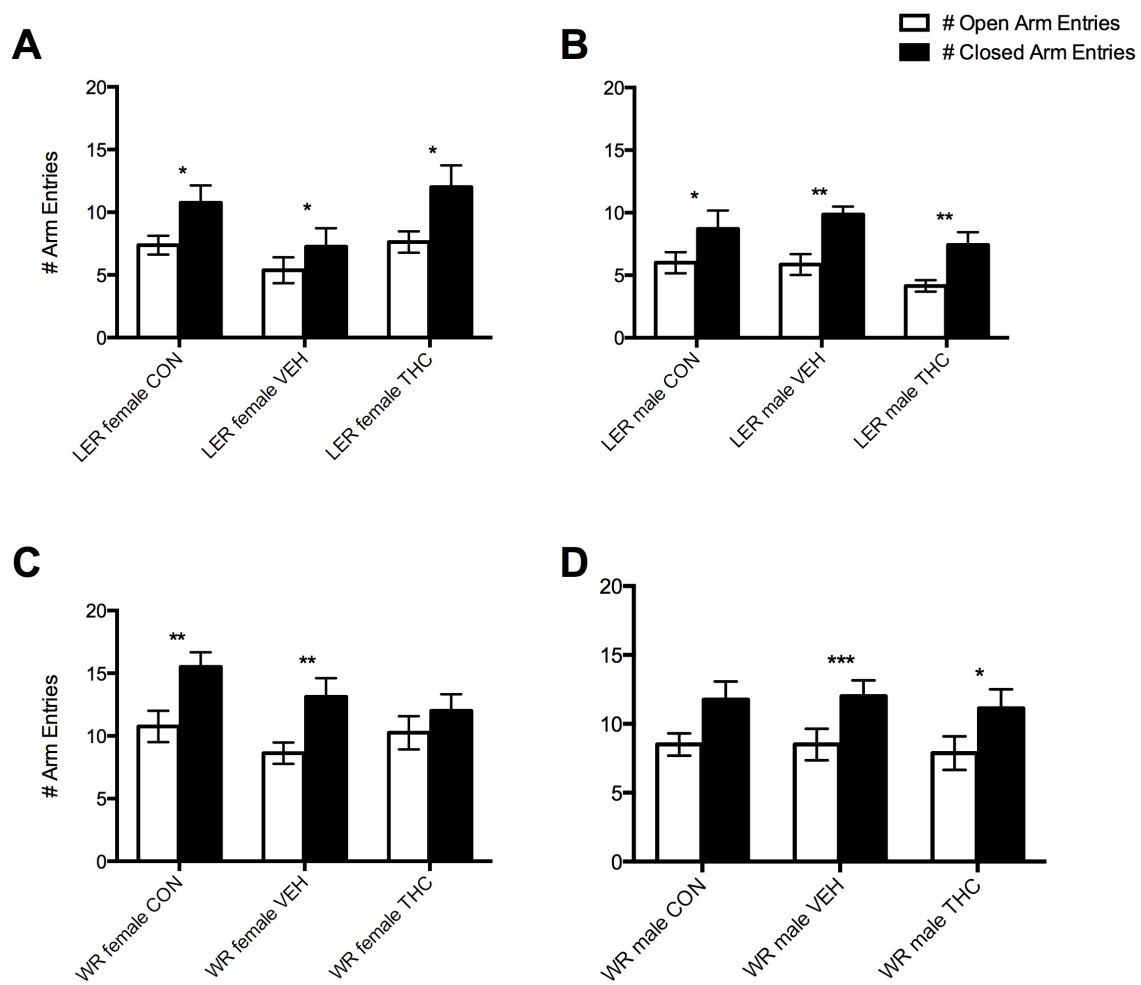
Figure 32: Effect of THC on arm entries in elevated plus maze. A. LER females. B. LER males. C. WR females. D. WR males. * indicates p<0.05. ** indicates p<0.01. *** indicates p<0.001.
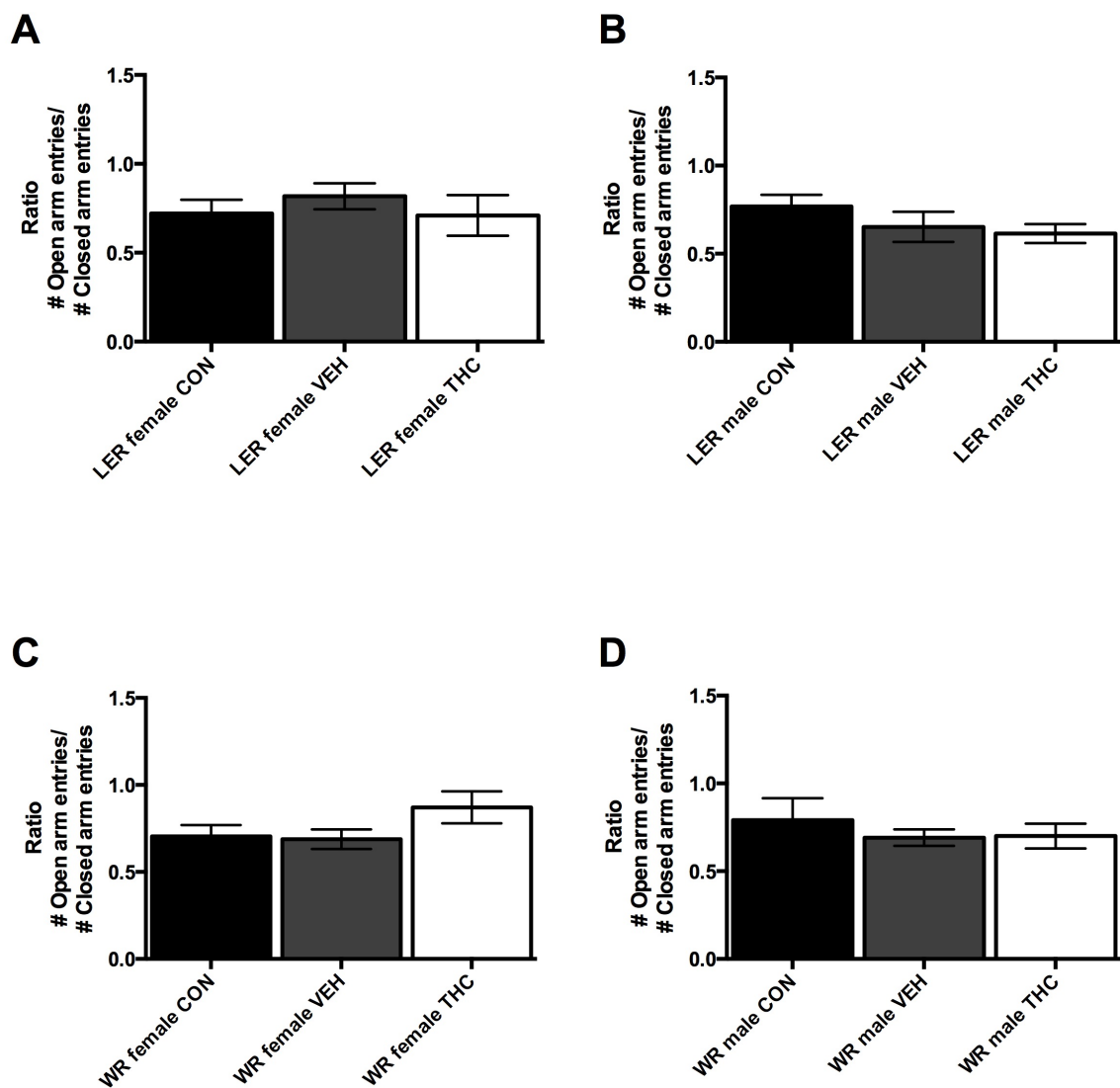
Figure 33: Effect of THC on ratio of open arm entries to closed. A. LER females. B. LER males. C. WR females. D. WR males
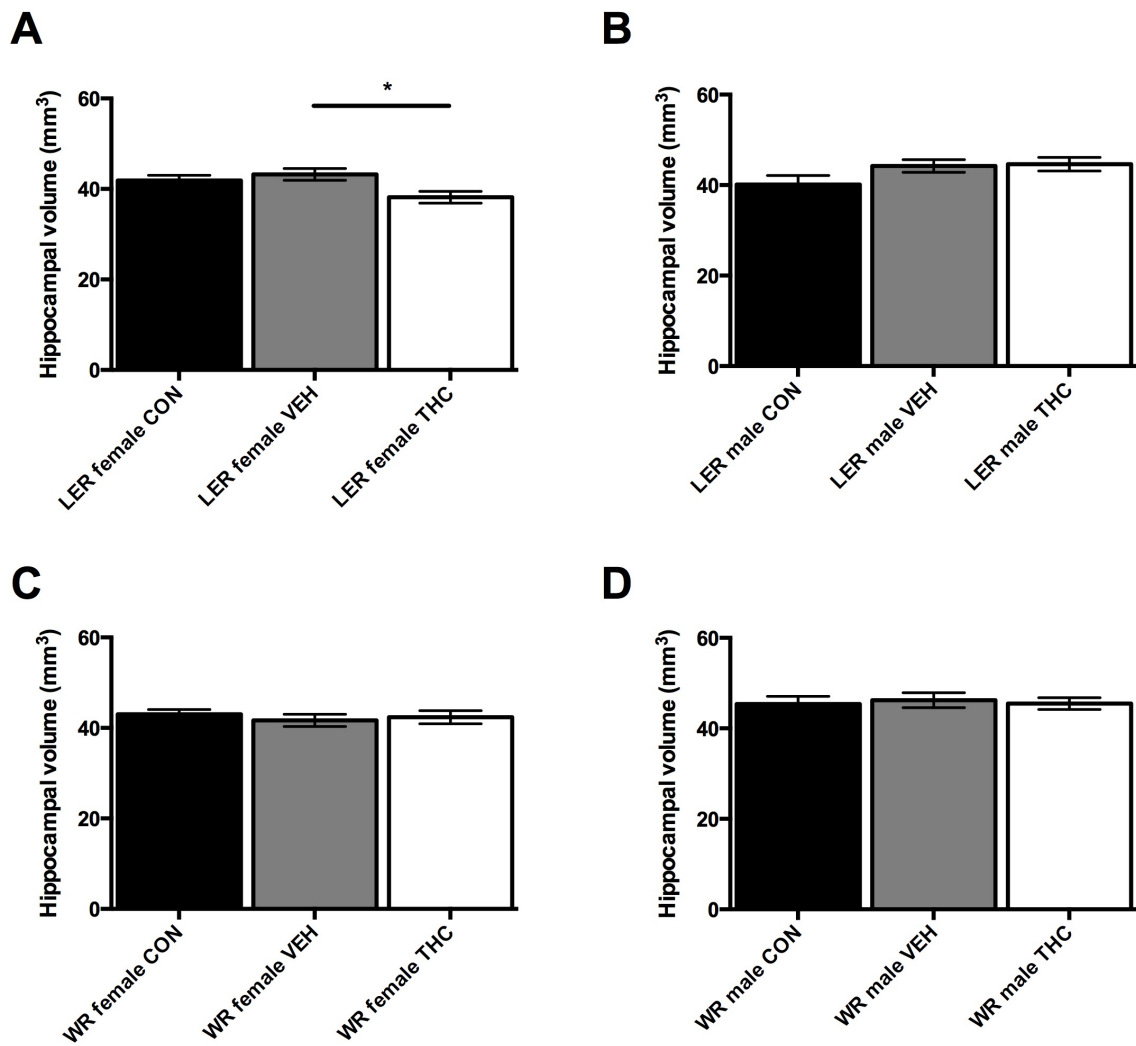
Figure 34: Effect of THC on hippocampal volumes. A. LER females. B. LER males. C. WR females. D. WR males. * indicates p<0.05.
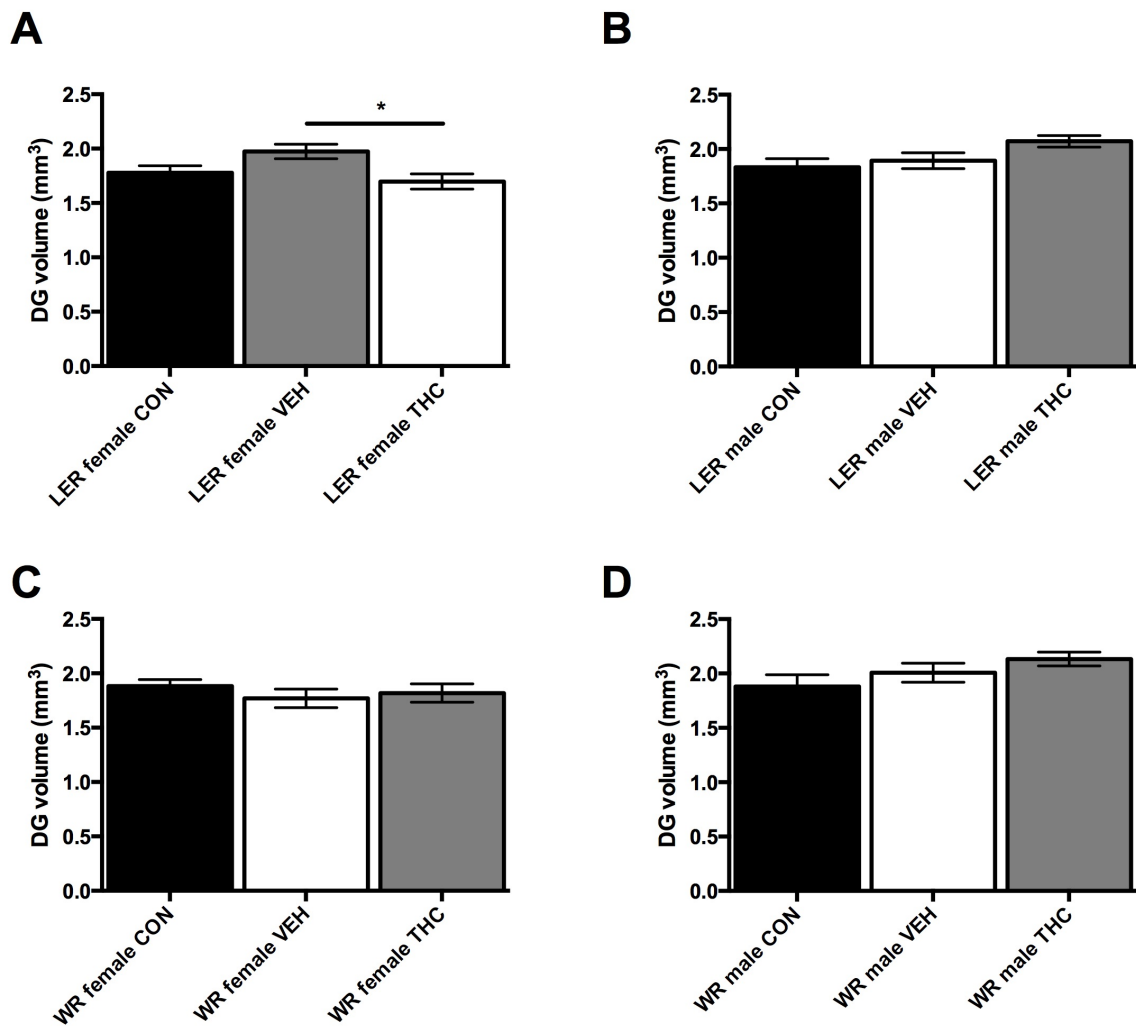
Figure 35: Effect of THC on DG volumes. A. LER females. B. LER males. C. WR females. D. WR males. * indicates p<0.05.
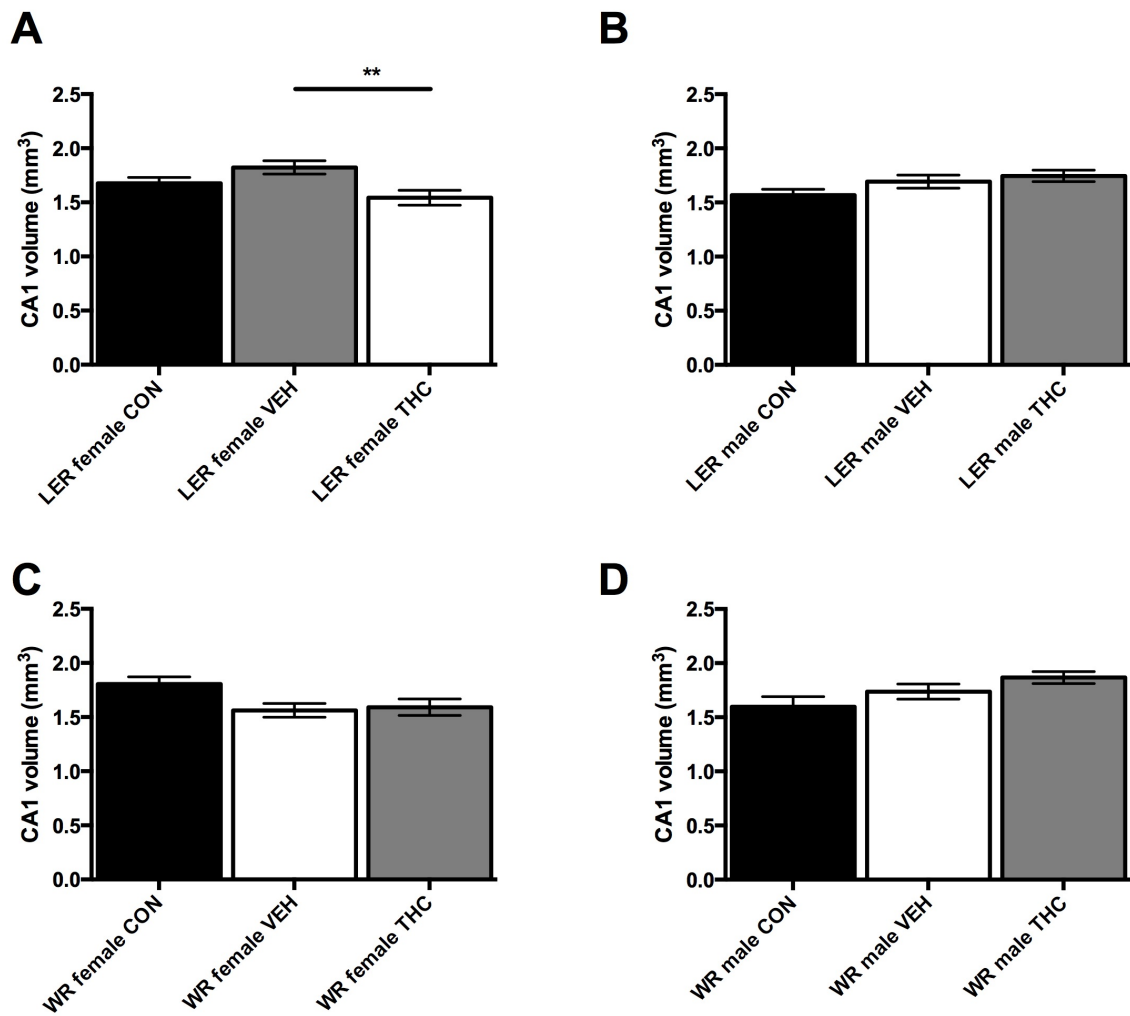
Figure 36: Effect of THC on CA1 volumes. A. LER females. B. LER males. C. WR females. D. WR males. * indicates p<0.05.
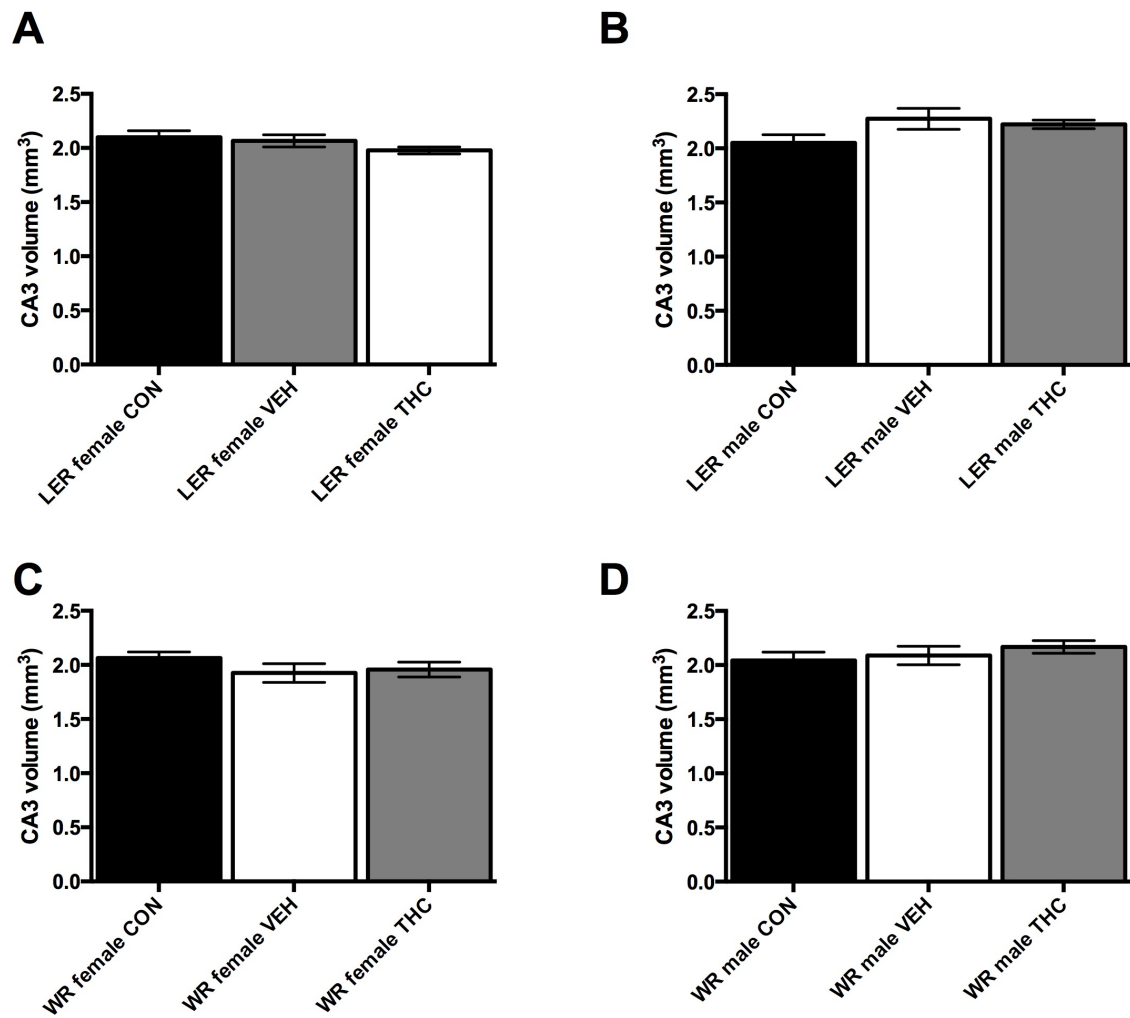
Figure 37: Effect of THC on CA3 volumes. A. LER females. B. LER males. C. WR females. D. WR males.
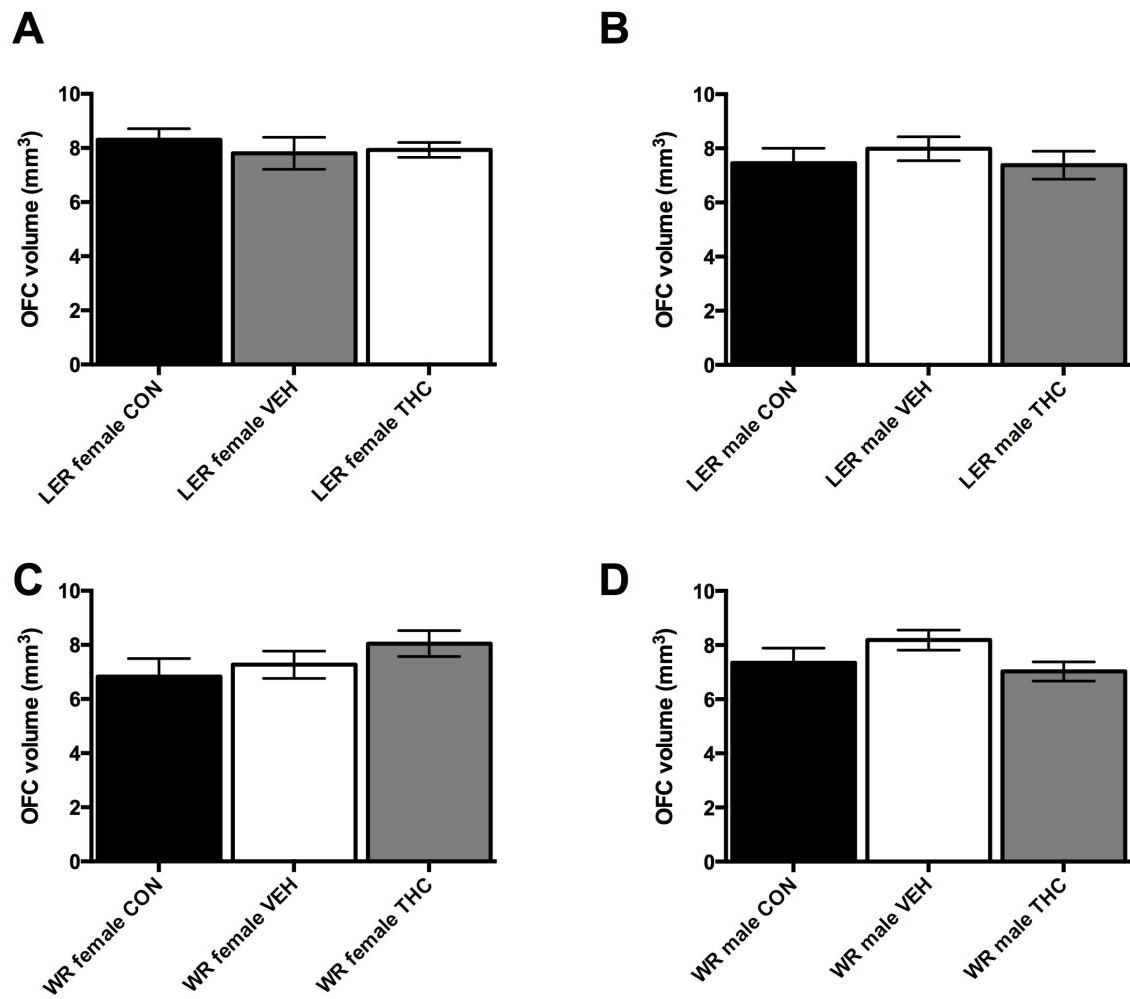
Figure 38: Effect of THC on OFC volumes. A. LER females. B. LER males. C. WR females. D. WR males.
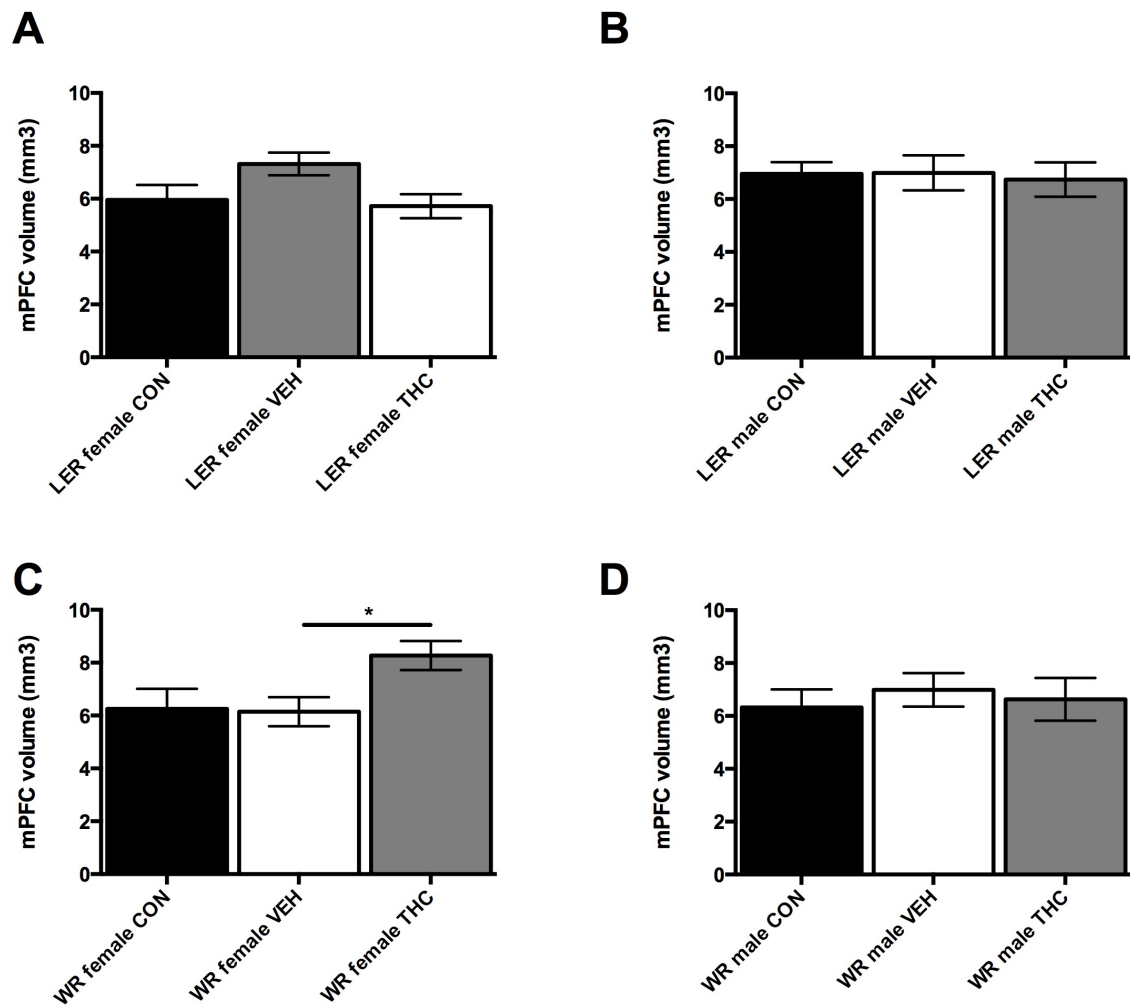
Figure 39: Effect of THC on mPFC volumes. A. LER females. B. LER males. C. WR females. D. WR males. * indicates p<0.05.
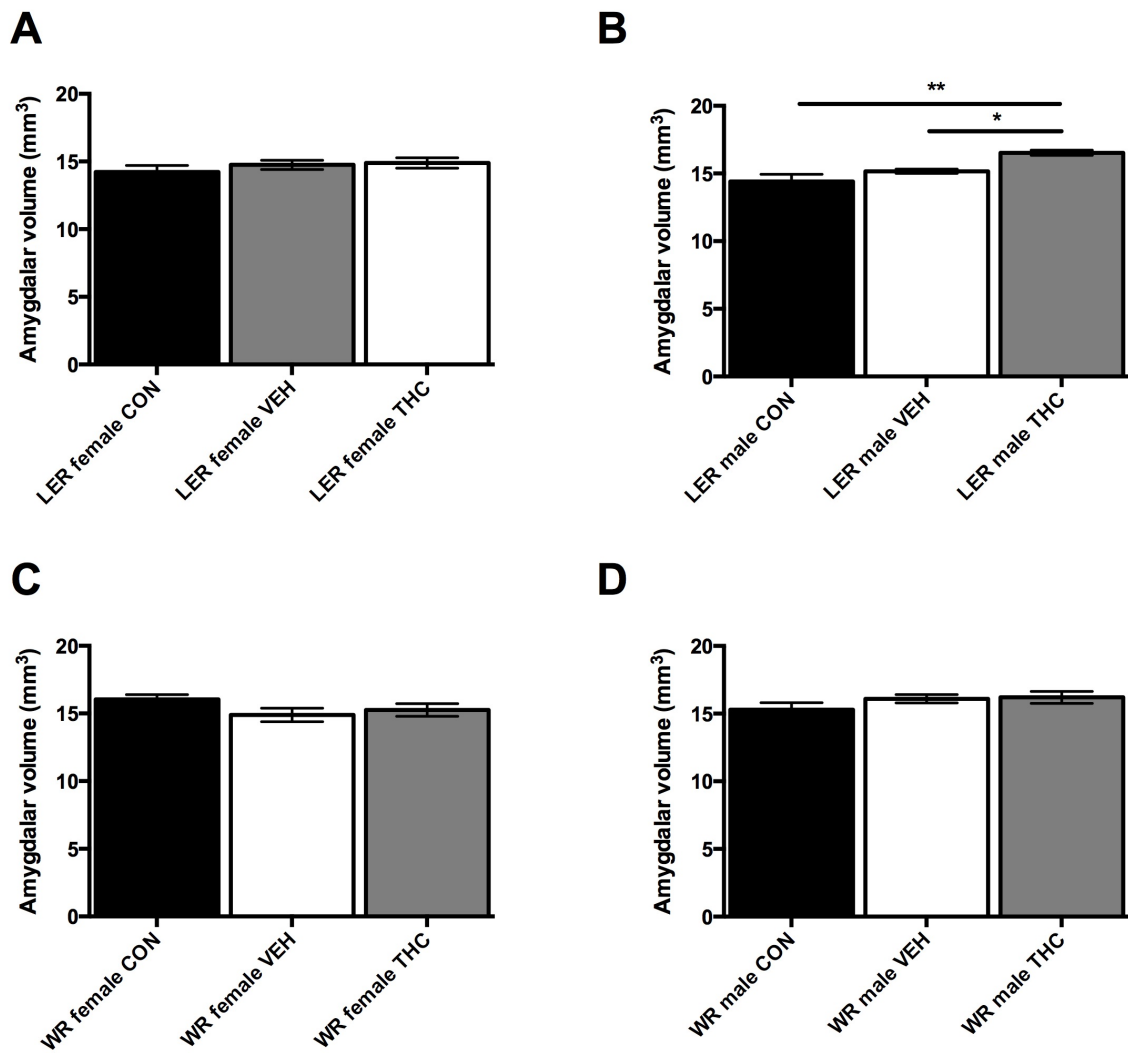
Figure 40: Effect of THC on amygdalar volumes. A. LER females. B. LER males. C. WR females. D. WR males. * indicates p<0.05. ** indicates p<0.01.
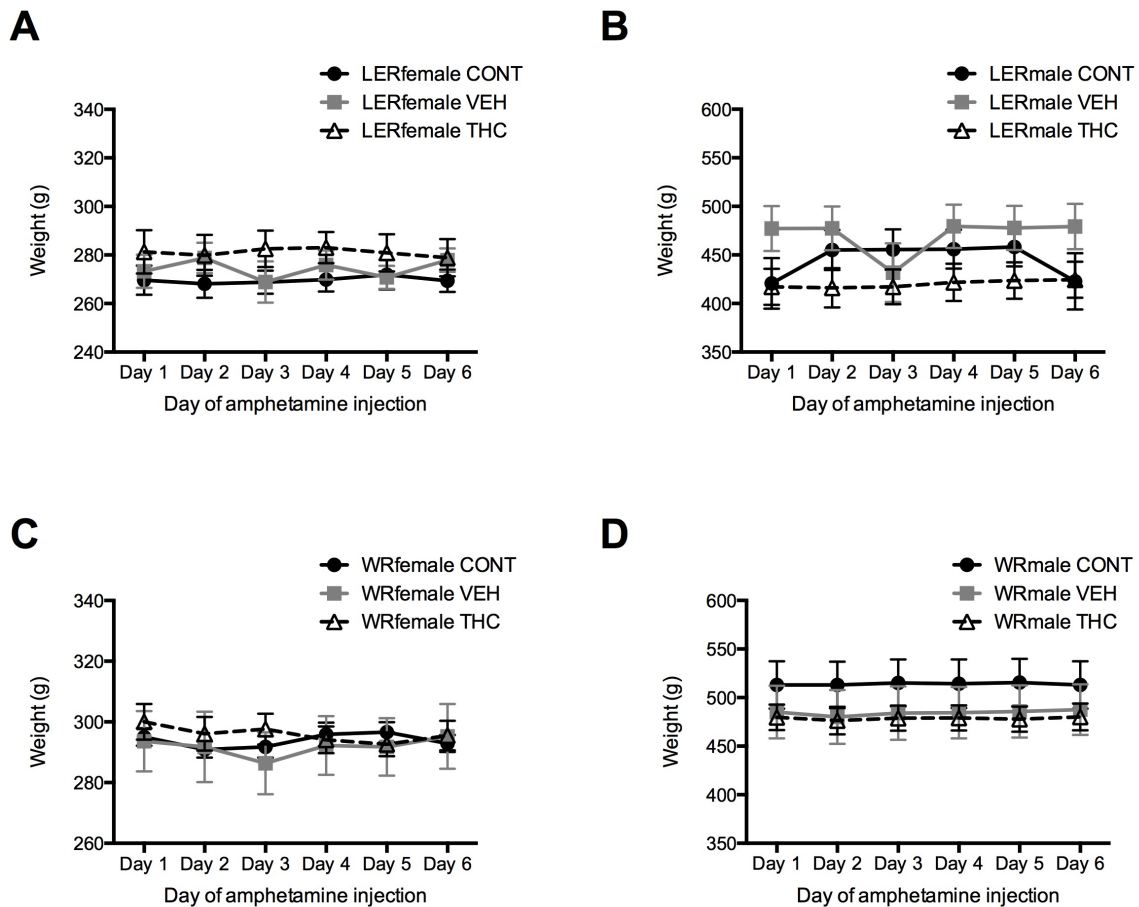
Figure 41: Weight during the injection period for A. LER females, B. LER males, C. WR females and D. WR males.
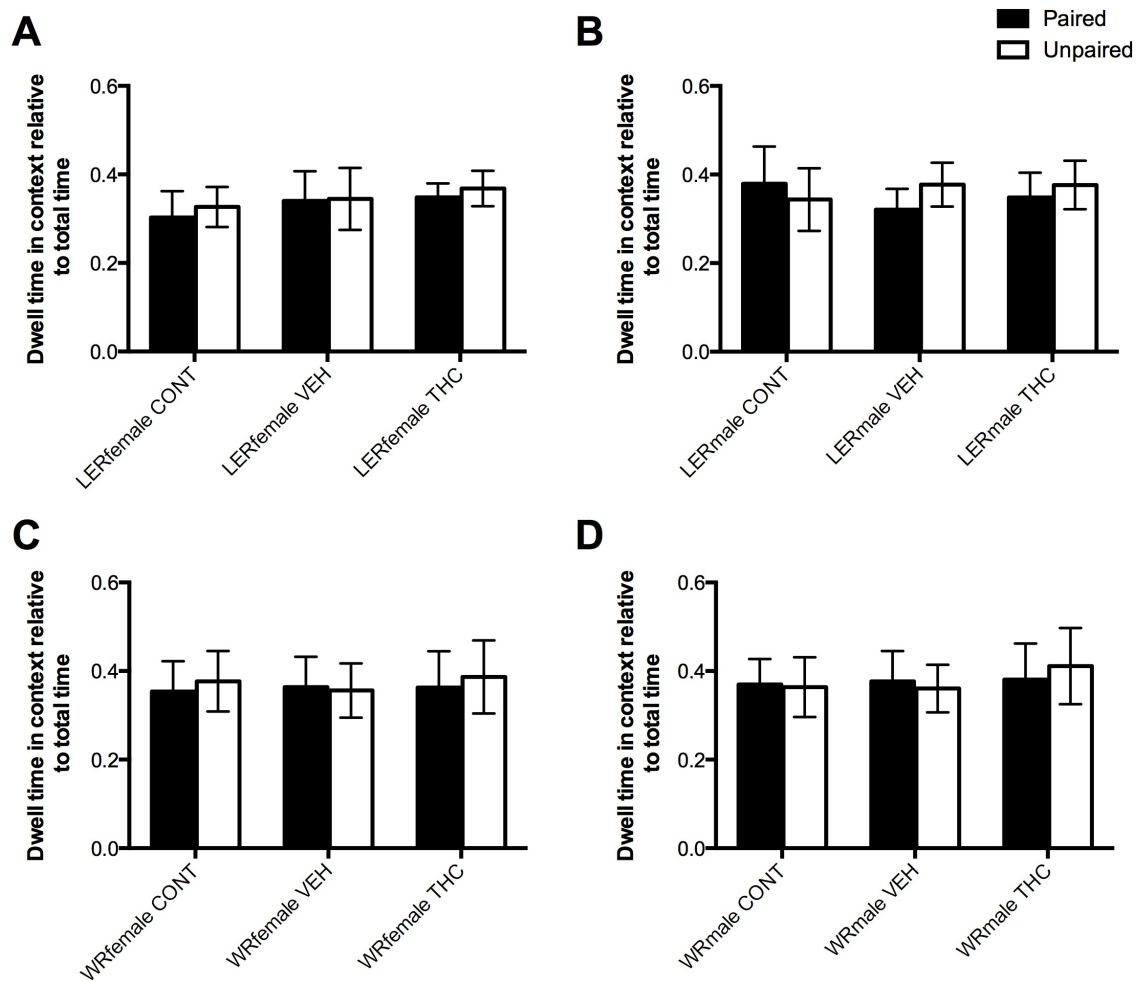
Figure 42: CPP pre-exposure for A. LER females, B. LER males, C. WR females and D. WR males.
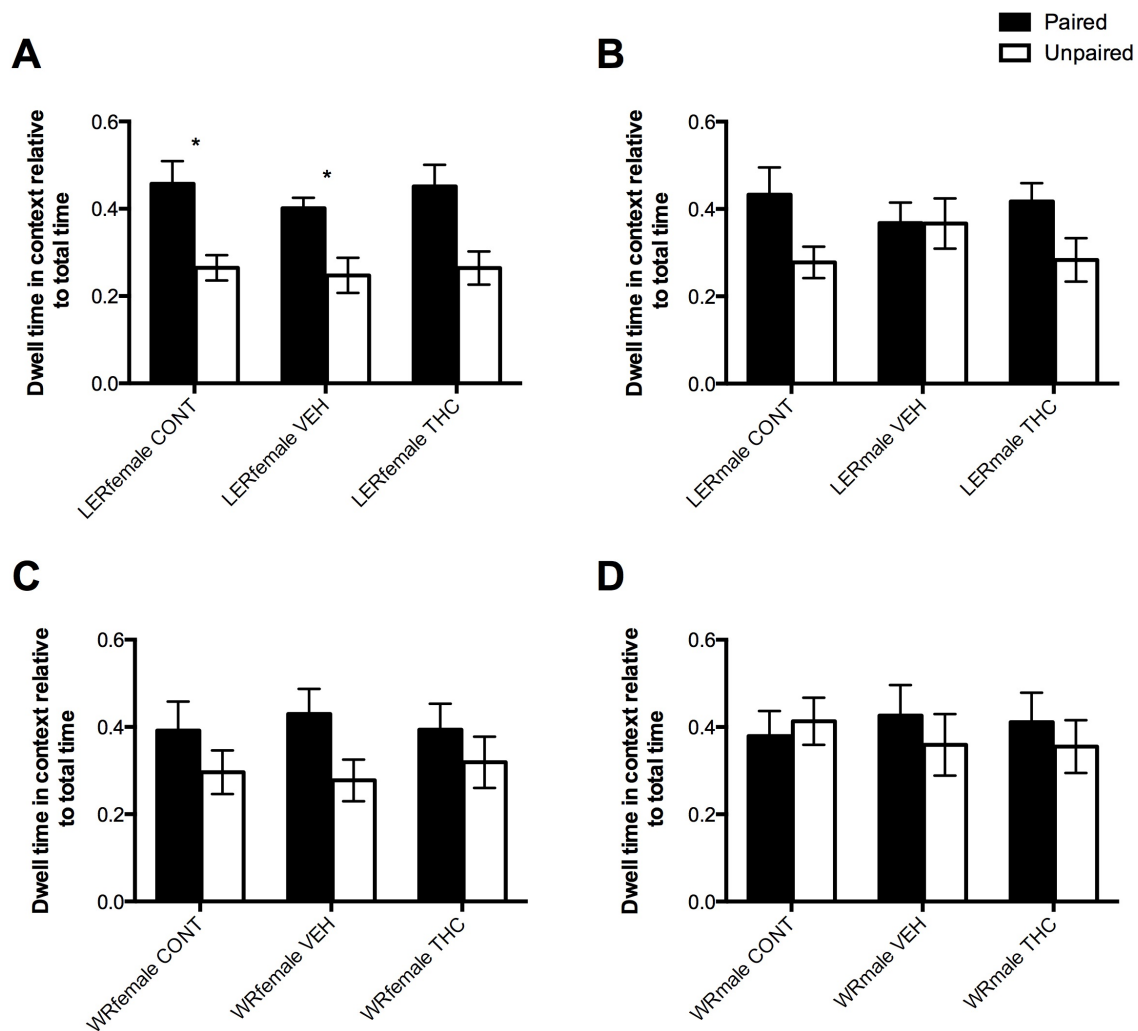
Figure 43: CPP preferences for A. LER females, B. LER males, C. WR females and D. WR males. * p < 0.05.
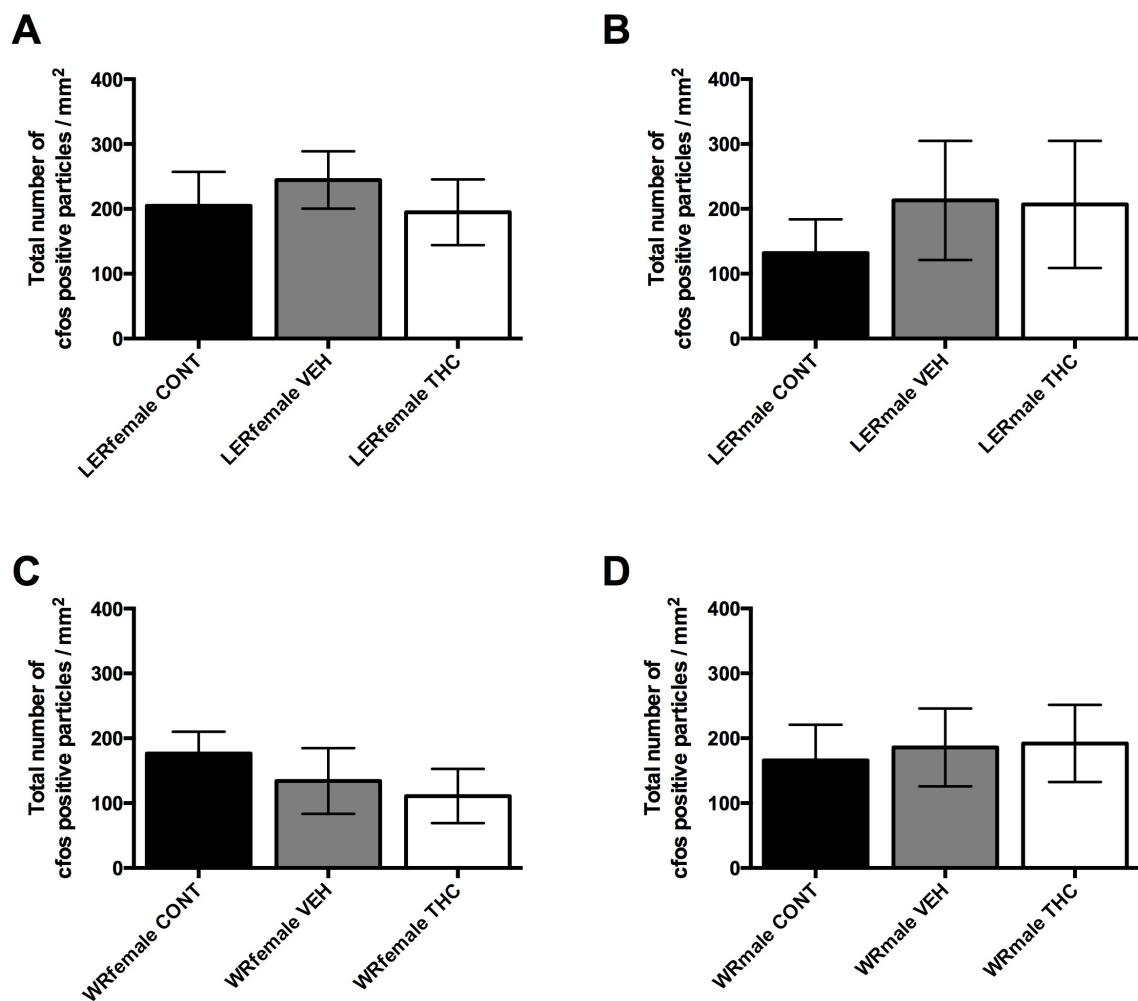
Figure 44: Number of cfos positive particles per area of a representative section of nucleus accumbens for A. LER females, B. LER males, C. WR females and D. WR males.
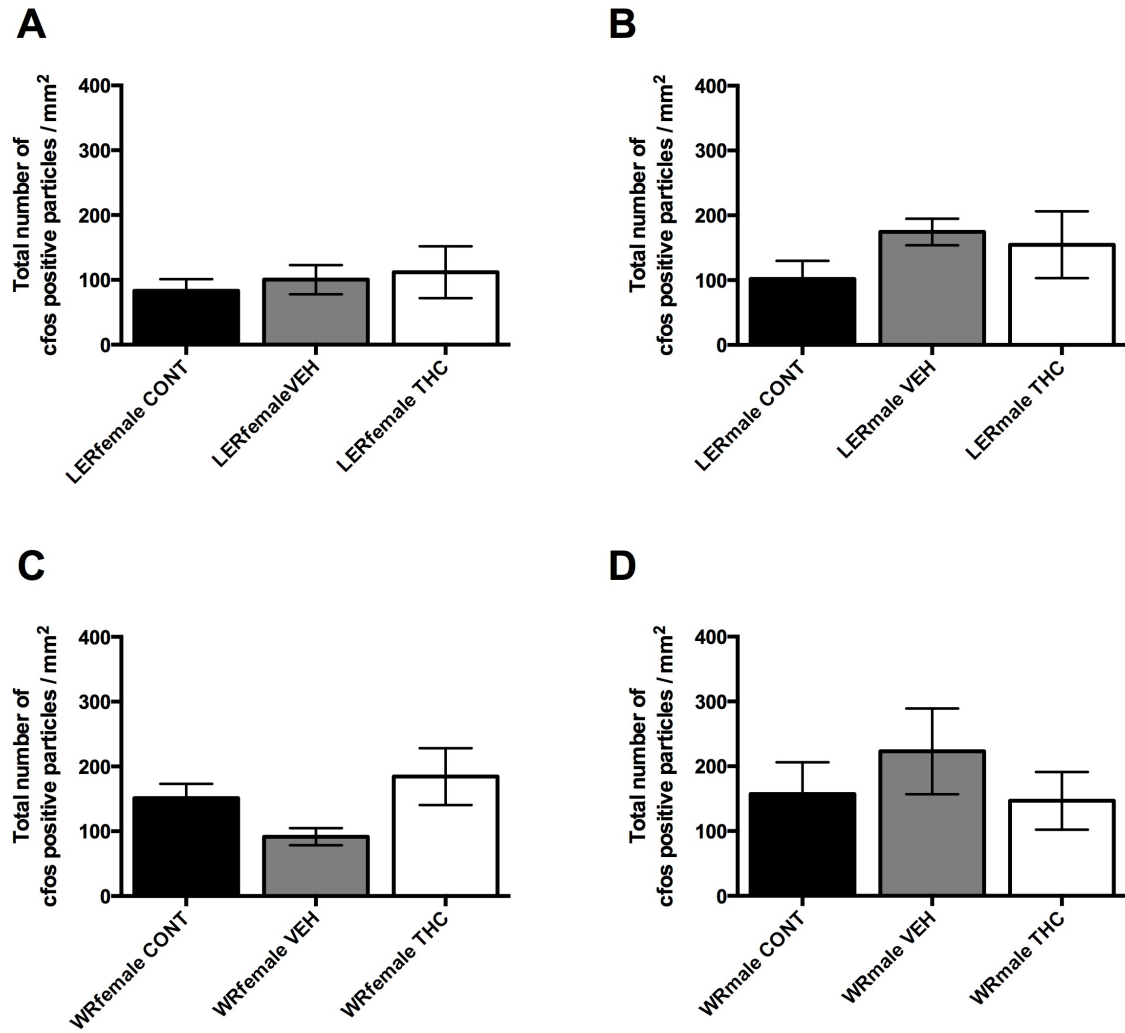
Figure 45: Number of cfos positive particles per area of a representative section of dorsal hippocampus for A. LER females, B. LER males, C. WR females and D. WR males.
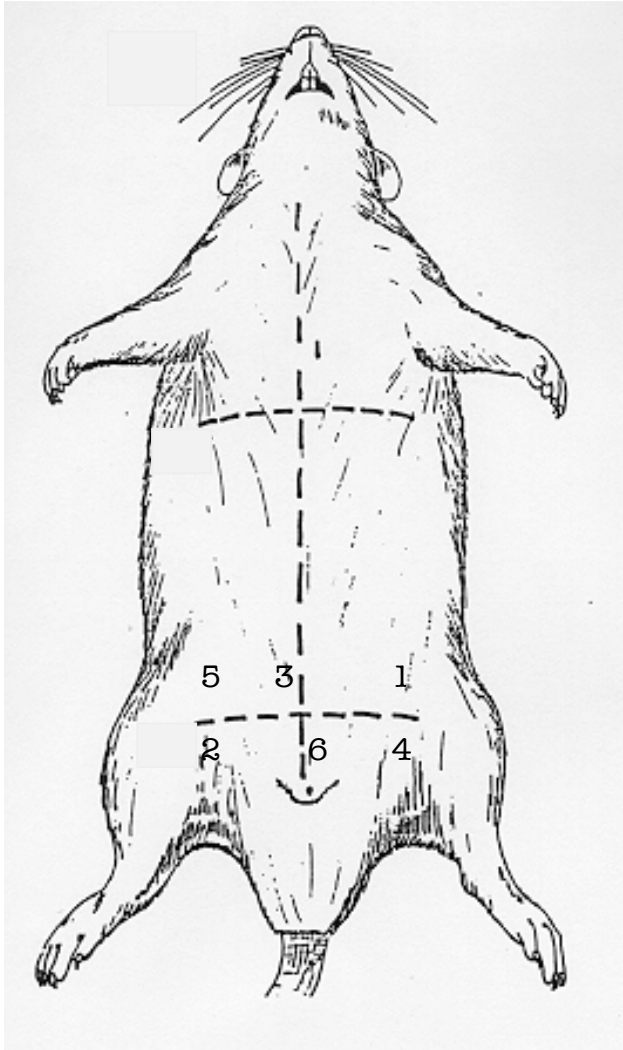
Injections sites varied every day, such that rats did not receive injections in the same area in 6 subsequent days. Rats were monitored for any signs of pain and distress as well as any signs of dermal abrasions due to injections.

APPENDIX 2 – REPRESENTATIVE IMAGES OF PHASES OF THE ESTROUS CYCLE
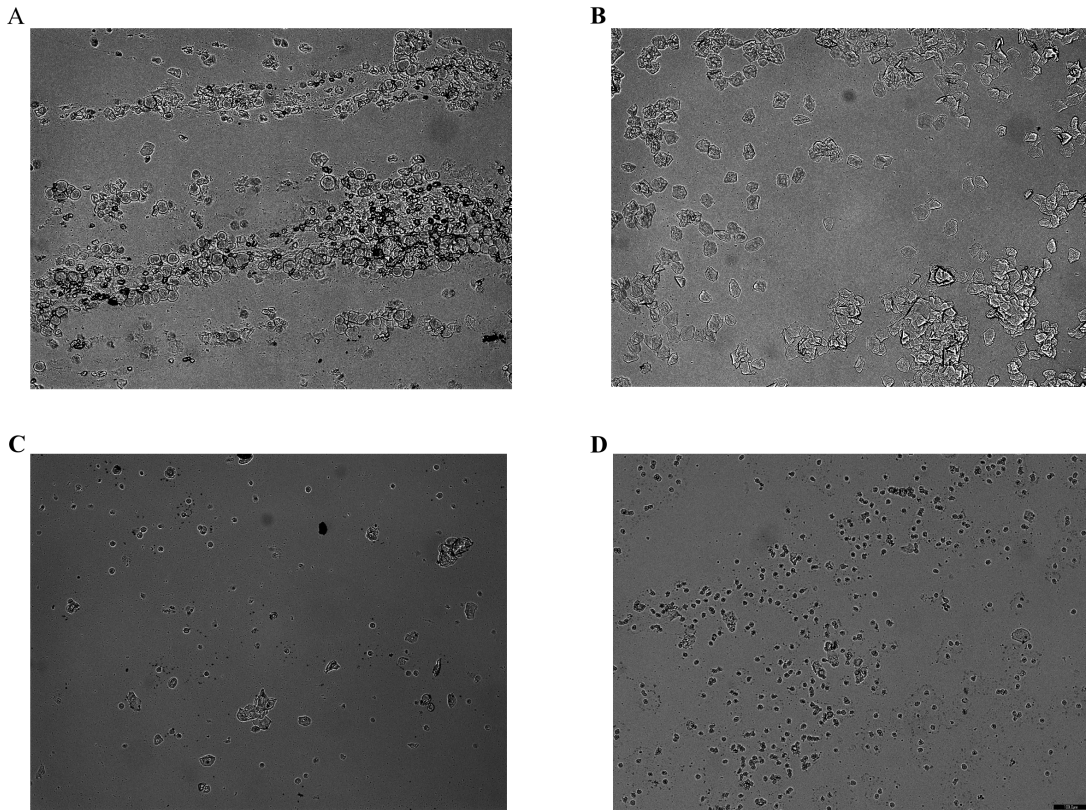
Representative images of estrous cycle pictures taken using a 10x objective. A. Proestrous. Proestrous was characterized by the presence of clumps or strands of nucleated epithelial cells. B. Estrous. Estrous was characterized by anucleated keratinized cells that appeared either needle-like or with jagged edges in large clumps. C. Metestrous. Metestrous was characterized as having a mix of nucleated and anucleated cells in combination with leukocytes. D. Diestrous. Diestrous was characterized as predominantly small, rounded leukocytes, occasionally in the presence of large rounded cells. All characterization was done in reference to images from (Marcondes et al., 2002; Goldman et al., 2007). Vaginal smears for multiple days were assessed in a qualitative fashion and compared to one another.

APPENDIX 3 – PLAY BEHAVIOUR FOLLOWING THC ADMINISTRATION

Briefly, unpublished work from our lab, in collaboration with Dr. Sergio Pellis and his students (Stephanie and Brett Himmler), examined the effects of THC during the injection period on play behaviour.

Methods
Subjects
A separate group of subjects was used and only LER males and females were studied. A total of 48 LER (female: N = 24; male: N = 24) were bred in house at the University of Lethbridge. All rats were weaned at postnatal day 21 and placed in quadrads of the same sex. Care was taken to not include more than 2 littermates per quadrad. Following weaning, rats were assigned to their groups: ultimate control (UC; N = 6), control (CON; N = 6), vehicle (VEH; N = 6) or THC (THC; N = 6). Pubertal onset and injections schedule was conducted as previously described (Chap 3.1).

Play behaviour
Play behaviour was assessed midway (after day 7) through the 14 day injection period. All rat play behaviour was compared to the UC group. On the 5th and 6th handling day, a quadrad was placed in the play apparatus for 30min approximately 1hr after the handling period. Twenty-four hours before the play bouts, play partners were separated and housed individually in the housing room in order to maximize the amount of play behaviour. Only 2 subjects participated in play bouts, and the order of play bouts with the UC was counterbalanced such that 2 individuals from each treatment group were exposed to the UC for the 1st play bout, 2 for the 2nd and 2 for the 3rd.

Play monitoring consisted of placing two play partners (the UC and an individual from another group) in the standard play apparatus (50 X 50 X 50cm clear Plexiglas box filled ~1-2cm with Betacob bedding) in a dark room and recorded using a video camera. Play partners were left in the dark in the play apparatus for 10min. Following play behaviour monitoring, rats were returned to their home cage. UC rats were allowed to remain with their cagemates for another 24hr before being isolated for additional play bouts. For example, on day 1 and 2, UC, CON, VEH and THC rats would be placed in the play apparatus for 30min approximately 1hr after handling or injection. On day 2, UC and VEH rats would be isolated. On day 3, UC and VEH rats would have their play behaviour recorded then would be returned to their home cage. On day 4, UC and CON rats would be isolated. On day 5, UC and CON rats would be monitored for play behaviour then returned to their home cage. On day 6, UC and THC rats would be isolated. On day 7, UC and THC would have their play behaviour monitored then returned to their home cage. Following all play behaviour and pre-exposure days, the play apparatus was cleaned using Virkon and fresh bedding was replaced for each session.

Play behaviour was quantified by a blind observer. Briefly, videos were analyzed frame-by-frame and the number and type of attacks and defences were recorded. The goal of play behaviour in rats is to achieve contact with the nape of the neck of the play partner. Therefore, play bouts consist of attacks and defence of the nape. The number of

attacks engaged by the treatment group, as well as the number of pins and defence indicate playful behaviour. Evasions are another metric of playfulness, such that a less playful rat will show a high number of evasions. The number and kind of rotations are characteristic of female- (complete) versus male- (partial) typical playful defences.

Results

The result of both the raw and probability of different attacks and defences can be found in the figures below. Both the frequency of playful behaviours and the proportion of these behaviours are shown. The frequency can give an indication of the amount of playful behaviour types, dependent on the activity levels of the individuals. The proportions of the playful behaviours give an indication of the relative amount of playful interactions, independent of the number of playful bouts. Briefly, both THC and VEH seemed to have a significant effect on play behaviour in male and female LER.
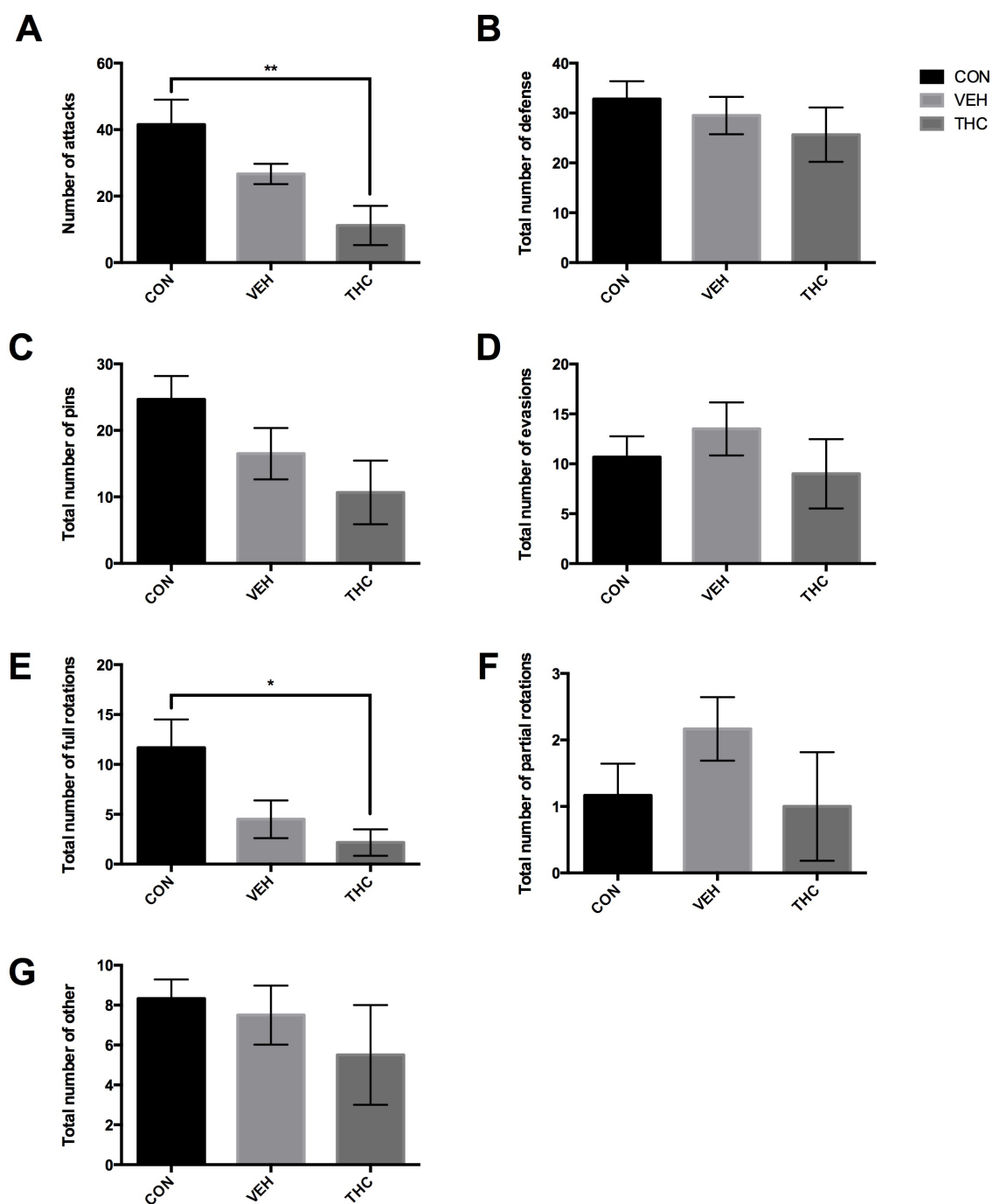
Raw data for play behaviour in LER males during handling/injection period. A. Total number of attacks. B. Total number of defence. C. Total number of pins. D. Total number of evasion. E. Total number of full rotations. F. Total number of partial rotations. G. Total number of other. * p<0.05. * p<0.01.
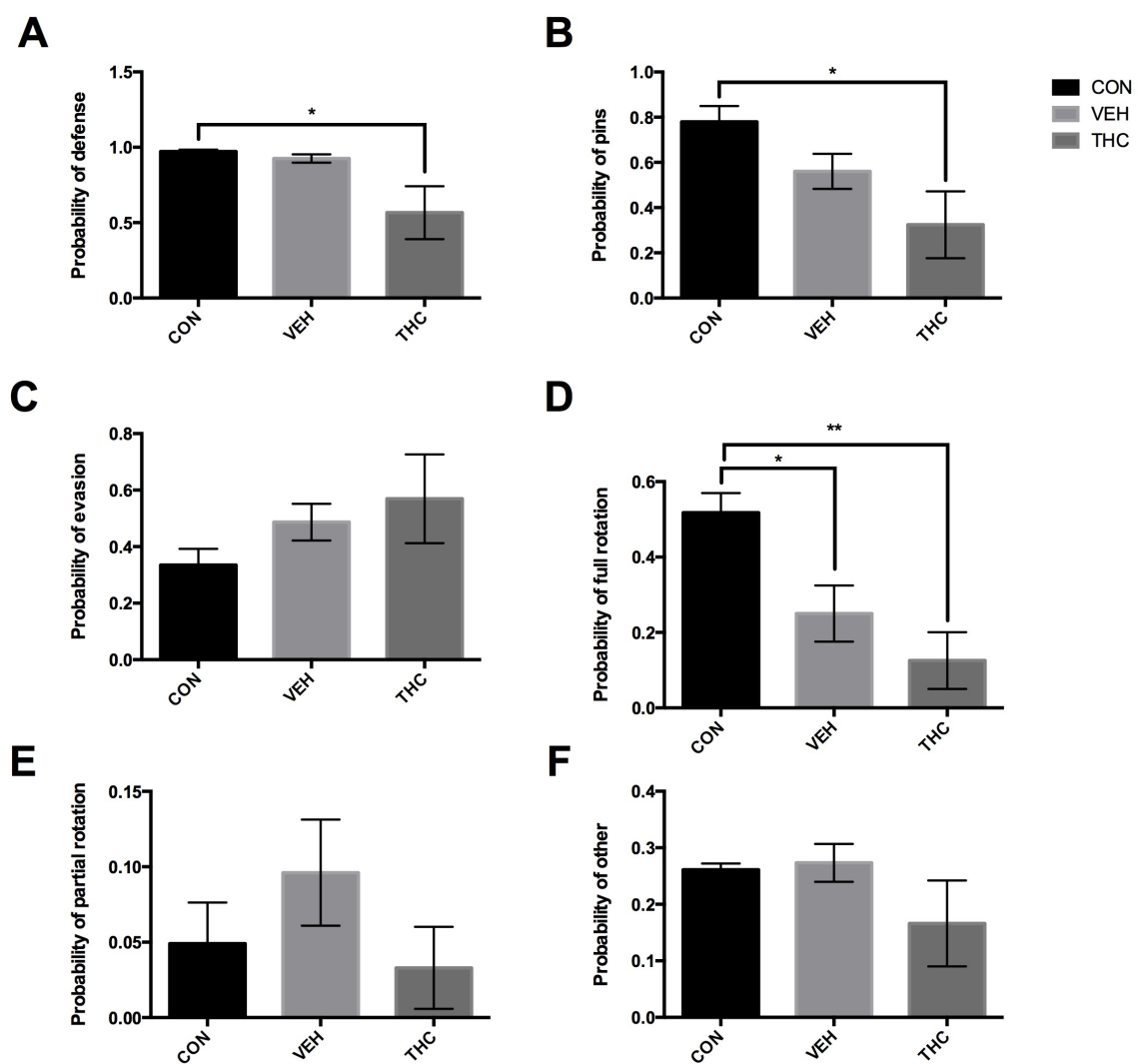
Probability of various play behaviours in LER males during handling/injection period. A. Probability of defence. B. Probability of pins. C. Probability of evasion. D. Probability of full rotation. E. Probability of partial rotation. F. Probability of other. * p<0.05.
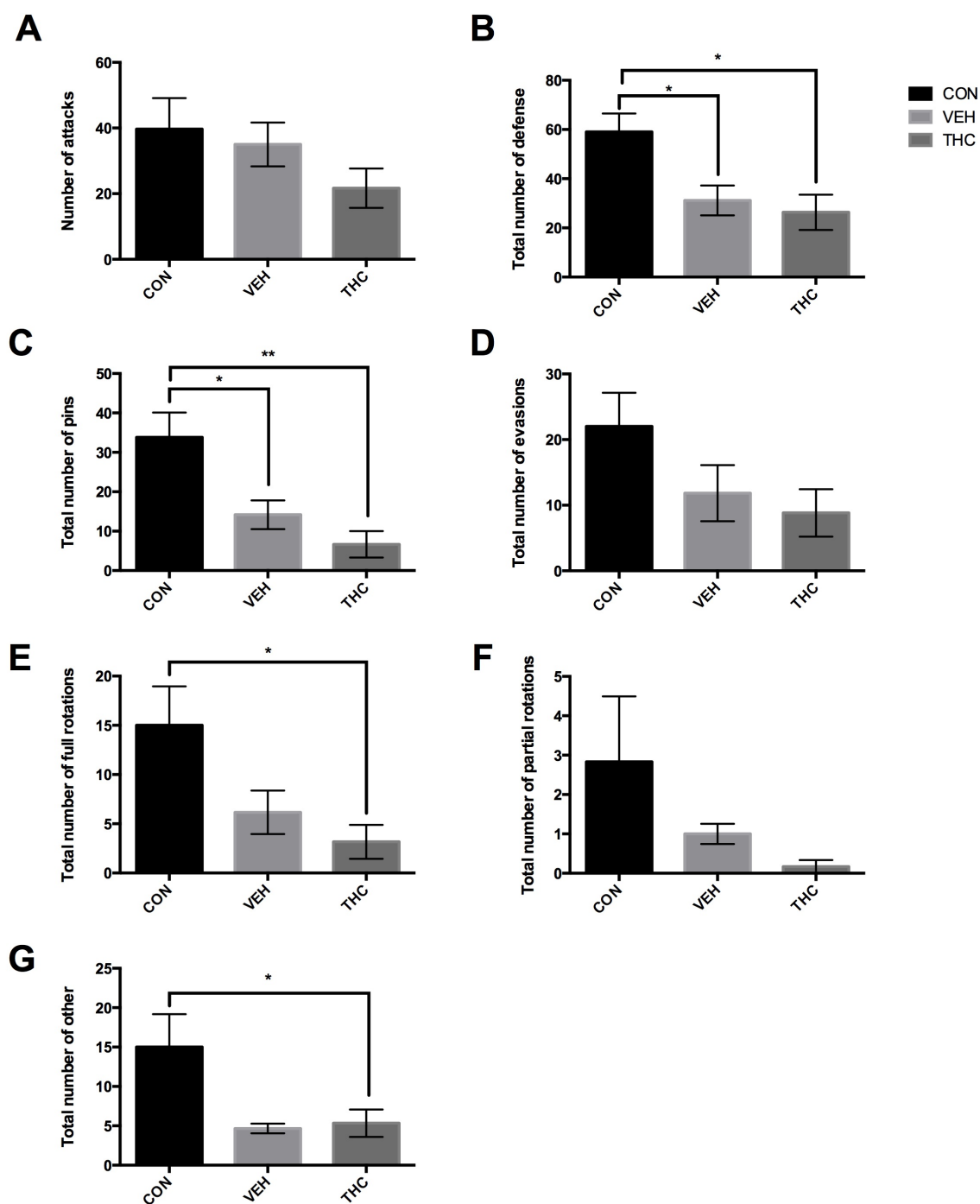
Raw data for play behaviour in LER females during handling/injection period. A. Total number of attacks. B. Total number of defence. C. Total number of pins. D. Total number of evasion. E. Total number of full rotations. F. Total number of partial rotations. G. Total number of other. * p<0.05.
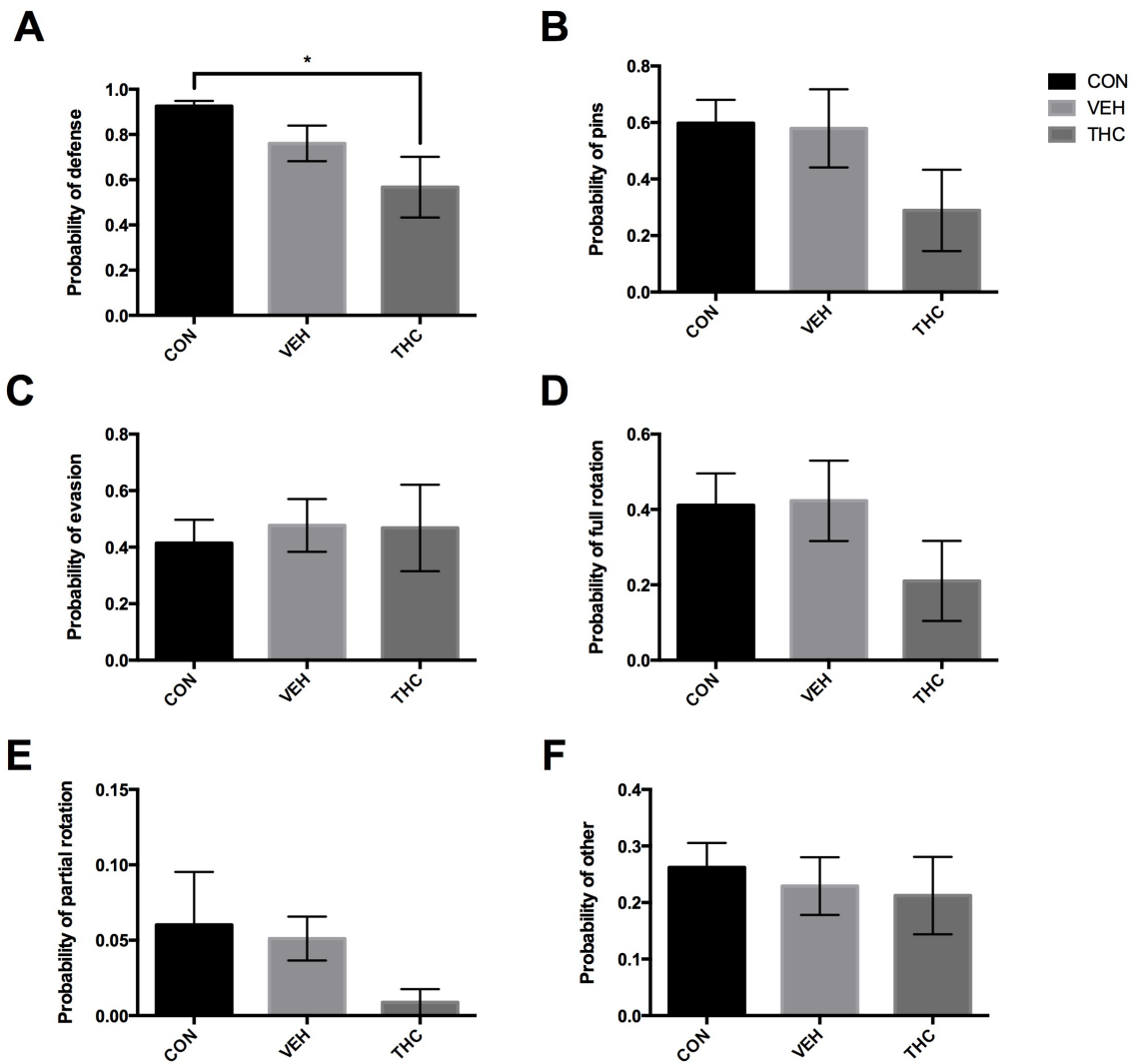
Probability of various play behaviours in LER females during handling/injection period.
A. Probability of defence. B. Probability of pins. C. Probability of evasion. D. Probability
of full rotation. E. Probability of partial rotation. F. Probability of other. * p<0.05. **
p<0.01.

# APPENDIX 4 – REPRESENTATIVE IMAGES OF ROIs

Representative images for all regions of interest (ROI) for volumetric estimates can be found in the following 2 pages. Note that only the right hemisphere volumes were taken for all measurements. Cresyl stained pictures of *R. norvegicus* were downloaded from BrainMaps.org (Mikula, Trotts, Stone, & Jones, 2007) and represented side-by-side with tracings at the same stereotaxic coordinates relative to Bregma from Paxinos & Watson (2007).

Representative images for ROIs for *cfos* staining, as pictured in Paxinos & Watson rat brain atlas (2007) can be found on the 3rd page of Appendix 4.
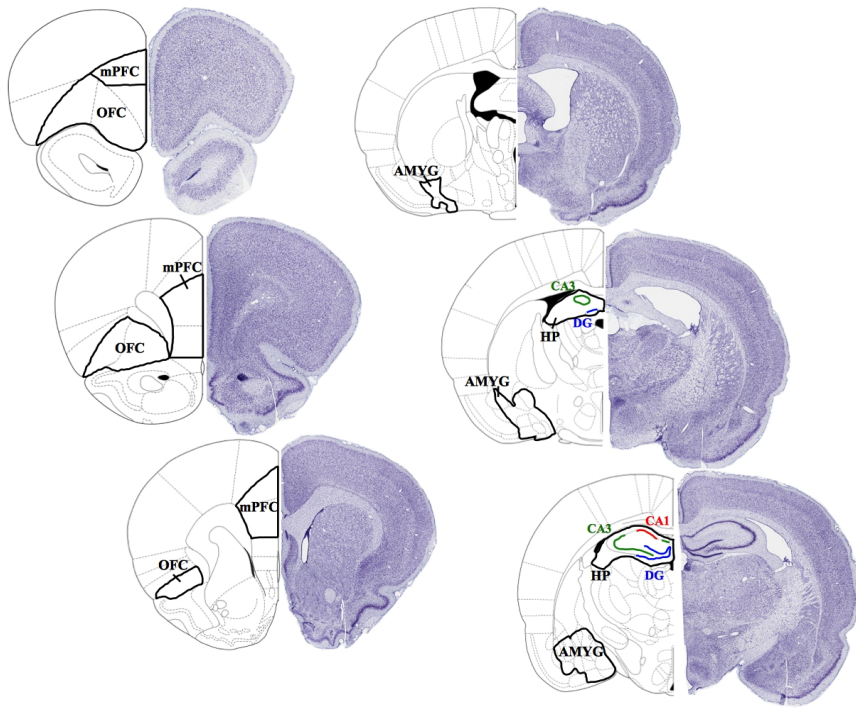
Representative images for ROIs for volumetric estimates (continued on next page)
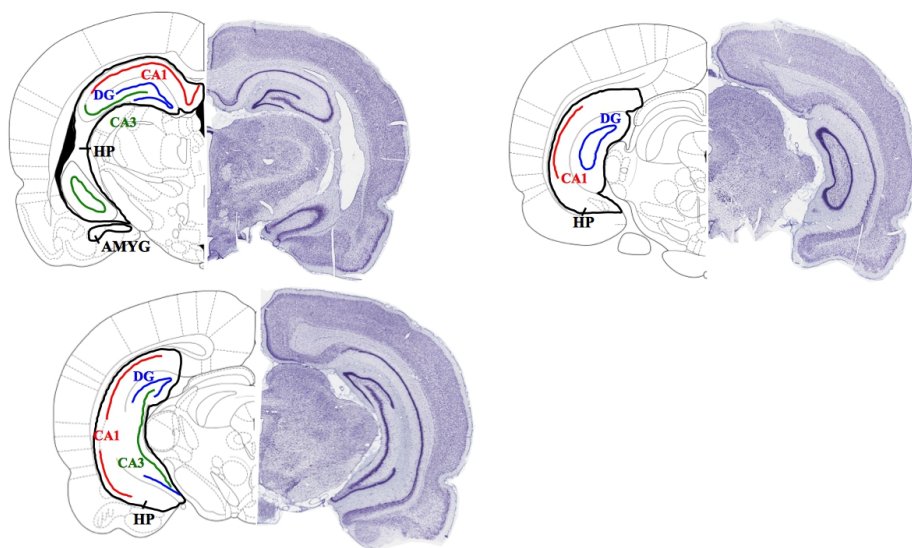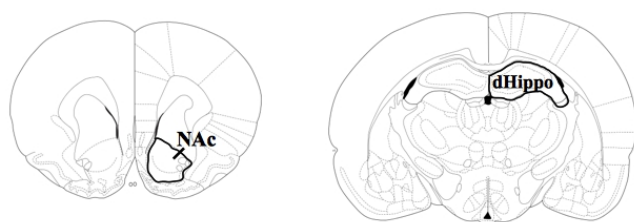
Representative images for ROIs for *cfos* quantification.