EXAMINING SEX DIFFERENCES IN HIPPOCAMPAL THETA ACTIVITY IN RELATION TO ANXIETY-LIKE BEHAVIOURS IN RATS

by

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Abstract

Hippocampal theta activity is an oscillatory, highly rhythmic (4-14 Hz) activity pattern generated by the hippocampal formation of mammals. Theta activity has been linked to anxiety states in rodent, based on a number of studies showing that anxiety-reducing pharmacological agents (anxiolytics) consistently decrease the frequency of theta activity in rats, leading to the influential “theta suppression model of anxiolysis.” To this date, very few studies have systemically compared anxiety-related defensive behaviour and hippocampal theta activity in males and females. The primary objectives of this thesis were to examine a possible association of theta frequency and behavioural levels of “anxiety” in individual rats, and to examine potential sex differences in anxiety, theta activity, and the anxiety-theta association. Female and male rats were tested on the elevated plus maze (EPM), a common paradigm to assess anxiety in rodents. Interestingly, the findings reveal that females exhibited higher amounts of open arm activity (number of open arm entries and open arm time) compared to males, indicative of reduced “anxiety” in females. Following the behavioral assessment, the same rats were anesthetized using urethane and underwent electrophysiological procedures to characterize hippocampal theta frequencies activity. Theta activity was recorded in the CA1 field of the hippocampus and was elicited by electrical stimulation of the brainstem reticular formation (consisting of 5-s trains of 0.1 ms duration pulses delivered at 100 Hz). Systemic administration of the clinically used anxiolytic drug buspirone, which acts as a partial agonist at 5-HT1A receptors, was shown to decrease the frequency of theta activity, a finding that is in agreement with prior work. These experiments showed that there was no sex difference in theta frequency, and that theta frequency in individual rats did not correlate with behavioral measures of anxiety in the EPM. Together, the results of this work show that theta frequency does not predict levels
of anxiety-like behavior in the EPM and challenge the predictive validity of hippocampal theta frequency as an index of anxiety levels.
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Lists of Abbreviations

5-HT: serotonin or 5-hydroxytryptamine
5-HT 1A: serotonin 1 A receptor subtype
ANOVA: Analysis of variance
βCCE: β- carboline-3-carboxylate ethyl-ester
CA1: Cornu ammonis subfield 1
EPM: Elevated plus maze
FG 7142: N-methyl-β-carboline-3-carboxamide
GABA: γ-aminobutyric acid
GABA A: GABA receptor A subtype
MS-DBB: Medial septum and diagonal band of Broaca
RF: Reticular formation
RPO: Reticularis pontis oralis
S.E.M: Standard error of the mean
SSRI: Selective serotonin reuptake inhibitors
SUM: Supramammillary nucleus
TSMA: Theta suppression model of anxiolysis
Yohimbine = 17-α-hydroxyl-yohimban-16-α-carboxylic acid methyl ester
Dedication

To all the lab rats and animals that have contributed to science, without them we would not be where we are today.
Chapter 1: Introduction

1.1 General Introduction

Hippocampal theta activity is an oscillatory, rhythmic activity pattern (4-14 Hz frequency range) generated by the hippocampal formation in rodents and other mammals (McNaughton et al., 2007). Theta activity is thought to play a role in movement, learning, and memory (McNaughton et al., 2007). Further, during fear conditioning and other anxiety-inducing situation, hippocampal theta appears, which is suggestive of a link between theta and “fear/anxiety” states. Studies have shown a highly consistent effect of anxiety-reducing pharmacological agents (e.g., benzodiazepines, fluoxetine) to decrease the frequency of theta activity in rats, leading to the influential “theta-suppression model of anxiolysis (TSMA)” (McNaughton et al., 2007). The TSMA predicts that a lower frequency of theta activity in the hippocampus is indicative of lower levels of anxiety, and vice versa (McNaughton et al., 2007).

While this model is based on pharmacological evidence, it leads to an extension that may predict that theta activity could constitute a physiological index of anxiety at the level of the individual, that is, rats with higher frequency of theta activity may be more “anxious” than animals with lower frequency theta activity. Levels of anxiety in rodents can be quantified by behavioural tests such as the elevated plus maze, a widely used, pharmacologically validated assay for anxiety in rats and mice (Lister et., 1987; Pellow et al., 1985; Pellow & File, 1986; Walf & Frye, 2007). The first, main objective of the thesis was to examine whether theta frequency and behavioural anxiety in rats are correlated.

It is of interest to note that, to date, the large majority of studies on anxiety and hippocampal theta activity have been conducted on male rats. There is a growing recognition that female mammals have long been neglected in biomedical research (Beery & Zuker, 2011). The
ratio of research articles using males versus females is highly skewed in favor of males (ration of about 5.5 to 1 in the neuroscience field. The fact that male rodents are used much more frequently than females may be particularly problematic in the area of anxiety research, given that anxiety is more prevalent in females (Beery & Zuker, 2011). Thus, conducting an explicit comparison of anxiety, and of the relation of anxiety to hippocampal theta frequency in male and female rats was the second, main objective of the experiments conducted for this thesis.

1.2. The Difference Between Fear and Anxiety

The terms fear and anxiety are often used synonymously, which can lead to confusion with regard to their precise definition and their psychological and physiological correlates. Fear and anxiety are both primitive, emotional states that are essential to the survival of an organism (Gray, 1982, p.2). Anxiety is a hyper-vigilant emotional state in anticipation of an imminent, adverse event or an uncertain outcome (Gray 1982, p.2). Anxiety (often also called ‘unresolved fear’) occurs from a threatening situation without effective means of coping (Öhman, 2008). Others have stated that the difference between fear and anxiety lies in the direction of the motivation of behavior (McNaughton & Coor, 2004). Thus, fear is characterized as an aversive reaction to a perceived threatening stimulus that results in defensive reactions, such as the fight-or-flight response (McNaughton & Coor, 2004).

Fear and anxiety states that become over-exaggerated or chronic can lead to the development of significant physiological and psychological pathologies. Behavioural responses to fear and anxiety include inhibition on going behaviours, scanning, and the avoidance of the perceived source of danger. Fear and anxiety states can be debilitating when responses to the perceived stimulus become exaggerated and maladaptive, such that they cause interference with
normal daily functioning and well-being (Pratt, 1992). Prolong maladaptive states of anxiety can cause detrimental effects to physical health, such as cardiovascular diseases and mental health issues (i.e., anxiety disorder, phobias, etc.).

Anxiety disorders affect about 5% of the population in Canada (CAMH, 2017). One in four Canadians will have at least one anxiety disorder in their life-time (CAMH, 2017). Mental illness alone cost the health care system approximately $7.9 billion dollars every year (CAMH, 2017). Anxiety can have a profound effect on daily activities, and can interfere with work or school performance, the establishment of successful relationships, sleep patterns, and many other aspects of general health and well-being. There has been a growing interest in understanding the neuronal and biochemical mechanisms of anxiety in clinical populations, but also in animal models of anxiety and fear.

1.3. Animal Models of Anxiety

Typically, anxiety is assessed using self-reports and formal diagnosis in clinical settings, which is not a feasible method for rodents. Thus, a number of animal models have been developed and used to study the neurobiology basis of anxiety, and also as screening tool for novel therapeutics. Animal models can be powerful tools that provide a gateway to understand the neural and pathophysiological mechanisms of anxiety. There are a number of behavioural approaches for the study of anxiety in animal models. Anxiety in animals can be measured using stress responses, such as measuring plasma corticosterone levels (endocrinology) or various autonomic (physiological) functions (e.g., heart rate, blood pressure, body temperature). Further, there is a wide variety of behavioural models that measure anxiety, mostly by assessing conditioned and unconditioned responses to some aversive, threatening stimulus or situation.
Many conditioned models (e.g., standard fear conditioning or conflict paradigms) involve stressful or painful stimuli and procedures (e.g., foot shock, food or water deprivation) and may require extensive training periods (Rodgers & Dalvi, 1997; Steimer, 2011). In contrast, unconditioned models place the animal in a new environment that evokes innate fear, curiosity, and avoidance behaviours without the use of painful stimuli (Steimer, 2011). Unconditioned models require no training and have been shown to be high in ethological validity (Rodgers & Dalvi, 1997). Together, conditioned and unconditioned models are powerful tools in helping to understand the mechanism of anxiety.

1.4. Ethological Relevance of Animal Models

Recently, a particular emphasis has been placed on developing and using animal models that are ethologically-based, that is, models that make use of the natural, behavioral repertoire of the animal. For example, anxiety in rats and mice is often measured through their innate tendency to avoid open space and bright lights (Adhikari, 2014; Calhoon & Tye, 2015). Often, these paradigms aim to exploit innate approach-avoidance conflicts, e.g., the need to explore open spaces to find food, water, or conspecifics vs. the avoidance of open spaces to limit the exposure to predators (Calhoon & Tye, 2015; Smith & Rudolph, 2012). The avoidance of open, exposed spaces is commonly studied using a paradigm called the elevated plus maze (EPM), which has been widely used and constitutes a pharmacologically validated assay for anxiety in rats and mice (Pellow et al., 1986; Walf & Frye, 2007). Paradigms such as the EPM, the elevated zero maze, open field tests, the light dark box, the elevated alley, and the hole board test all examine approach-avoidance conflicts in rodents.
There are many benefits in using ethological-based assays to measure anxiety. The EPM has been shown to have a high degree of face validity. Face validity is defined as the ability of a task to measure what it is suppose to measure. The EPM is an approach avoidance assay which measures anxiety or fear of open spaces thus showing a great degree of face validity. In addition, anxiolytic drugs have been shown to reduce anxiety-like behaviours in these tests (Hogg, 1996; Moser, 1989; Pellow et al., 1986; Rodger & Dalvi, 1997; Treit et al., 1993; Walf & Frye, 2007). Further, one of the more practical benefits of utilizing ethological assays is that, often, there is no training required prior to the animal performing in the test. Also, the tasks are often relatively simple and do not involve large amounts of expensive equipment. Lastly, many ethologically-based assays are able to detect bi-directional changes in anxiety, that is, they are able to measure both increases and decreases in anxiety levels in rodents.

1.5. The Elevated Plus Maze

Early studies by Montgomery (1955) demonstrated that rats display avoidance responses during the first few minutes when placed on an elevated, open alley, Y-shaped apparatus. Moreover, it was revealed that rodents tend to spend more time in the closed, rather than the open space of the Y maze (Montgomery, 1955). The EPM test, a now widely used test to assess anxiety in rodents, is based on procedures similar to those used for the Y maze tests by Montgomery (1955).

The EPM consists of a junction of four (two open and two enclosed) arms. The EPM is able to measure anxiety by utilizing the rats’ unconditioned avoidance of heights and open spaces. Importantly, the EPM has been pharmacologically validated, in that clinically effective anxiolytics reduce the avoidance of the open arms, while anxiogenic compounds lead to a further
increase in open arm avoidance (Cheeta et al., 2000; Pellow et al., 1985; Hogg, 1997; Treit et al., 1993). Exposure to the EPM has been shown to increase plasma corticosterone concentration, freezing responses, and the production of fecal boli, all of which are indicative of anxiety-like responses (Pellow et al., 1985). In a typical EPM test, rodents are allowed to freely explore the maze for five minutes and anxiety-like behaviours are quantified by examining the time spent on open arms relative to the time spent in closed arms, and the number of open entries relative to the number of closed arm entries (Pellow et al., 1985). An open arm entry is defined as an event when at least half of the rat’s body enters an open arm. Open arm time is defined as the total time in which half of the rat’s body is located in the open arm. Other behavioural measures such as rearing, head dips (a downward movement of the animal’s head towards the floor from the open arm), and stretch attends (the animal’s head is placed at least 50% more forward compared to the natural position, while keep their paws back) can also be quantified to provide additional measures of the behavior of the animal on the EPM.

The behaviours observed in the EPM can be quantified to provide information regarding general activity levels and exploration, as well as the inferred “anxiety” state experienced by the animal. Total number of arm entries or of closed arm entries can be used to assess general locomotor activity (Walf & Frye, 2007). Risk assessment can be examined by determining the frequency and/or duration of rearing, stretch attends, and head dips (Hogg, 1996; Pellow et al., 1986; Walf & Frye, 2007). Increases in the time spent in the open arms is a widely used index of lower anxiety levels (Pellow et al., 1985). Time spent in the open arms is generally reported as a percentage of total arm time (Hogg, 1996). Percentage of open arm entries and percentage of open arm time are the most common methods to score anxiety-related behaviours in rats.
Percentage of open arm entries and percentage of open arm time can be calculated as follows:

(for the formulas below, you need to multiply x 100 to get percentage)

\[
\text{Percentage of open arm entries} = \frac{\text{number of open arm entries}}{\text{number of open + closed arms}} \times 100\%
\]

\[
\text{Percentage of open arm time} = \frac{\text{amount of time in open arms (s)}}{\text{open arm time (s) + closed arm time (s)}} \times 100\%
\]

There are several other behavioral tasks that examine anxiety in rodents, which often rely on novel, highly aversive stimuli such as electric shocks, food/water deprivation, or exposure to predator odor in order to elicit heightened levels of anxious behaviors (Walf & Frye, 2007). The EPM, on the other hand, relies on natural, species-specific behaviors, since rodents prefer the safety of dark and enclosed space, but also rely in exploration of their environment to locate food sources, water, or conspecifics (Walf & Frye, 2007). Many studies have demonstrated that the EPM is an effective behavioral tool for assessing the involvement of limbic regions, including the hippocampus and amygdala, in defensive and anxiety-related behaviors (Adhikari, 2015; Rodgers & Dalvi, 1997; Hogg, 1997; Treit et al., 1993). Thus, the EPM is a valuable tool for studies designed to expand our understanding of the neurobiology of anxiety and defense, since it allows researchers to examine anxiety by assessing naturalistic, ethologically valid behaviors of the animal.

1.6. **Sex Differences in Anxiety on the Elevated Plus Maze**

Despite the fact that the incidence of anxiety disorders the prevalence rate is higher in females (30.5%) than males (19.2%) (McLean et al., 2011). The current animal research on
anxiety is heavily biased toward the use of male subjects (Beery & Zuker, 2011), a trend that is also present in the larger field of behavioural neuroscience research (Beery & Zuker, 2011; Clayton, 2016; Clayton & Collins, 2014). It was thought that hormonal changes during the estrous cycle in females introduces variability in the data set, thus increasing the need to run more subjects (and associated costs) in order to detect possible effects (Palanza, 2001). However, the under-representation of females in neuroscience research is problematic and could hinder further advances in our understanding in anxiety. Since males and females often differ in their physiology and behaviour, it is important to incorporate both sexes in research designs to provide a more complete understanding of brain-behavior relations, including those that mediate states of anxiety (Clayton, 2016). Thus, my thesis will explore a potential sex difference in anxiety behaviours on the elevated plus maze in male and female rats.

In fact, the few investigations that have compared male and female rats suggest that there may be a sex difference in their behavioural profile on the EPM (File & Johnson, 1991; Kelly et al., 1999). Female rats have been shown to exhibit less aversion to the open arms of the EPM when compared to males (File & Johnson, 1991; Zimmerberg & Farley, 1992). Further, female rats display a greater level of motor activity and lower levels of defecation than males (Grey, 1979). Thus, it appears that female rats exhibit lower levels of anxiety-related behaviours compared to their male counterparts.

The apparent sex difference in behaviour on the EPM may be related to the fluctuation in the ovarian hormones during the estrous cycle. (Gray & Levine, 1964; Mora & Diaz-Veliz, 1997; Palanza, 2001). Female rats in estrus or proestru show a reduction in anxiety-like behaviours when compared to metestru and diestru (Mora & Diaz-Veliz, 1997). Similarly, another study found that rats in proestru spent more time on the open arms and showed more open arm entries.
than rats in diestrus and metestrus (Frye, Petralia & Rhodes, 2000; Marcondes et al., 2001). Furthermore, female rats in diestrus and male rats tend to spend less time on the open arms than female rats that are in proestrus (Walf & Frye, 2007). Interestingly, ovariectomized female rats show higher indices of anxiety than intact female rats (Mora & Diaz-Veliz, 1997; Zimmberberg & Farley, 1993). It has been suggested that falling levels of progesterone in diestrus could attribute to an increased responsiveness to stress and anxiety (Fernandes et al., 1999; Fernandez-Guasti & Picazo, 1997; Lovick, 2012). Together, the findings provide strong support for the hypothesis that estrogen plays a role in modulating anxiety-like behaviours in rats.

1.7. The Hippocampal Theta Rhythm

To further our understanding of anxiety, it is important to identify neurobiological systems (i.e., limbic structures, GABA system, serotonin system) that mediate anxiety and defensive behaviours. Complex cognitive functions and emotional processes require the involvement of many neurons in a variety of brain regions (Bland, 1986). Brain rhythms (also referred to as oscillations) link the ensemble of neurons to a particular activity (Vanderwolf, 1969). Theta activity is a prominent oscillatory activity pattern that is present in the hippocampus and surrounding cortical tissue in humans and non-animals, and its role is widely debated in the literature (Burges, 2008; Buzsáki, 2005; O’Keefe & Burgess, 2005). Theta rhythms were first discovered by Jung and Kormuller (1938) in rabbits. Research at that time did not succeed in clarifying the functional importance of theta activity, but it led to a rapidly growing interest in the mechanism and possible functions of theta (and other) rhythms across a variety of mammalian species (i.e. rodents, cat, rabbit, monkey) (Green & Arduini, 1954; Vanderwolf, 1969).
Theta activity is present in awake, behaving animals, but can also be experimentally elicited with high frequency stimulation in the midbrain reticular formation (Green & Arduini, 1954). Theta activity of large amplitude (around 1000 µV) can be recorded from electrodes placed in the entorhinal cortex or hippocampal formation (Green & Arduini, 1954; McNaughton et al., 2007; Vertes & Koecs, 1997) and has been suggested to play a role in sensorimotor integration (Blande & Oddie, 2002). In behaving animals, hippocampal theta can be categorized into two types: Type 1 and Type 2. Type 1 hippocampal theta has an overall frequency range of 6 to 12 Hz (Lever et al., 2014; Sainsbury, 1986). It is correlated with “voluntary” movements such as walking, rearing, swimming, jumping, digging, or manipulation of objects with the forelimbs (Whishaw et al., 1976). Type 1 theta does not habituate and it is always present during voluntary movement (Lever et al., 2014; Whishaw et al., 1976). Further, type 1 theta is not sensitive to drugs that block muscarinic acetylcholine receptors. Moreover, type 1 theta is resistant to atropine sulfate, and sensitive to urethane, ethyl ether, and alcohol (Lever et al., 2014; Sainsbury, 1986). In contrast, type 2 theta frequency range from 4 to 9 Hz and is atropine sensitive (Sainsbury et al., 1986). Type 2 theta activity occurs during behavioral immobility and is mediated by cholinergic inputs (i.e., it is sensitive to muscarinic receptor blockers) to the hippocampal formation from the medial septum (Bland, 2002; Bland et al., 2007; Lever et al., 2014; Sainsbury, 1998). Unlike type 1 theta, type 2 theta is abolished by muscarinic receptor antagonists (Lever et al., 2014). When an animal is in an “aroused” state, type 2 theta can occur as a response to a sensory stimulus (Sainsbury et al., 1986). Interestingly, it has been suggested that the hippocampus and theta activity play a role in defensive behavior and anxiety states in animals, a hypothesis that is reviewed in more detail in a subsequent section.
1.8. Mechanism of Theta Rhythm Generation

The medial septum and diagonal band of Broca (MS-DBB) are located in the basal forebrain. MS-DBB are the main generators of hippocampal theta activity, and stimulation of the MS-DBB is sufficient to elicit theta activity (Bland, 2001; Bland, Konopacki, & Dyck, 2002; Buzsáki, 2002; Colgin, 2013; Vertes et al., 1997; Vertes et al., 2004). Further, it has been demonstrated that lesions or temporary inactivation of MS-DBB disrupt theta activity in hippocampus (Green & Arduini, 1954; Petsche et al., 1962). Both GABAergic and cholinergic neurons in the MS-DBB play a role in theta generation by modulating excitatory and inhibitory transmission in the hippocampus (Buzsáki, 2002; Colgin, 2013). MS-DBB sends cholinergic and GABAergic projections to the hippocampus. The cholinergic projections from MS-DBB to the hippocampus excites both pyramidal cells and interneurons that promote theta rhythmic firing, while the GABAergic cells inhibit GABAergic interneurons (Bland, 2001; Bland, Konopacki, & Dyck, 2002; Buzsáki, 2002; Colgin, 2013). GABAergic inhibitory neurons in the MS act as important pacemaker cells for theta activity, thus controlling the frequency of theta activity (Bland & Oddie, 2001; Buzsáki, 2002).

Another important region that also plays a role in generating theta activity is the reticular formation (RF) of the brainstem (Green & Arduini, 1954). Electrical stimulation of the reticular formation increases firing rates of MS-DBB pacemaker cells and elicits theta activity in the hippocampus (Petsche et al., 1962). One component of the reticular formation that is involved in theta activity appears to be the nucleus reticularis pontis oralis (RPO), since it has been shown that the intensity of activity in the RPO is related to the frequency of hippocampal theta (Bland & Oddie, 2001; Colgin, 2013). The RPO has ascending projections to the supramammillary nucleus (SUM), which then innervates the MS-DBB to elicit theta activity (Bland & Oddie,
Inactivation of the SUM has been shown to abolish theta activity in the hippocampus, which supports the notion that this ascending pathway (RPO-SUM-MS-DBB-hippocampus) plays an important role in theta generation (Bland & Oddie, 2001).

### 1.9. The Theta Suppression Model of Anxiolytics (TSMA)

There is a large body of experimental evidence suggesting that suppression of theta frequency in the hippocampus is a valid, predictive assay of anxiolytic drug action. In urethane-anesthetized rats, it has been demonstrated that anxiolytic drugs such as the 5-HT1A agonist buspirone (Coop & McNaughton, 1991), the selective serotonin reuptake inhibitors (SSRI) fluoxetine (McNaughton et al., 2007), and benzodiazepines suppress the frequency of brainstem-elicited hippocampal theta activity and alter GABA-A receptor function, without affecting the amplitude of theta (McNaughton et al., 2007). Importantly, other psychoactive drugs (e.g., neuroleptics) do not exert this pattern of effects on theta activity, suggesting a specific link between theta frequency suppression and anxiolytic effects on behavior (McNaughton et al., 2007).

While the theta suppression hypothesis of anxiety reduction has received considerable experimental support, it is noteworthy that several studies have challenged the association of theta frequency and anxiety (Chee et al., 2014; Chee et al., 2015; Yeung et al., 2013; Yeung et al., 2015). It has been shown that theta frequency and not theta power has been linked to anxiety and anxiolytic drug action; thus we focused the analysis on theta frequency and not power. For example, one can hypothesize that, if anxiolytic drugs suppress theta frequency, then anxiogenic drugs should exert the opposite effect and increase theta frequency (Yeung et al., 2013). In contrast to this prediction, Yeung and colleagues (2013) found that drugs such as FG7142,
βCCE, and yohimbine produce anxiogenic-like effects in the EPM (i.e., less time in open arms), but do not increase hippocampal theta frequency. Further, Chee and colleagues (2015) found that, while infusions of muscimol (a GABA<sub>A</sub> receptor agonist) into the lateral septum increased theta frequency, these infusions also resulted in decrease in anxiety-like behaviours on the EPM, thus challenging the TSMA. A similar effect was seen with histamine application in the lateral septum, which also increased theta frequency, but decreased anxiety-like behavioural responses in the EPM and the novelty-induced suppression of feeding test (Chee et al., 2014). Finally, Yeung and colleagues (2016) showed that infusion of histamine in the ventral hippocampus produced an increase in theta frequency and exerted anxiolytic-like effect in the EPM (Yeung et al., 2016). Together, these findings challenge the theta suppression model of anxiolysis, and further investigations are required to examine the relation between theta activity in the hippocampus and levels of anxiety expressed in behavioral tests.

2.0. **Objective and hypothesis**

The primary objectives of the current study are: 1) to examine a possible association of theta frequency and behavioural levels of “anxiety” in rats; and 2) to examine potential sex differences in anxiety, theta activity, and anxiety-theta association.

Consistent with the theta suppression hypothesis of anxiolysis, I hypothesize that rats expressing higher levels of “anxiety-like” behaviours in the EPM (e.g., reduced open arm exploration) will exhibit higher frequencies of hippocampal theta activity. Also, consistent with prior work, I hypothesize that female rats will show less anxiety on the EPM compared to their male counterparts. Following from these hypotheses, one would also expect that male rats (who are more anxious than females) exhibit higher-frequency theta activity compared to female rats.
Chapter 2: Methods

2.1 Subjects

The experiments were conducted on experimentally naïve male and female Long-Evans rats (Charles River Laboratories, Inc., Quebec, Canada) weighing 250-320 g at the time of surgery. All rats were housed in groups of up to four animals in polycarbonate cages (51 x 40 x 21 cm). The colony room was on a reversed 12:12 dark/ light cycle (lights off at 7:00 h) and temperature was approximately 21 ± 1°C, with food and water available ad libitum. The rats were allowed to acclimatize to the colony for at least one week before any experimental procedure. All procedures were in compliance with regulations by the Canadian Council Animal Care and were approved by the Queen’s University Animal Care Committee.

2.2. Estrous cycle monitoring

The estrous cycle in female rats was monitored by collecting vaginal smears (between 9:00h to 10:00h) for the five consecutive days leading up and including the day of testing in the EPM (i.e., the fifth/last smear was obtain on the test day, after behavioral testing was completed). A sterilized cotton swab was submerged in 0.9% saline solution for 20 seconds and then gently inserted into the rat’s vagina. Vaginal fluid was then applied to glass slides and stained with cresyl violet solution (Sigma-Aldrich, Oakville, Ontario, Canada; 0.1 g cresyl violet dissolved in 100 mL of distilled water). Immediately after straining, slides were examined using a standard light microscope (10x and 40x magnification) to identify cells types associated with different stages of the estrous cycle in female rats (Marcondes et al., 2002; Cora et al., 2015).
2.3. Elevated plus maze

The EPM was a wooden, plus-shaped maze consisting of two opposing closed arms (50 x 10 x 50 cm) and two opposing open arms (50 x 10 cm). The arms of the maze did not have roofs and were elevated 50 cm above the floor. The testing room was dimly lit with a desk lap placed on the floor along one of the walls of the room. All tests were conducted between 10:00h and 14:00h and the maze was cleaned with a 5% alcohol solution after each test session to minimize odor cues.

Rats (n = 25; n = 12 for males and n= 13 females) were individually transported from the colony to the testing room in a clean polycarbon cage (51 x 40 x 21 cm) containing fresh bedding material. The experimenter placed each rat in the center of the maze and left the testing room, while the rat was given 30-minute to freely explore the maze. The experimenter observed the rat on a computer monitor in an adjacent room and all sessions were video-recorded using a digital camcorder for the subsequent scoring of the behavior using Observer VideoPro (Noldus, MA). At the end of the 30-minute test session, the experimenter entered the room, removed the rat from the maze, and returned it back to the colony room.

The following behaviors were scored offline by an experimenter blind to the sex of the animal: a) total time spent in open arms; b) total time spent in closed arms; c) percentage of open arm entries (% of open arm entries = open arm/ open + closed arm x 100%); d) percentage of open arm time (% of open arm time = open arm time/ open arm time + closed arm time x100%); and e) total arm entries. The percentage of open arm time and open arm entries in the EPM are commonly used measures to provide an index of “anxiety” of rats (Pellow et al., 1985; Chee et al., 2014). An arm entry is defined as having all four feet into one entry (Pellow et al., 1985). It is important to note that this is just one method of defining an arm entry.
2.4. Surgical preparation

Surgical and electrophysiological procedures were conducted one week following behavioral testing on the same rats (n = 25) that were used for EPM testing. An additional group of male rats (n = 7) were used only for the electrophysiological procedures in order to validate the stimulating and recording techniques employed for the present experiments.

Rats were deeply anesthetized with urethane (total dose of 1.5 g/kg; administered as three 0.5 g/kg intraperitoneal (i.p.) injections every 15 minutes, supplements given as necessary). Following anesthesia induction, the local anesthetic marcaine (2 mg/kg) was injected subcutaneously (s.c.) along the incision site under the skin of the skull and the rat was placed in the stereotaxic instrument. A rectal thermometer was inserted to monitor (every 15 minutes throughout the entire experiment) body temperature, which was maintained at approximately 37°C by a fleece insulating blanket and an electrical heat pad.

An incision was made along the midline of the head to expose the skull. Small burr holes were drilled over the CA1 (−4.0 mm anterior-posterior [AP], + 2.0 mm medial-lateral [ML]; all measurements from bregma point) and the reticular formation (−7.0 mm AP, +1.6 mm ML; same coordinates as used by [McNaughton & Coop, 1991; Chee et al., 2014]). An additional hole was drilled in the frontal bone to allow the insertion of the ground connection (jeweler screw attached to a miniature connector). A bipolar recording electrode (two 125-µm diameter Teflon-insulated stainless steel wires, vertical tip separation about 1 mm) was lowered into the hippocampus (from dura: −3.0 to −3.5 mm). A stimulating electrode (Series 100 concentric bipolar electrode; Rhodes Medical Instruments, David Kopf, Tujunga, CA) was lowered into the reticular formation (from dura −7.2 mm).
2.5. **Electrophysiology and buspirone treatment**

The bipolar recording electrode was connected at a differential amplifier (BioAmp, AD Instruments, Toronto, Ontario, Canada) and A/D Converter (PowerLab 4/s system running Chart Software v.5.4: AD Instruments) that digitized (500 Hz) and stored the raw signal for subsequent offline analysis. Further, Chart software was used to apply a band-pass filter (4 to 10 Hz) to isolate activity in the theta frequency band.

The concentric, bipolar stimulating electrode was connected to a stimulus isolation unit (ML 180 Stimulus Isolator; AD Instruments) that provided constant current stimulation to the reticular formation, consisting of 5-s trains of 0.1 ms duration pulses delivered at 100 Hz (Chee et al., 2015, 2014; McNaughton & Coop, 1991).

Initially, the threshold current intensity to elicit theta activity during stimulation of the reticular formation was established for each rat. Reticular formation stimulation was applied at consecutively increasing intensities (0.02 – 0.20 mA in 0.02 mA increments; one stimulation episode for each intensity with an inter-stimulus interval of 30 seconds) and the lowest intensity that elicited theta activity was taken as the threshold intensity for that rat. For the subsequent, formal data collection, stimulation intensities of 2 x threshold, 3 x threshold, and 4 x threshold were applied in a pseudo-randomized order (i.e., random order, but each intensity was applied only once for each drug condition; see below for further details.

For rats that only underwent the electrophysiological experiments (see section 2.4), hippocampal theta activity during reticular formation stimulation (at 2, 3 and 4 x threshold) was recorded at three different time points/treatment phases of the experiment: a) prior to drug treatment; b) 30 min. after the administration of 0.9% saline (2 ml/kg, i.p.); and c) 30 min. after the administration of buspirone hydrochloride (10 mg/kg, i.p.; dissolved in 0.9% saline, freshly
prepared for each experiment; Tocris Bioscience/R&D System, Inc., Minneapolis, MN). Saline injections did not exert significant effects on theta activity (see Results section). Thus, for the electrophysiological experiments on rats that were first tested in the EPM, theta activity was recorded only prior to, and 30 min following an injection of buspirone (same dose).

2.6. Histology

At the end of the experiment, rats were given an additional 1-2 mL of urethane (i.p.). Subsequently, rats were perfused through the heart with 120 mL of 0.9% saline, followed by 120 mL of phosphate-buffered formalin. The brain was removed and stored in formalin for at least 48 hours prior to being sectioned (40 µm coronal sections) using a freezing cryostat. Sections were mounted onto gelatin coated glass slides and standard histological techniques were used to assess the accuracy of electrode placements by an experimenter who was blind of the results of individual animals. Data obtained with inaccurate electrode placements were excluded from the data analyses.

2.7. Data analyses

Data are presented as mean ± standard error of the mean (S.E.M.). Statistical analyses (analysis of variance [ANOVA] and correlational analysis between behavioral and electrophysiological measures) were carried out using the SPSS software package (v. 19; IBM, Inc.).
Chapter 3: Results

3.1 Validation of theta frequency as a measure of pharmacological anxiolysis

The first experiment assessed the effect of treatment with the clinically used anxiolytic agent buspirone (Rickels, 1990) on theta frequency in the CA1 of male, urethane-anesthetized Long-Evans rats (n = 7). Stimulation of the reticular formation (5 second trains, 100 Hz, 0.1 ms pulses) reliably elicited theta activity in all animals (Fig. 1). Typically, theta activity occurred throughout the entire stimulation periods and ceased soon (< 1 s) after the end of stimulation; spontaneous theta activity was never observed outside the stimulation periods. Further, as shown in Fig. 2 (No Drug condition), the frequency of theta activity increased with higher stimulation intensities (2 x, 3 x, and 4 x threshold intensity to elicit theta activity; delivered in a pseudo-randomized fashion), a finding that is consistent with prior research (McNaughton et al., 2007; Chee et al., 2014, 2015).

After recordings of hippocampal activity were obtained in the No Drug condition, all rats received an injection of saline (2 ml/kg, i.p.) and stimulation resumed 30 min following the injection. As shown in Figs. 1 and 2, saline administration did not result in clear changes in hippocampal theta frequency at any of the three stimulation intensities.

Next, rats received an injection of buspirone (10 mg/kg, i.p.) and stimulation was continued 30 min following the drug administration. Buspirone treatment resulted in a decrease in hippocampal theta frequency (Fig. 1), an effect that was most pronounced at stimulation intensities of 3 x and 4 x threshold (Fig. 2).

Theta frequencies data were analyzed with a repeated-measures ANOVA to assess the effects of treatment condition (no drug, saline, buspirone) and stimulation intensities (2.0 x, 3.0 x, and 4.0 x threshold) on theta frequency. There was a significant main effect of stimulation
intensity, $F(2, 12) = 6.7, p = 0.01$, and a main effect of drug condition, $F(2, 12) = 9.29, p < 0.01$. However, the treatment x intensity interaction was not significant, $F(4, 24) = 2.2, p = 0.16$.

Subsequent pairwise comparisons showed that there was a significant difference between the no drug and buspirone condition ($p < 0.01$), as well as the saline and buspirone condition ($p < 0.01$), confirming that buspirone lowered theta frequency relative to the two other treatment conditions.

Together, these data confirm prior work that electrical stimulation of the reticular formation effectively induces theta activity in the hippocampus of urethane-anesthetized rats, and that systemic administration of the anxiolytic agent buspirone results in a reduction of theta frequency under these experimental conditions (McNaughton et al., 2007; Chee et al., 2014, 2015).

Figure 1: Representative example of unfiltered (top) and filtered (4 to 12 Hz, bottom) hippocampal theta activity recorded in the CA1 that was elicited electrical stimulation of the reticular formation (black bars: consisting of 5-s trains of 0.1 ms duration pulses delivered at 100Hz, 3 x stimulation threshold) in no drug (left) and buspirone (10 mg/kg, right).
Figure 2: Mean (± S.E.M.) peak hippocampal theta frequency elicited by reticular formation at three stimulation intensities (2.0 x threshold, 3.0 x threshold, 4.0 x threshold) in male Long Evans (n= 7) in three drug condition. The * denotes that there was a significant difference between no drug and buspirone condition and saline and buspirone (p <0.01).

3.2 Results for the elevated plus maze

As shown in Table 1 and Fig. 3, both male (n = 12) and female rats (n = 13) spend some of the test time (30 min) on the elevated plus maze exploring the open arms of the maze. However, as expected, both sexes spend much less time in the open arms compared to the closed arms (about 25% and 10% open arm time for females and males, respectively; Fig. 3A).

Interestingly, there appeared to be a sex difference in behavior on the elevated plus maze, with females displayed a greater number of open arm entries than males during the first 5 minutes of testing, and also over the entire 30 min test period (Table 1 and Fig. 3B). Similarly, female rats spent more time in the open arms than males at the 5 minutes and 30 minutes time point (Table 1 and Fig. 3A). Thus, it appears that females show less avoidance of open arms, a
common measure of anxiety in rodents, than their male counterparts. It is also noteworthy that, at the 5 min test interval, the total number of arm entries (a measure of general motor activity) was very similar for males and females (Table 1, top), but females displayed more arm entries than males over the entire 30 min test session (Table 1, bottom).

To confirm the description of the results provided above, independent t-tests were conducted to examine potential differences in elevated plus maze behaviours between female and male rats.

**Percentage of Open Arm Time, 5 min:** A Levene’s test found that assumption of homogeneity of variance was not significant, $F(1, 23) = 25.02, p < 0.05$; therefore equal variance cannot be assumed and, as a consequence, the Welsh t-test was conducted. Using Welsh t-test, it was revealed that there was a significant difference in the percentage of open arm time between males (M= 5.5%) and females (M= 19.1%) during the first five-minute interval (Fig. 3A); $t(16.13) = 2.53, p = 0.022$. Further, Cohen’s effect size value ($d = 0.99$) suggests there is a sex difference in percentage of open arm time during the first five minutes on the elevated plus maze.

**Percentage of Open Arm Time, 30 min:** Next, another independent t-test was conducted to compare the open arm time in males and females over the entire 30 min test (Fig. 3A). A Levene’s test revealed that homogeneity of variance can be assumed, $F(1, 23) = 3.82, p = 0.063$; therefore group variance can be treated as equal. There was a significant difference between females (M= 23.3%) and males (M= 8.4%) in terms of percentage open arm time during the thirty minutes of testing on the elevated plus maze (Fig. 3A), $t(23) = 4.19$, $p < 0.05$. Furthermore,
Cohen’s d was computed to examine the effect size, which revealed a value of \( d = 1.69 \), suggesting that there was a sex difference in percentage of open arm time.

**Percentage of Open Arm Entries, 5 min:**

An independent t-test was conducted to compare the percentage open arm entries between females and males during the first 5 min of testing. Levene’s test of homogeneity of variance revealed that equal variance cannot be assumed, \( F(1, 23) = 9.82, p = 0.05 \), therefore, a Welsh t-test was conducted. Welsh t-test revealed that there was a significant difference between females (M= 28.8%) and males (M= 9.7%) in the percentage of open arm entries (Fig. 3B); \( t(16.13) = 2.94, p = 0.01 \). Furthermore, Cohen’s d was computed to examine the effect size, which yielded a value of \( d = 1.16 \), suggesting that there was a sex difference in percentage open arm entries at the five minute time point.

**Percentage of Open Arm Entries, 30 minutes:** Finally, an independent t-test was conducted to compare open arm entries between females and males at the 30 minutes time point. Levene’s test of homogeneity of variance revealed that equal variance can be assumed, \( F(1, 23) = 3.35, p = 0.08 \). The independent t-test revealed that there was a sex difference between female (M= 35.2%) and male (M= 15.5%) rats in the percentage of open arm entries (Fig. 3B); \( t(23) = 5.14, p < 0.005 \). Furthermore, Cohen’s d was computed to examine effect size, which yielded a value of \( d = 2.04 \), suggesting that there was a sex difference in percentage open arm entries at the thirty minutes time point.
Table 1: Mean (± S.E.M) number of open and closed arm entries and duration of open arm time in the elevated plus maze displayed by male (n = 12) and female (n = 13) rats. The top and bottom sections display activity during the first 5 minutes and the entire 30 minutes of testing, respectively. The * in the table denote that p < 0.05.

<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th>Male</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>5 minutes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td># Closed Arm</td>
<td>7.15 ± 0.49</td>
<td>9.08 ± 0.81</td>
<td>t (23) = 2.07, p = 0.05</td>
</tr>
<tr>
<td># Open Arm</td>
<td>3.38 ± 0.81</td>
<td>1.00 ± 0.28</td>
<td>t (23) = 2.69, p = 0.01*</td>
</tr>
<tr>
<td># Total Number of Entries</td>
<td>10.54 ± 0.79</td>
<td>10.08 ± 0.94</td>
<td>t (23) = 0.37, p = 0.71</td>
</tr>
<tr>
<td>Duration open (s)</td>
<td>57.29 ± 14.83</td>
<td>16.54 ± 6.28</td>
<td>t (16.13) = 2.53, p = 0.02*</td>
</tr>
<tr>
<td><strong>30 minutes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td># Closed Arm</td>
<td>40.38 ± 2.78</td>
<td>34.50 ± 2.62</td>
<td>t (23) = 1.53, p = 0.14</td>
</tr>
<tr>
<td># Open Arm</td>
<td>22.77 ± 2.54</td>
<td>7.58 ± 1.91</td>
<td>t (23) = 4.72, p &lt; 0.01*</td>
</tr>
<tr>
<td># Total Number of Entries</td>
<td>63.15 ± 4.68</td>
<td>42.08 ± 3.95</td>
<td>t (23) = 3.41, p = 0.02 *</td>
</tr>
<tr>
<td>Duration open (s)</td>
<td>419.27 ± 51.26</td>
<td>150.77 ± 37.05</td>
<td>t (23) = 4.18, p &lt; 0.01*</td>
</tr>
</tbody>
</table>
Figure 3: Bar graphs displaying the mean (± S.E.M.) percentage of open arm time (figure 3A) and the percentage of open arm entries (figure 3B) of male (n= 12) and female (n= 13) rats. Rats were placed on the elevated plus maze for thirty minutes. The graph compares behavior during the first 5 minutes of testing to the entire 30 minutes of the test. The * denotes p < 0.05 when comparing females and males at different time points.
Finally, I also examined the potential influence of the estrous cycle on behavior in the elevated plus maze and hippocampal theta frequency in female rats (n = 13; same animals as reported in the previous sections). However, the influence of the estrous cycle was not a primary focus of this thesis, and the sample sizes for the different conditions (i.e., estrous cycle stages) were very small, particularly for the electrophysiological data (see Tables 2 and 3). Thus, I merely provide a descriptive summary of the trends observed in these experiments, without a formal, statistical analysis of these data sets.

Interestingly, at both the 5 min and 30 min time points, it appeared that rats in metestrus showed a great number of open arm entries and time in the open arms relative to rats in proestrus (Table 2). Total arms entries, a measure of general motor activity, were similar in both groups over the first 5 minutes of testing, but appeared to be somewhat higher for rats in metestrus over the entire 30-min test session (Table 2).
Table 2: Mean (S.E.M.) number of closed arm entries, number of open arm entries, number of total arm entries, and time spend in the open arm (s) of female rats (n = 13) during different phases of the estrous cycle. Means for the first 5 minutes of testing and the entire 30-minute test period are presented.

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Proestrus (n = 3)</th>
<th>Metestrus (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td># of Closed Arm Entries</td>
<td>8.33 ± 0.67</td>
<td>6.8 ± 0.57</td>
</tr>
<tr>
<td># of Open Arm Entries</td>
<td>1.67 ± 0.88</td>
<td>3.90 ± 0.98</td>
</tr>
<tr>
<td># of Total Arm Entries</td>
<td>10.00 ± 0.58</td>
<td>10.70 ± 1.02</td>
</tr>
<tr>
<td>Time in the Open Arms (s)</td>
<td>39.39 ± 38.94</td>
<td>62.67 ± 16.31</td>
</tr>
</tbody>
</table>

3.3 Examining Sex Differences in Reticular-Elicited Hippocampal Theta Frequency Before and After Buspirone Administration

One week after the completion of behavior testing, all rats were anesthetized with urethane and electrophysiological experiments were conducted to examine the frequency of theta activity in the hippocampus elicited by electrical stimulation of the reticular formation (5 second trains, 100 Hz, 0.1 ms pulses, intensity of 2 x, 3 x, and 4 x of threshold to elicit theta).

Similar to the observations reported for Experiment 1, theta activity occurred throughout the entire stimulation period and ceased soon (< 1s) after the end of the stimulation. Spontaneous theta activity was never observed outside the stimulation periods. Further, as shown in Fig. 4, the frequency of theta increased with higher stimulation intensity (2, 3, 4 x threshold), a finding that is consistent with results of Experiment 1. Importantly, there was no indication of a systematic difference in theta frequency between male (n = 12) and female rats (n = 13), and both sexes showed a similar increase in theta frequency with higher stimulation currents (Table 2; Figure 7).
Similar to Experiment 1, after recording hippocampal theta activity in the No Drug condition, all rats received an injection of buspirone (10 mg/kg, i.p.) and stimulation was continued after a 30 minutes absorption-period (given that saline injections did not exert obvious effects on theta frequency in Experiment 1, saline was not administered in Experiment 2). As shown in Fig. 7, buspirone decreases hippocampal theta frequency in both female and males, similar to the effects observed in Experiment 1 (in male rats only).

Electrophysiological data were analyzed with a mixed-model ANOVA to assess the effects of sex (between subjects variable), drug condition (No Drug vs. buspirone; repeated-measures variable), and stimulation intensity (2, 3, and 4 x threshold; repeated-measures variable) on the frequency of hippocampal theta activity. There was a main effect of intensity, $F(2, 42.80) = 9.18, p < 0.01$, and a main effect of drug condition, $F(1, 42.80) = 5.87, p = 0.017$, but no main effect of sex, $F(1, 42.80) = 2.54, p = 0.11$, on theta frequency. Further, none of the interactions effects reached statistical significance (intensity x drug condition, $p = 0.51$; intensity x sex, $p = 0.07$; treatment x sex, $p = 0.78$; intensity x drug x sex, $p = 0.33$). Subsequent pair-wise comparisons showed that there was a significant difference between the No Drug and buspirone condition ($p < 0.01$), again confirming that buspirone lowers theta frequency.
Figure 4: Bar graph comparing the mean (± S.E.M.) hippocampal theta frequency in male (n=12; denoted in grey colour) and female (n=13; white colour) Long-Evans rats in the No Drug and Buspirone condition during reticular formation stimulation (2 x, 3 x, and 4 x threshold intensity).

Table 3: Averaged Hippocampal Theta Frequencies (± S.E.M) in the No Drug and Buspirone condition collapsed across stimulation intensity: 2.0 x threshold, 3.0 x threshold, and 4.0 x threshold.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Drug</td>
<td>5.31 ± 0.14</td>
<td>5.25 ± 0.13</td>
</tr>
<tr>
<td>Buspirone</td>
<td>4.88 ± 0.11</td>
<td>5.00 ± 0.11</td>
</tr>
</tbody>
</table>

3.4 **Estrous cycle and hippocampal theta frequency**

I also conducted a preliminary analysis regarding the possibility that the stage of the estrous cycle has an effect on hippocampal theta frequency in female rats. As shown in Table 4,
prior to drug treatment (No Drug condition), theta frequencies were in the range of about 4.4-6.1 Hz and generally increased with higher stimulation intensities. There appeared to be a trend toward slightly higher theta frequency in rats during diestrus, but the small sample sizes preclude any firm conclusions.

Buspirone treatment (10 mg/kg, i.p.) reduced theta frequency of rats in diestrus and proestrus, but not in metestrus and estrus (Table 4); again, the small sample sizes make it impossible to draw firm conclusions regarding the reliability of these observations.

Generally, the data presented in Table 4 do not indicate a major influence of different phases of the estrus cycle and associated hormonal fluctuations on the frequency of hippocampal theta activity elicited by stimulation of the reticular formation.

Table 4: Mean (± S.E.M) frequency (in Hz) of hippocampal theta activity elicited by reticular formation stimulation (2, 3, and 4 x threshold) in the No Drug and Buspirone condition of female rats (n = 13) during different phases of the estrus cycle.

<table>
<thead>
<tr>
<th>Stimulation Intensity</th>
<th>Metestrus</th>
<th>Diestrus</th>
<th>Proestrus</th>
<th>Estrus</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Drug</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0 x threshold</td>
<td>4.64 ± 0.19</td>
<td>5.17 ± 0.18</td>
<td>4.36 ± 0.08</td>
<td>4.48 ± 0.61</td>
</tr>
<tr>
<td>3.0 x threshold</td>
<td>5.20 ± 0.29</td>
<td>5.78 ± 0.29</td>
<td>4.95 ± 0.14</td>
<td>5.13 ± 0.65</td>
</tr>
<tr>
<td>4.0 x threshold</td>
<td>5.74 ± 0.26</td>
<td>6.10 ± 0.25</td>
<td>6.10 ± 0.38</td>
<td>5.45 ± 0.47</td>
</tr>
<tr>
<td>Buspirone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0 x threshold</td>
<td>4.60 ± 0.45</td>
<td>4.56 ± 0.18</td>
<td>4.18 ± 0.08</td>
<td>4.76 ± 0.31</td>
</tr>
<tr>
<td>3.0 x threshold</td>
<td>5.17 ± 0.48</td>
<td>5.09 ± 0.32</td>
<td>4.61 ± 0.03</td>
<td>4.76 ± 0.43</td>
</tr>
<tr>
<td>4.0 x threshold</td>
<td>5.90 ± 0.47</td>
<td>5.61 ± 0.25</td>
<td>5.22 ± 0.15</td>
<td>5.90 ± 0.25</td>
</tr>
<tr>
<td>No Drug</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Averaged Across</td>
<td>5.19 ± 0.20</td>
<td>5.68 ± 0.18</td>
<td>5.14 ± 0.25</td>
<td>5.02 ± 0.32</td>
</tr>
<tr>
<td>Stimulation Intensity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buspirone</td>
<td>5.22 ± 0.30</td>
<td>5.09 ± 0.20</td>
<td>4.67 ± 0.14</td>
<td>5.14 ± 0.25</td>
</tr>
</tbody>
</table>
3.5 **Correlations of Elevated Plus Maze Behaviour**

Finally, I examined whether there is a relation between behavior in the elevated plus maze and the frequency of theta activity in individual rats. As outlined in the Introduction, the theta suppression hypothesis of anxiety assumes that a lower frequency of theta activity is an index of (pharmacologically) reduced anxiety levels in rodents (McNaughton et al., 2007). Thus, I hypothesized that rats that display more anxiety-like behavior (e.g., greater open arm avoidance) in the maze would have higher frequencies of theta activity in the hippocampal formation during stimulation of the reticular formation.

In order to test this hypothesis, a series of Pearson correlational analyses were computed between behavioral measures and hippocampal theta frequencies. In contrast to the hypothesis, these analyses failed to reveal any significant associations between anxiety-like behavior and theta frequency under the present, experimental conditions. As shown in Figs. 5 to 8, none of the behavioral measures (open arm time, number of open arm entries) showed a significant association with theta frequencies, regardless of the stimulation intensity used to elicit theta (2 x, 3 s, and 4 x threshold intensity). Further, this lack of association between behavior and theta frequency was apparent during both the no drug and buspirone condition.

*Statistics:*

Open Arm Entries over 5 Minutes (Fig. 5): No Drug condition, 2 x threshold, \( r = 0.022, p = 0.92 \); 3 x threshold, \( r = -0.054, p = 0.80 \); 4 x threshold, \( r = 0.056, p = 0.79 \); Buspirone condition, 2 x threshold, \( r = 0.130, p = 0.54 \); 3 x threshold, \( r = -0.079, p = 0.71 \); 4 x threshold, \( r = -0.032, p = 0.88 \).
Open Arm Entries over 30 Minutes (Fig. 6): No Drug, 2 x threshold, \( r = -0.12, \ p = 0.57 \); 3 x threshold, \( r = -0.006, \ p = 0.98 \); 4 x threshold, \( r = -0.20, \ p = 0.34 \); Buspirone, 2 x threshold, \( r = 0.24, \ p = 0.24 \); 3 x threshold, \( r = -0.094, \ p = 0.66 \); 4 x threshold, \( r = 0.17, \ p = 0.41 \).

Open Arm Time over 5 Minutes (Fig. 7): No Drug condition, 2 x threshold, \( r = 0.14, \ p = 0.51 \); 3 x threshold, \( r = -0.014, \ p = 0.95 \); 4 x threshold, \( r = -0.019, \ p = 0.93 \); Buspirone condition, 2 x threshold, \( r = 0.19, \ p = 0.37 \); 3 x threshold, \( r = 0.057, \ p = 0.79 \); 4 x threshold, \( r = -0.035, \ p = 0.87 \).

Open Arm Time over 30 Minutes (Fig. 8): No Drug condition, 2 x threshold, \( r = 0.093, \ p = 0.66 \); 3 x threshold, \( r = 0.042, \ p = 0.84 \); 4 x threshold, \( r = -0.19, \ p = 0.38 \); Buspirone condition, 2 x threshold, \( r = 0.29, \ p = 0.16 \); 3 x threshold, \( r = 0.04, \ p = 0.84 \); 4 x threshold, \( r = 0.077, \ p = 0.72 \).

Together, these results suggest that there is no clear relationship between open arm exploration in the elevated plus maze and the frequency of hippocampal theta activity in male and female rats.
Figure 5: Scatterplots displaying the relationship between percentage open arm entries over 5 minutes of testing on the elevated plus maze and hippocampal theta frequency for male (n = 12; denoted by the gray triangle) and female (n= 13; denoted by the black diamond). The following conditions are shown: A. 2.0 x threshold stimulation intensity, no drug condition; B. 2.0 x threshold intensity, buspirone condition; C. 3.0 x threshold, no drug condition; D. 3.0 x threshold, buspirone condition; E. 4.0 x threshold, no drug condition; F (4.0 x threshold, buspirone condition. The line shown in the figures is the regression line.
Figure 6: Scatterplots displaying the relationship between percentage open arm entries over 30 minutes of testing on the elevated plus maze and hippocampal theta frequency for male (n = 12; denoted by the gray triangle) and female (n = 13; denoted by the black diamond) rats. The following conditions are shown: A. 2.0 x threshold stimulation intensity, no drug condition; B. 2.0 x threshold intensity, buspirone condition; C. 3.0 x threshold, no drug condition; D. 3.0 x threshold, buspirone condition; E. 4.0 x threshold, no drug condition; F (4.0 x threshold, buspirone condition). The line shown in the figures is the regression line.
Figure 7: Scatterplots displaying the relationship between percentage open arm time over 5 minutes of testing on the elevated plus maze and hippocampal theta frequency for male (n = 12; denoted by the gray triangle) and female (n= 13; denoted by the black diamond) rats. The following conditions are shown: A. 2.0 x threshold stimulation intensity, no drug condition; B. 2.0 x threshold intensity, buspirone condition; C. 3.0 x threshold, no drug condition; D. 3.0 x threshold, buspirone condition; E. 4.0 x threshold, no drug condition; F (4.0 x threshold, buspirone condition. The line shown in the figures is the regression line.
Figure 8: Scatterplots displaying the relationship between percentage open arm time over 30 minutes of testing on the elevated plus maze and hippocampal theta frequency for male (n = 12; denoted by the gray triangle) and female (n = 13; denoted by the black diamond) rats. The following conditions are shown: A. 2.0 x threshold stimulation intensity, no drug condition); B. 2.0 x threshold intensity, buspirone condition); C. 3.0 x threshold, no drug condition; D. 3.0 x threshold, buspirone condition; E. 4.0 x threshold, no drug condition; F (4.0 x threshold, buspirone condition. The line shown in the figures is the regression line.
Discussion

4.1. **Experiment 1: Validation of theta frequency as a measure of pharmacological anxiolysis**

In the first experiment, rats were anesthetized with urethane to assess the effects of buspirone on theta frequency in the CA1 hippocampus elicited by stimulation of the brainstem reticular formation. In the no drug condition (i.e., prior to systemic administration of saline and buspirone), theta frequency increased with increasing stimulation intensities (2, 3, 4 x threshold). Saline administration did not elicit clear changes in hippocampal theta activity. However, buspirone treatment resulted in a decrease of hippocampal theta frequency, an effect that was most evident at stimulation intensities of 3 x and 4 x threshold.

The results of this experiment confirm that electrical stimulation in the reticular formation induces hippocampal theta activity in urethane-anesthetized rats (Chee et al., 2014; Chee et al., 2015; McNaughton et al., 2007). Prior research has shown that theta frequency increases when stimulation intensity increases, and that systemic saline treatment has no obvious effects on theta frequency (Chee et al., 2014; Chee et al., 2015), observations that are consistent with the findings in Experiment 1.

Prior research has reported that the anxiolytic agent buspirone significantly decreases hippocampal theta frequencies elicited by brainstem stimulation (McNaughton et al., 2007; McNaughton & Coop, 1991; Chee et al., 2014). Thus, the data presented here provide further support for the notion that pharmacological agents that produce anxiolytic effect in behavioural tests reliably act to decrease hippocampal theta frequencies, in accordance with the influential theta suppression model of anxiolysis (McNaughton et al., 2007; Yeung et al., 2012). The theta suppression model postulates that all clinically effective, anxiolytic drugs (e.g., benzodiazepines,
such as diazepam; 5-HT1A agonists, such as buspirone; SSRIs, such as fluoxetine) suppresses reticular-elicited theta frequencies in urethane anesthetized and awake, freely moving rats (McNaughton et al., 2007). Thus, theta frequency can be viewed as a neurophysiological assay of anxiety, as well as a tool to assess potential, anxiolytic (and also anxiogenic) properties of novel, pharmacological compounds. The observations in Experiment 1 that buspirone lowers theta frequency provide further support for the theta suppression model; importantly, they also verify that the electrophysiological methods used for the current experiments are able to detect the typical, neurophysiological effect seen with anxiolytic drug treatments.

4.2. **Experiment 2: Examination of potential sex differences on the elevated plus maze**

In the second experiments, rats were placed on the elevated plus maze for a thirty-minutes period and a variety of defensive, anxiety-like behaviours were subsequently analyzed. It is worthwhile to note that a thirty-minute test period is much longer than the typical five-minute duration employed in the large majority of studies using the elevated plus maze to measure anxiety in rodents. Thus, data were scored for both the initial five minutes of the test, as well as the entire thirty-minute test period. One of the reasons for placing the rats on the elevated plus maze for thirty-minutes was to increase the sensitivity of the task to detect individual differences in exploratory behaviour on the maze, as well as differences between the sexes. Thus, I aimed to examine behaviour over a longer time interval to compare anxiety-related behaviours during the first five minutes to a longer test duration.

As expected, both sexes spent more time exploring the closed arms, rather than the open arms of the maze. Interestingly, female rats displayed a greater number of open arm entries and longer time in the open arms than their male counterparts for the first five minutes and the entire
thirty-minutes of the task, with the statistical analyses confirming significant sex differences for both of these anxiety-related measures at both time points of testing. In addition to examining sex differences, I also collected pilot data on the potential influence of the estrus cycle on the behaviour of female rats in the elevated plus maze. It appeared that rats in metestrus showed a greater number of open arm entries and a longer duration in the open arms than rats in proestrus over the first five minutes and the entire 30 minutes of the test.

The data presented here confirm that there are sex differences in anxiety-related behaviours on the elevated plus maze. Our findings are similar to previous studies reporting that female rats show a greater percentage of open arm entries than males (Johnson & File, 1991; Imhof et al., 1993). In addition, our study also confirms previous findings that females show a reduced aversion to open arms and spend a greater percentage of time in the open arms compared to male rats (Johnson & File, 1991; Kelly et al., 1999; Lovick, 2012; Marcondes et al., 2001; Walf & Fry, 2013).

In contrast, the pilot data on the influence of different stages of the estrous cycle do not support previous findings in literature (Marcondes et al., 2001; Palanz, 2001). Most studies have reported that female rats in proestrus show a larger number of open arm entries than rats in metestrus (Kelly et al., 1999; Lovick, 2012; Marcondes et al., 2001; Palanz, 2001). Similarly, rats in proestrus show an increase in the number of open arm entries and open arm time when compared to rats in diestrus and metestrus (Díaz-Véliz et al., 1997; Marcondes et al., 2001; Zimmerberg & Farley, 1993). It has been reported that female rats in proestrus and estrus showed a greater percentage of open arm entries and spent more time in the open arms than rats in diestrus under low-light test conditions (Mora et al., 1996). Thus, it appears rather that I found
that metestrus rats exhibited more open arm entries and spent a longer time in the open arms than females in proestrus.

One explanation for discrepancies between the data presented here and the results of prior studies could be the timing of the experiment. Rats were placed on the elevated plus maze between 10 AM and 2 PM. In the morning of proestrus, progesterone levels tend to be low (Marcondes et al., 2001). The proestrus phase typically last 12 to 14 hours (Marcondes et al., 2001). Levels of progesterone rise during the afternoon and peak at night time (Marcondes et al., 2001). If the female rats were placed on the elevated plus maze during the afternoon when progesterone levels are higher, we would expect more open arm entries and longer open arm duration. Another potential explanation for the discrepancy is the small sample size in the proestrus group, with only three rats in proestrus on the day of the elevated plus maze test, when most rats were in metestrus (n= 10). Thus, it is difficult to draw any conclusion regarding hormonal influences on plus maze behaviour from the data collected for this thesis, even though future studies could examine this question in more detail.

4.3. **Sex differences in hippocampal theta**

Most research on hippocampal theta activity in relation to anxiety has been conducted on male rodents (Chee et al., 2014; Chee et al., 2015; Coop & McNaughton, 1991; McNaughton & Coop, 1991; Yeung et al., 2011; Yeung et al., 2012, Yeung et al., 2013; Yeung et al., 2016; Zhu & McNaughton, 1991). In Experiment 2, hippocampal theta activity was recorded in male and female rats before and after systemic administration of the anxiolytic drug buspirone. Similar to the findings of Experiment 1, theta frequency increased with higher stimulation intensities (2, 3, 4 x threshold). Importantly, there did not appear to be any systematic differences in hippocampal
theta frequency between male and female rats and buspirone lowered hippocampal theta frequency, an effect seen in both sexes.

In addition to examining sex differences, I also collected pilot data on the potential influence of the estrous cycle on hippocampal theta frequency. In the no drug condition, females in proestrus exhibited the lowest theta frequency at the 2 x and 3 x threshold stimulation intensities when compared to metestrus, diestrus, and estrus. Following buspirone treatment, female rats in proestrus had a tendency to exhibit lower theta frequency when compared to rats in estrus, metestrus, or diestrus. Females in diestrus showed the highest theta frequency (at 2 x and 3 x threshold stimulation) compared to the other stages of the estrus cycle; it is important to emphasize that all of these trends were small and often apparent at only some of the stimulation intensities used to elicit theta activity in the hippocampus.

Overall, the data suggest that there appear to be no major sex differences in hippocampal theta frequency under the present, experimental conditions. It was surprising that the pilot data showed that hippocampal theta frequency appears to be lowest in proestrus and highest in diestrus when compared to the other phases of the estrus cycle. It is important to keep in mind that the groups for each phase of the cycle were only 3 to 4 rats. Hippocampal theta frequencies vary across individual rats, making it possible that the differences between various stages of the estrus cycle are heavily influenced by differences between individual animals. Importantly, the data presented here confirm previous findings of a lack of a sex difference in hippocampal theta activity elicited by stimulation of the medial septal area in rats (Drewett et al., 1977). Thus, data on hippocampal theta frequency (and the effects of anxiolytic drugs) obtained using male rats may well generalize to both sexes, at least for this species.
4.4. **Correlation of elevated plus maze behaviour and hippocampal theta frequency**

There were no correlations between any of the anxiety-related behaviours on the elevated plus maze and the frequencies of hippocampal theta activity elicited by stimulation of the reticular formation at intensities between two and four times the threshold intensity to elicit theta. Further, this lack of association between behaviour and theta frequency held up for both test intervals on the elevated plus maze (5 and 30 minutes) and both pharmacological theta conditions (before and after buspirone administration). Thus, it appears that the frequency of hippocampal theta activity in individual rats is not associated with indexes of anxiety at the behavioural level.

It was unexpected to find this lack of association between theta frequency and behaviour, since numerous studies have provided evidence that a reduction in theta frequency is a reliable, physiological measure of lower anxiety levels (McNaughton et al., 2007). It is worthwhile to note, however, that this evidence stems largely from pharmacological studies, rather than using theta as a predictor of anxiety in individual, pharmacologically untreated animals.

To the best of our knowledge, only one prior study has taken an approach similar to the one used here in terms to examining a possible link between theta activity and anxiety without the use of pharmacological manipulations. Horváth et al. (2015) compared theta frequency in two strains of inbred mice that show pronounced differences in anxiety-related behavior, such as exploration on the elevated plus maze. Interestingly, these authors did not detect differences in theta frequency (or power) between the anxious and non-anxious mouse strain (Horváth et al., 2015), results that are similar to our observations that male and female rats, scoring high and lower on anxiety in the elevated plus maze, did not exhibit differences in theta frequency. Horváth et al. (2015) did not perform correlational analyses using individual animals and,
importantly, did not study female mice; thus, the current thesis differs from prior work in its scope and analytical approach. However, both studies clearly challenge the assumption of a link between theta frequency and behavioural measures of anxiety in rodent species.

There is additional evidence that questions the theta-anxiety association. In order to provide support for the theta suppression model, a novel anxiolytic compound should elicit a reduction in anxiety-related behaviours and in hippocampal theta frequency, while anxiogenic drugs should exert the opposite effect on theta (McNaughton et al., 2007). In contrast to this hypothesis, three compounds that exerted clear, anxiogenic effects in the elevated plus maze (FG7142, βCCE, and yohimbine) all failed to elicit an increase in hippocampal theta frequency (Yeung et al., 2013). Moreover, another studied showed that when histamine was infused into the lateral septum, it produced an anxiolytic-like effect in the elevated plus maze and novelty-induced suppression of feeding test (Chee & Menard, 2013; Chee et al., 2014), but also led to an increase in theta frequency (Chee et al., 2014). A similar dissociation of behavioural and electrophysiological actions (i.e., reduced anxiety, but increased theta frequency) was seen with infusions of the GABA_A receptor agonist muscimol into the lateral septum (Chee et al., 2015). Together, these studies provide examples of a dissociation of the behavioural and hippocampal (theta) effects of manipulating anxiety level in rodents and challenge the assumption that theta frequency is a physiological “read-out” of the anxiety state on an animal.

Although the data presented in this thesis do not support the influential theta suppression model, there are a large number of studies that support the link between theta frequency and anxiety, at least with regard to drug effects on these two variables (Engin et al., 2008; Coop & McNaughton, 1991; McNaughton & Coop, 1991; McNaughton et al., 2007; Yeung et al., 2014; Yeung et al., 2016). Buspirone, benzodiazepines, and selective serotonin reuptake inhibitors, all
of which are clinically used anxiolytics, reduce defensive behaviours in rodent anxiety tests and also suppress hippocampal theta frequency in freely behaving or urethane-anesthetized rats (Engin et al., 2008; McNaughton & Coop, 1991; McNaughton et al., 2007; Yeung, 2012). Thus, it appears that the present experiment does not confirm the proposed link between behavioural anxiolysis and suppression of hippocampal theta frequencies.

It is unlikely that the specific electrophysiological methods used for the present experiments were insufficient to detect a possible association between theta frequency and behavioural levels of anxiety. The electrode placements, stimulation parameters, and anesthesia type and dose were chosen to conform to those used in previous work (Chee et al., 2014; Chee et al., 2015; Coop & McNaughton, 1991; McNaughton & Coop, 1991), and I was able to detect the well-known decrease in theta frequency seen following systemic administration of the clinically effective anxiolytic agent buspirone (Coop & McNaughton, 1991; McNaughton & Coop, 1991; McNaughton et al., 2007). Thus, it appears unlikely that the failure to observe correlations between the behavioural results in the elevated plus maze and theta activity are due to issues with the electrophysiological recordings or related, experimental procedures.

4.5. Limitations and some future directions

One of the limitations of the study is that we did not have a sufficient sample size to systematically assess the effects of different stages of the estrous cycle on behaviour and hippocampal activity. As discussed, a number of published papers have examined sex differences (Johnson & File, 1991; Kelly et al., 1999; Palanza, 2001) and the effects of the estrus cycle on behaviour in the elevated plus maze (Frye et al., 2000; Marcondes et al., 2001; Mora et al., 1997; Palanza, 2001). However, little is known with regard to the influence of the fluctuations of
various hormones over the estrus cycle on hippocampal activity; to date, it appears that only one study has examined this question and showed that the estrous cycle did not have an effect on hippocampal theta in freely moving rats (Dewitt et al., 1977). However, it is possible that more advanced electrophysiological recording techniques (e.g., multichannel recordings) and analytical methods (e.g., current-source density analysis) could detect changes in hippocampal activity during the different stages of the estrous cycle. Also, future studies might want to correlate other behavioural anxiety assays (e.g., shock probe burying test, Vogel conflict test, light dark exploration test) with theta frequency to assess if there are correlations between these behavioural assays and hippocampal activity in order to further test the theta suppression model of anxiety.

Numerous studies have used urethane as anesthesia to study pharmacological manipulation of elicited theta (Chee et al., 2014; Horváth et al., 2015; McNaughton et al., 2007; Yeung et al., 2013; Yeung et al., 2016). However, it is important to acknowledge that urethane anesthesia could alter theta properties and perhaps obscure a potential link between behavioural data and theta frequency (McNaughton et al., 2007). In this experiment, type 2 theta activity was observed under urethane anesthesia. Type 2 theta is dependent on acetylcholine, which means that there could be differences between urethane anesthetized rats and freely moving rats, which exhibit both Type 1 and Type 2 theta during natural behaviours. Thus, future work could replicate the present experiments, but record theta in freely-behaving animals. It is worthwhile noting, however, that my finding did show a decrease in theta frequency following treatment with buspirone, which aligns with what has been commonly found in literature (McNaughton et al., 2007).
4.6 Conclusions

To conclude, my study contributes new information to our current understanding of sex differences in anxiety, theta activity, and the anxiety-theta association. The study demonstrates that there appears to be a sex difference in percentage open arm time and percentage of open arm entries in the elevated plus maze, with females being less anxious compared to their male counterpart. The first electrophysiology study confirms that the anxiolytic agent buspirone decreases hippocampal theta frequency elicited by stimulation of the brainstem reticular formation. These data are in agreement with prior work showing that a variety of anxiolytics decreases theta thus initially frequency, evidence that constitutes an important cornerstone of the theta suppression model of anxiety. There was no evidence of an obvious sex difference in theta frequency when comparing theta activity in male and female rats. Finally, there were no associations between open arm exploration on the elevated plus maze and the frequency of hippocampal theta activity, which challenges the theta suppression model. Thus, my study challenges the predictive validity of the theta suppression model by showing that behavioural levels of anxiety do not correlate with hippocampal theta activity. Future studies should correlate hippocampal theta activity with other behavioural measures of anxiety to assess whether this lack of correlation is a more general phenomenon that applies to a wide range of behavioural anxiety models.
References


