

Novel targets, biomarkers, and models in the neurobiology of anxiety

by

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## ABSTRACT

Despite considerable research progress towards characterizing the neurobiology of anxiety, the focus on putative behavioral markers and the absence of a common behavioural/neurobiological signature for the different classes of anxiolytic drugs have potentially slowed research into the neural mechanisms of anxiolytic drug action. As such, current research efforts are dedicated towards identifying neural indices that yield universal signatures across all anxiolytic compounds. This, in turn, may facilitate the development of therapeutics for clinical anxiety disorders.

The general purpose of this thesis is to identify and characterize putative compounds with anxiolytic potential and to critically evaluate an emerging neurophysiological model of anxiolytic drug action. Chapter 1 of this thesis will provide a systematic overview of current animal behavioral models of anxiety, summarize the role of several neurotransmitter systems in the biochemical basis of anxiety, describe the seminal work characterizing the neuroanatomical correlates of anxiety, characterize the role of hippocampal theta in the neurobiology of anxiety, and identify novel pharmacological targets. In chapter 2, the anxiolytic properties of somatostatin following intra-amygdala and intra-septal microinfusions and the receptor specificity of these effects will be summarized. Here, we found that a selective sst2 receptor antagonist, PRL2903 can reverse the anxiolytic effects of somatostatin in two well established behavioral models of anxiety, the elevated plus-maze and shock probe burying test. Chapter 3 will provide a critical assessment of the hippocampal theta suppression model of anxiolytic drug action. Here, we found that the bradycardic agent ZD7288 significantly suppresses reticularly activated theta frequency and produces corresponding anxiolytic effects in the elevated plus-maze. In chapter 4,

three well established anxiogenic agents FG7142, yohimbine, and  $\beta$ CCE will be used to assess the construct validity of the theta model, (i.e. to evaluate the functional role of theta frequency in anxiety *per se*). Here, we found that all three anxiogenic agents reliably produced anxiogenic-like effects in the elevated plus-maze but had no effects on theta frequency. Chapter 5 of this thesis will explore the regional and functional differences of the dorsal and ventral hippocampus in relation to the theta suppression model using histamine, an important neurotransmitter in the brain. Paradoxically, we found that histamine produced marked increases to theta frequency following ventral hippocampal microinfusions while eliciting robust anxiolytic effects in the elevated plus-maze. Finally, chapter 6 will summarize the major results and conclusions of these studies, describe their limitations and propose future directions for research on novel models and mechanisms in the neurobiology of anxiety.

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## **CHAPTER 1**

# **GENERAL INTRODUCTION: AN OVERVIEW OF CURRENT ANIMAL MODELS, NEUROCHEMICAL AND NEUROANATOMICAL CORRELATES, AND NOVEL PHARMACOLOGICAL TARGETS IN THE NEUROBIOLOGY OF ANXIETY**

## **1.) General Introduction**

“Emotion” refers to a state of feeling that arises spontaneously or in reaction towards a particular object. This conscious (and sometimes unconscious) mental state results in changes in physiological arousal, the modulation of the appraisal of a situation or stimuli, and a characteristic expression of a set of behavioral responses, is at once hard to describe, yet hard to ignore. The evolution of emotions as a byproduct of natural selection has been a topic of interest since Darwin’s 1872 book on the “The Expression of Emotions in Man and Animals.” With regards to this, fear is considered a universal and primal emotion which has evolved among our pre-mammalian ancestors and is associated with the most ancient parts of our brain. “Fear” refers to a set of emotional responses resulting from perceived immediate threats, while “anxiety” typically refers to a persistent, and potentially maladaptive/pathological expression of these emotional responses which extends beyond the existence of an immediate threat. As such, fear is typically characterized as being a ‘normal’ behavioral response, while anxiety may represent an abnormal expression of this emotion. Nevertheless, both states share a common set of behavioral and physiological responses arising from the perception of a harmful stimulus or its context (“A dog bit me in this house”). These visceral responses can be accompanied by a number of physiological responses such as heart rate acceleration, vasoconstriction, pupil dilation, piloerection, glucocorticoid release, as well as defensive behaviors such as fleeing, freezing or fighting, which are all adaptive and vital to the survival of all mammals. In view of this, both “fear” and “anxiety” might be viewed as an adaptive correlates of defensive behavior, essential for species survival and a by-product of natural selection, while their over-expression may extend these behaviors to levels that are harmful and disadvantageous to species survival. Accordingly, we should not be surprised to see evolutionary conservation of the neural

correlates, mechanisms and circuitry, associated with defense and anxiety across animal species. As such, homologous brain structures and physiological/neurochemical mechanisms should be expressed in many different mammals, from rats to humans (LeDoux, 2000). Research in the neurobiology of fear and anxiety has expanded rapidly in recent years and extends across many levels of analysis ranging from the molecular level in basic organisms to human cognitive neuroimaging tests.

Another important reason for conducting research on the neurobiology of fear and anxiety is the role of these emotional processes in a number of mental illnesses. Anxiety disorders in humans are defined as the abnormal expression of fear-related cognition and behavior, and are debilitating illnesses with widespread effects in many segments of the population including adolescents, women, and aging adults. Lifetime prevalence rates for anxiety disorders are estimated to be approximately 16.6% but have been reported in the range upwards of 25% depending on the population examined (Starcevic, 2006; Wittchen et al., 1994). The DSM-V recognizes many classes of anxiety disorders including generalized anxiety disorder, obsessive-compulsive disorders, phobias, panic disorders, and post-traumatic stress disorders (Wittchen et al., 2014). Additionally, many anxiety disorders have high levels of co-morbidity with other affective disorders such as depression.

While each anxiety disorder is characterized by a unique set of criteria, the diagnostic criteria for generalized anxiety disorder remains a benchmark standard given that it has seen very few changes since the DSM-IV (2000). Generalized anxiety disorder is characterized by excessive worry or anxiety that is difficult to control, causes significant distress in day to day functions and cannot be attributed to the physiological effects of a medication/substance or another medical condition/disorder. It is also associated with symptoms including restlessness, fatigue, difficulty

concentrating, anhedonia, irritability, muscle tension, and sleep disturbances. Given the wide array of diagnostic criteria associated with anxiety disorders, animal models are necessary to extend our knowledge about the biochemical basis and neuroanatomical circuitry mediating these symptoms. By furthering our understanding of the neural mechanisms of using ethologically valid animal models of anxiety, we may be able to facilitate the development of novel therapeutics for the clinical disorders of anxiety and related depression.

## **2.) Behavioral Animal Models of Anxiety**

The development of various animal models of anxiety has been driven historically by the widespread use of animal models in biomedical research (e.g., (Markou et al., 2009)). Like many medical models, the primary impetus for the development of an animal model of anxiety has been the identification or “screening” of potential therapeutic targets in the treatment of human anxiety disorders. Accordingly, a well-developed animal model of anxiety has representations of the symptomatology, etiology, and treatment of clinical anxiety. A second and closely related motivation is to use these same behavioral models in order to further our understanding of the neural mechanisms of anxiety. Within this domain, animal behavior models, lesions, and microinfusion studies have been fundamental to mapping out specific neurotransmitter pathways and brain regions involved in fear and anxiety.

Nevertheless, because of the phylogenetic distance between human subjects and animal subjects, methods for validating animal models of anxiety have been paramount. Furthermore, several types of validity need to be considered when evaluating the usefulness of any particular model. One important validation criterion involves establishing the predictive or correlational validity of an animal model. An animal model of anxiety that has predictive validity can



accurately detect the anxiolytic potential of a manipulation, whether it be a novel drug with no known effects on anxiety or another neurobiological intervention. Isomorphic and homologous validity of a model should also be considered. A model is isomorphic if the model elicits a similar behavioral response akin to those typically seen during human fear/anxiety (e.g. fleeing, freezing). A model is homologous if the underlying causes of anxiety are similar to those in humans (e.g., painful stimuli, and the parts of the peripheral and central nervous systems which enable the perception and memory of that pain). Finally, it is also important for a model to have construct validity. Construct validity essentially asks whether the “model” measures what it is purported to measure (e.g., does it index fear-reduction or anxiety, or does it reflect more general inhibitory processes?).

Because a variety of fearful stimuli can produce anxiety in humans, a number of different animal models have been developed to assay different kinds of anxiety or fear. Broadly, animal models of fear and anxiety are categorized according to whether they are unconditioned assays with ethological endpoints evaluating spontaneous or innate anxiety, or conditioned paradigms associated with an explicit learning component in relation to a fear/anxiety provoking stimulus. Unconditioned models indexing a natural state of anxiety typically introduce the organism to novel test environments (e.g. elevated plus-maze, light-dark box, social interaction test, and novelty induced suppression of feeding test). Conversely, conditioned models rely on learned cues associated with an aversive stimulus or situation (e.g. contextual fear conditioning, fear potentiated startle, shock-probe test).

The following sections of this introduction will revisit two widely used behavioral models of anxiety, the elevated plus maze (EPM) and shock probe burying test (SPB). Each of

these models has been extensively validated as a measure of anxiety, at the behavioral, physiological, and pharmacological levels (File et al., 1990; Pellow et al., 1985; Treit, 1985).

*2.1 The elevated plus-maze (EPM) test* is one of the most widely used animal models of unconditioned anxiety (Pellow et al., 1985; Walf and Frye, 2007). The EPM has four arms, arranged in the shape of a plus sign (+), with two opposing open and two opposing arms enclosed with walls. All arms have an open roof and are elevated by 40-70 cm above the floor (50 cm is the standard elevation). Rodents are allowed to explore freely for the duration of the test (typically 5 minutes). The model relies on a rodent's intrinsic aversion of open spaces (thigmotaxis), leading to a preference for the enclosed arms (Treit, 1993). When rats are confined to the open arms by blocking their access to the enclosed arms, they show increased plasma corticosterone, defecation, and freezing behavior (Walf and Frye, 2007). An anxiolytic-like behavioral profile is reflected by an increase in the proportion of open arm entries and time spent in the open arms of the maze. Clinically effective anxiolytic drugs (e.g. diazepam) increase both measures of open arm activity, while anxiogenic drugs such as Yohimbine, inverse GABA<sub>A</sub> agonists, and beta carbolines decrease the same measures (Cole et al., 1995; Johnston and File, 1989; Walf and Frye, 2007). The number of total arm entries and closed arm entries serve as indices of general activity and locomotion (for reviews, see (Hogg, 1996; Walf and Frye, 2007)).

*2.2 Shock-probe burying test (SPB)*. In this model, rats are shocked following contact with a stationary, electrified probe protruding into one of the four walls of a transparent Plexiglas chamber. Rats typically make contact with the probe within the first five minutes of the test. The test duration lasts for fifteen minutes following initial contact with the probe and rats can explore

the testing chamber freely. The chamber floor is covered with bedding material, which the rat can use to spray and cover aversive stimuli such as the electrified shock-probe (Treit and Fundytus, 1988; Treit et al., 1981). Rats use their forepaws to rapidly push bedding material towards the shock probe, which is commonly characterized as “burying behavior” (Treit and Fundytus, 1988; Treit et al., 1981). Rats shocked from the probe also show increased plasma corticosteroids and adrenaline, and carefully avoid further contact with the probe while they bury it. A decrease in the duration of probe-burying, in the absence of a reduction of general activity, is the primary measure of anxiolysis (Treit, 1985; Treit et al., 1981). Additionally, significant increases in shock contacts with the electrified probe serve as a secondary measure of anxiety-reduction. These fear responses, both physiological and behavioral, can be suppressed by standard anxiolytic drugs (e.g., diazepam) and enhanced with anxiogenic drugs (e.g., Yohimbine) (Treit, 1985; Treit et al., 1981). Resting quietly on the chamber floor is taken as an inverse measure of general activity (for review, see (Treit, 1985)).

The next section outlines the involvement of several neurotransmitter systems involved in the biochemical basis of anxiety.

### **3.) Biochemical Basis of Anxiety**

#### *3.1 Benzodiazepines and GABA*

Several neurotransmitter systems have been well documented in the biochemical basis of anxiety. The role of  $\gamma$ -aminobutyric acid (GABA), the major inhibitory neurotransmitter in the mammalian brain has been at the forefront of research in this area. Within this domain, the anxiolytic action of benzodiazepines (a class of psychoactive drugs targeting the GABA<sub>A</sub> receptor, e.g., diazepam) and the involvement of GABAergic mechanisms have received

considerable attention. The benzodiazepine site on the GABA receptor was discovered in 1977 (Nutt and Malizia, 2001) and there is substantial genetic, pharmacological, and clinical evidence to suggest that its modulatory role on GABAergic neurotransmission is central to its anxiolytic action (Nutt and Malizia, 2001). Benzodiazepines have a strong affinity for their receptor site and the clinical potency of a benzodiazepine has been associated with increased affinity levels (Nutt and Malizia, 2001). Structurally, the GABA<sub>A</sub> receptor, in conjunction with the chloride ion channel, forms a large pentameric complex typically composed of two  $\alpha$ , two  $\beta$ , and one  $\gamma$  subunit by which neuronal inhibition is produced. The benzodiazepine binding site is located between the  $\alpha$  and  $\gamma$  of the GABA<sub>A</sub> receptor. Benzodiazepines are positive allosteric modulators of the GABA<sub>A</sub> receptor and lower the concentration of GABA required for chloride conductance, thereby potentiating chloride flux and producing a larger inhibitory effect. Chloride levels also provide negative feedback to this complex by regulating GABA and benzodiazepine binding. This reciprocal regulation is the critical key to the anxiolytic action of benzodiazepines (Nutt and Malizia, 2001).

### *3.2 Serotonin (5-HT)*

The role of serotonin (5-Hydroxytryptamine (5-HT) in fear and anxiety has also received much attention. This has been driven largely by the clinical efficacy of serotonin modulators in the treatment of anxiety disorders i.e., selective serotonin reuptake inhibitors (SSRIs, e.g., fluoxetine). Nevertheless, anxiolytic drugs targeting serotonergic mechanisms are often pharmacologically non-selective, making it difficult to separate the effects on serotonin from effects on other neurotransmitters. With regards to this, buspirone, a selective 5HT-1<sub>A</sub> agonist that is particularly effective in the treatment of generalized anxiety disorders, has been a useful

tool of study. Buspirone, in conjunction with its  $G_i$  protein complex, inhibits secondary messenger system adenylyl cyclase, thereby facilitating hyperpolarization through increased outward potassium conductance. Conversely, the activation of 5-HT<sub>2</sub> and 5-HT<sub>3</sub> receptors has been linked to anxiogenesis and increased inward potassium conductance (Murphy, 2000) While the GABA-benzodiazepine receptor complex has been associated with the control of anxiety and anxiolytic mechanisms, serotonergic mechanisms have been proposed to regulate defense and coping mechanisms in response to fear. This theory suggests that 5-HT projections from the dorsal raphe nucleus regulate the avoidance of threats and mediate behavioral inhibition during anticipatory environmental threats (Deakin, 1998).

### *3.3 Noradrenaline and other neurotransmitter systems*

Finally, noradrenergic mechanisms, particularly those in the locus coeruleus, have long been implicated in the neural mechanisms of anxiety. The locus coeruleus has widespread inputs and interacts with a number of neurotransmitter systems including GABA, corticotrophin releasing hormone (CRH), and 5-HT. Fear evoking stimuli increase the firing of cells in the locus coeruleus, thereby increasing the turnover of norepinephrine, which can be inhibited by anxiolytic agents (Bremner et al., 1996). Additionally, physiological responses to fear such as piloerection, are also associated with increased firing of the locus coeruleus.

Taken together, the biochemical basis of anxiety involves highly complex interactions of multiregulatory neurotransmitter systems and requires further exploration. A definitive examination would include a summary of the neuroanatomical circuitry involved in the expression of fear responses and their maintenance.

#### **4.) Neuroanatomical correlates of anxiety**

A number of different neural structures and neurotransmitter pathways are involved in regulating a variety of behavioral and physiological responses that are associated with fear and anxiety. Specifically, bidirectional innervation between the periaqueductal grey, raphe nuclei, locus coeruleus, hypothalamus and limbic system structures (such as the amygdala, septum and hippocampus) play important regulatory roles. For example, the periaqueductal grey (PAG) has a regulatory role in heart rate, respiration, and freezing behaviors (Keay and Bandler, 2001). Additionally, the reticularis pontis oralis and ventral tegmental area have been implicated in the regulation of arousal, attention, and vigilance. (Lang et al., 2000). Finally, the raphe nuclei and the locus coeruleus have long been implicated in fear and anxiety (Lechin et al., 2006). The raphe nuclei are a main source of serotonergic output to the forebrain, while the locus coeruleus sends widespread noradrenergic innervation to the PAG, hypothalamus, and limbic system structures including the amygdala and septum (Vertes, 1991; Vertes et al., 1999). Within the diencephalon, the hypothalamus also plays a critical role in fear responses by mediating the release of stress related hormones (i.e. corticosteroids) through the hypothalamic-pituitary-adrenal (HPA) axis (Mislin, 2003). While these brainstem structures play an important role in generating many of the behavioral and physiological responses related to fear and anxiety, there is a plethora of evidence indicating that “higher level” forebrain structures are critical to the integration, organization and expression of an emotional experience.

Historically, the so-called limbic system (including the hypothalamus, cingulate and parahippocampal gyrus, mammillary bodies, fornix, hippocampus, amygdala, and septum) have been at the forefront of research on the “emotional brain” since Paul MacLean conceptualized it

as part of the triune brain including the reptilian complex (basal ganglia) and neocortex. The following sections of the thesis will focus on three limbic system structures (the amygdala, the septum, and the hippocampus), which are critical to the neural mediation of fear and anxiety.

#### *4.1 Amygdala*

The seminal work by Kluver and Bucy (1937) on rhesus monkeys set the framework for the role of sub-cortical temporal lobe structures and their involvement in fear and anxiety. Following recovery from bilateral lesions of the temporal lobes, Kluver and Bucy noted that they displayed a peculiar pattern of behaviors. The monkeys seemed “blind” to the biological significance of a wide range of stimuli, many of which they previously avoided (e.g., humans, snakes). “Inappropriate” or indiscriminant behavior that emerged after temporal lobe lesions included trying to eat inedible objects such as rocks, or to copulate with other species (Kluver and Bucy, 1937). More importantly, lesioned primates appeared to be extremely tame throughout their explorations and instead of running away and hiding from the experimenter, they approached him, made physical contact with him, and even tolerated being stroked and picked up. Although Kluver and Bucy at first conceptualized these bizarre behaviors in terms of “psychic blindness,” or a kind of “agnosia,” in their later writing (Kluver and Bucy, 1939) they acknowledged that the much of the “syndrome” could be also explained in terms of “emotional indifference” or a complete absence of normal fear reactions.

While these early studies stimulated and focused later research on the specific role of the amygdala in anxiety and fear (e.g., LeDoux, 2000), Paul MacLean argued for the central role of the hippocampus in emotional processing (MacLean, 1949). Part of the impetus for this view was MacLean’s observations of the behaviors of patients with temporal lobe epilepsy. These patients

showed severe emotional and psychological disturbances prior to and between seizures, possibly due to the abnormal activity of the hippocampus. MacLean was also aware of the importance of the hippocampus in previous theories of the neuroanatomical bases of emotion, particularly the Papez neural circuit (1937) of emotional expression. This proposed circuit included connections between the sensory cortex, cingulate cortex, thalamus, hypothalamus, and hippocampus. Sensory messages from stimuli were directed to the thalamus and then processed accordingly by the cortex (stream of thinking) or hypothalamus (stream of feeling). The cingulate cortex integrated this information from the sensory cortex and hypothalamus and directed this output to the hippocampus to generate an emotional experience. Consistent with the psychodynamic theories of the time, MacLean proposed that emotional disorders occurred as a result of impaired integration of the “visceral brain” (e.g., subcortical structures) with the “word brain” (neocortex).

Modern research has supported and refined these early conceptualizations of the “emotional brain.” For example, many studies have shown that amygdala lesions reduce both fear behaviors in rats, such as the freezing in response to signaled foot-shock, as well as concomitant increases in sympathetic nervous system outflow (Hitchcock and Davis, 1991; LeDoux et al., 1990; Sananes and Campbell, 1989). The results of these studies, however, have often been influenced more by the method of lesion induction (e.g., electrolytic versus excitotoxic), and the size of the lesion, than the exact site of the lesion. For example, when the amygdala lesions are relatively small, or unilateral, anxiolytic effects rarely occur in standard tests (Riolobos and Martin Garcia, 1987). Similarly, when the serotonergic innervation of the amygdala is eliminated with a neurotoxin such as 5,7 dihydroxytryptamine, anxiolytic effects fail to occur in the elevated plus maze, whereas direct amygdala lesions with an excitotoxin like N-Methyl-D-Aspartate (NMDA) produce fairly reliable anxiolytic effects (Sommer et al., 2001;



Strauss et al., 2003). The limitations and vagaries of standard lesioning techniques can be contrasted with the effects of direct microinfusions of GABA<sub>A</sub> receptor agonists and NMDA receptor antagonists into the amygdala. Despite variations in dose and cannulae placements, these microinfusions result in clear and consistent anxiolytic effects in a variety of animal models of anxiety (for a review see (Engin and Treit, 2008b)). Specific targeting of receptor subtypes by reversible and selective receptor ligands eliminates many of the interpretational problems posed by permanent lesions. Permanent lesions broadly disrupt neuronal integrity of the brain and over time induce compensatory changes such as edema, microglial proliferation and neuronal reorganization, all of which confound the interpretation of the behavioral findings.

For the most part, intra-amygdala microinfusion of benzodiazepines produce anxiolytic effects in a number of rat anxiety models, presumably through positive allosteric modulation of the GABA<sub>A</sub> receptor, which in turn increases neural inhibition through increasing the frequency of chloride channel openings. These general findings have been corroborated using different benzodiazepine receptor agonists, such midazolam, (Green and Vale, 1992; Zangrossi and File, 1994) and direct GABA<sub>A</sub> agonists such as muscimol (Moreira et al., 2007). More complex findings show that microinfusions of midazolam into the central nucleus of the amygdala decreased passive avoidance of the electrified probe in the shock probe burying test, but not open-arm avoidance in the plus-maze, whereas microinfusions of midazolam into the basolateral amygdala decreased open arm avoidance in the plus-maze but had no significant effects on passive avoidance of the electrified shock-probe in the burying test (Pesold and Treit, 1994; Pesold and Treit, 1995). The pharmacological specificity of these site-by-response dissociations were confirmed by co-infusions of the benzodiazepine receptor antagonist flumazenil, which reversed both of these behaviorally selective anxiolytic effects. Finally, recent studies infusing

neuropeptides into the amygdala (i.e. somatostatin) have yielded promising results for new potential therapeutic targets, although further exploration is needed (Yeung et al., 2011; Yeung and Treit, 2012).

Overall, it seems that much of the primary focus of recent research on the neurobiology of fear and anxiety has been centered on the amygdala, often presuming that this region is central to the neural mediation of fear-related responses. However, there is evidence that suggests that the amygdala may not be critical to mediating all aspects of behavioral and physiological functions related to fear and anxiety. For example, some studies have shown that lesions of the amygdala do not significantly affect levels of open arm activity in the elevated plus-maze (Sommer et al., 2001; Treit and Menard, 1997). Other studies have shown that amygdala lesions have no effect on specific unconditioned fear responses such as the duration of burying behavior in the shock-probe test (Kopchia et al., 1992; Treit and Menard, 1997; Treit et al., 1993). Thus, other limbic system structures such as the hippocampus and the septum may also play crucial roles in the regulation of fear and anxiety.

#### *4.2 Septum*

Gray (1982) provided early evidence that the septum and hippocampus play important roles in anxiety by showing similarities in the behavioral effects of septo-hippocampal lesions and anxiolytic drugs, in a battery of behavioral tests. For the most part, however, Gray relied on traditional behavioral models of learning and memory in rats, which could have little to do with anxiety and more to do with learning and memory. Nevertheless, some of the commonalities he found in aversive learning paradigms (e.g., active avoidance) provide indirect evidence for the idea that the septo-hippocampal system is involved in anxiety.

There are, however, a number of specific shortcomings in the evidence Gray uses to support his “neuropsychological” theory of anxiety. For example, “septal rage” and hyper-reactivity is often observed in rats during the first days after septal lesions (Albert and Chew, 1980). It is not clear whether Gray took into consideration the confounding effects of “septal rage” in behavioral experiments, or even whether it was quantified. Septal rage or hyper-reactivity can be easily eliminated by systematically handling rats before behavioral testing (Gotsick and Marshall, 1972), but the effects of septal lesions on food- motivated learning (e.g., punishment, conditioned suppression) are more problematic (Donovick et al., 1970). Changes in food motivation could easily confound the interpretation of behavioral changes that occur in these paradigms after septal lesions or anxiolytic drugs (for details see (Treit, 1985)). Whether or not food motivation is a confounding factor, in every behavior paradigm that Gray used to support his theory, it is difficult (if not impossible) to separate the effects of septal lesions on learning and memory from their effects on anxiety. At the same time, these limitations underline the advantages of modern models such as the plus-maze or shock probe test, which do involve food motivation or acquisition of a learned response.

Indeed, subsequent research has shown that bilateral electrolytic or excitotoxic lesions of the septum produce robust anxiolytic effects in the elevated plus maze and in the shock probe test, in the absence of hyper-reactivity (e.g., (Treit and Pesold, 1990)). More importantly, intra-septal microinfusions of GABA<sub>A</sub> agonists, which do not have the disadvantages of permanent lesions (see above), produced the same pattern of effects, increasing open arm exploration in the elevated plus maze and decreasing burying in the shock probe test. The receptor specificity of these effects was confirmed by co-infusion of flumazenil, a benzodiazepine receptor antagonist that reversed the behavioral effects of the agonists (Pesold and Treit, 1994; Pesold and Treit,

1996). These results have been replicated a number of times, with both direct (e.g., muscimol; (Degroot et al., 2001)) and indirect agonists (e.g., midazolam; (Pesold and Treit, 1996)), and seem to occur when either the medial septum or the lateral septum are targeted. It should be pointed out, however, that the medial and lateral divisions of the septum co-exist in a globular area that is approximately 2 millimeters in diameter at its equatorial plane (see Paxinos and Watson Atlas plates #13-18), so that even small volumes (1  $\mu$ l) of lipid soluble drugs (e.g., midazolam) could diffuse at least a millimeter or more away from their initial site of infusion (Pesold and Treit, 1996). Thus, it is quite possible that the behavioural effects of agents infused into either the medial *or* the lateral septum could ultimately be due to actions at nearby structures.

The effect of neuropeptides on anxiety is generally variable, but some of the results found in the septum are relatively straight forward. For example, neuropeptide Y produced anxiolytic effects in the elevated plus-maze and social interaction tests, while NPY antagonists were without effect. Similarly, galanin (the endogenous agonist) produced anxiolysis in the shock probe burying test, while a galanin receptor antagonist did not (Echevarria et al., 2005; Kask et al., 2001). Additionally, corticotropic releasing factor agonists produced potent anxiogenic effects in the elevated plus maze and social interaction tests after intra-septal microinfusion. The receptor specificity of these results, however, is still under investigation (Tezval et al., 2004). Septal microinfusions of the CRF2 receptor agonist urocortin significantly increased anxiety related behaviours in the light and dark box, an effect reversed by a selective CRF2 antagonist (but see (Henry et al., 2006)). Finally, recent studies have suggested a role for somatostatin in anxiety. These studies demonstrated that septal infusions of both biologically active isoforms

produced robust anxiolytic effects in the elevated plus maze and shock probe burying paradigms (Yeung et al., 2011; Yeung and Treit, 2012).

### *4.3 Hippocampus*

Although Papez (1937) and MacLean (1949) saw the hippocampus as a critical center of emotional experience, later researchers such as Brenda Milner provided evidence showing the hippocampus as primarily involved in explicit memory (Milner, 1972). At around the same time, experimental evidence for the involvement of the hippocampus in spatial memory was also emerging (Morris et al., 1982). And by the end of the 20<sup>th</sup> century, the role of the hippocampus in cognitive (mostly mnemonic) functions was the dominant focus of neuroscientific interest.

Nevertheless, a number of studies have found that lesions of the hippocampus, like lesions of the septum, produce clear anxiolytic effects (Pesold and Treit, 1992; Menard and Treit, 1996). Pharmacological inhibition of hippocampal function through microinfusion of GABA<sub>A</sub> agonists, GABA reuptake inhibitors, and Na<sup>+</sup> channel blockers also produces anxiolytic effects in the elevated plus maze, shock probe burying test, and social interaction test (Bannerman et al., 2002; Degroot and Treit, 2004; McEown and Treit, 2009; Menard and Treit, 2001). Bilateral intra-hippocampal infusions of CCK-8 produced robust anxiogenic effects in the elevated plus maze while a selective CCK2 receptor antagonist reversed these effects (Rezayat et al., 2005). Additionally, it has been shown that NPY infusions into the hippocampus produced anxiolysis in the elevated plus maze, while both NPY1 and NPY2 receptor antagonists reversed these effects (Smialowska et al., 2007). More recently, it has also been demonstrated that intra-hippocampal microinfusion of vasopressin receptor antagonists produce anxiolytic effects that vary as a function of both sub-region (dorsal or ventral hippocampus) and receptor sub-type (v1b vs. v1a)

(Engin and Treit, 2008a). Additionally, Engin et al. (2009) found that intra-ventricular microinfusion of somatostatin produced robust anxiolytic effects in the elevated plus-maze, while Yeung et al., (2011) found that both isoforms of somatostatin (SST 14 and SST 28) were anxiolytic in the amygdala and in the septum, in both the elevated plus-maze and the shock-probe burying tests. Finally, many of these behavioral results were corroborated in a new, electrophysiological model of anxiety, in which suppression of hippocampal theta is the index of anxiety-reduction. McNaughton's theta suppression model will be discussed in further detail in the next part of this chapter reviewing well established behavioral models of anxiety and discussing novel models of the neurobiology of anxiety.

## **5.) A novel neurophysiological model: the role of hippocampal theta in anxiety research**

### *5.1 Hippocampal Theta Rhythm*

The hippocampal theta rhythm is a large-amplitude, near-sinusoidal rhythm defined by its fundamental peak frequency ranging from 4 Hz to 14 Hz. Hippocampal theta has been suggested to play a number of roles in a variety of behavioural, cognitive and physiological functions, including voluntary movement (Skaggs et al., 1996; Vanderwolf, 1969), sensorimotor integration (Bland and Oddie, 2001), cognition and memory (Cohen and Eichenbaum, 1991; Hasselmo, 2005; Hasselmo, 2009; O'Keefe and Conway, 1978), spatial navigation (O'Keefe, 1999) and affective responses. (Engin et al., 2008; Gray and McNaughton, 2000; McNaughton et al., 2007; Yeung et al., 2012).

Two types of theta (Type 1 and Type 2) have been characterized according to their distinct pharmacological and behavioral properties. Type 1 theta resistant to antagonists of the

muscarinic acetylcholinergic receptor, has a higher frequency than type 2 theta, and occurs during locomotion. Conversely, Type 2 theta is “atropine sensitive”, has a frequency between 4-7 Hz, and occurs during immobility, REM sleep, and under urethane anaesthesia (Kramis et al., 1975; Sainsbury et al., 1987a; Sainsbury et al., 1987b). During voluntary behavior, both subtypes are thought to be co-active (Vanderwolf, 1988; Bland, 1986).

The medial septum and the diagonal band of Broca, (MSDB) play a critical role in theta generation, oscillation and synchrony and send cholinergic, glutamatergic and GABAergic projections directly to the hippocampus (Amaral and Kurz, 1985; Bland et al., 1999; Vertes and Kocsis, 1997). The majority of septohippocampal neurons are cholinergic and ipsilaterally innervate the CA1 pyramidal cells and granule cell layers of the dentate gyrus in the hippocampal formation via the fimbria, fornix, and supracallosal striae (Dutar et al., 1995). The septohippocampal GABAergic projections primarily innervate GABAergic interneurons in the hippocampal formation, which are involved in the feedback inhibition of principal cells in the hippocampus (Andersen, 1980). Finally, dense glutamatergic projections from the medial septum to the hippocampal formation suggest that they may play an important role in theta generation, though these mechanisms may be independent of cholinergic/GABAergic projections (Colom et al., 2005). A dynamic balance between cholinergic, glutamatergic, and GABAergic mechanisms is essential to the generation of theta oscillations (Colom et al., 1991; Smythe et al., 1991). Cholinergic afferents provide excitatory drive to the hippocampal cells, while GABAergic projections attenuate GABAergic mechanisms by inhibiting the activity of hippocampal GABA neurons. This interactive mechanism of theta generation is substantiated by pharmacological evidence using carbachol, a cholinergic agonist, and bicuculline, a GABA<sub>A</sub> receptor antagonist (Colom et al., 1991; Smythe et al., 1991). While it has been shown that the isolated hippocampus

can intrinsically generate sustained theta for up to 120 minutes (Goutagny et al., 2009), in the septally deafferented hippocampus and *in vitro* cell preparations, co-infusions of carbachol and bicuculline can produce theta oscillations (Colom et al., 1991; Konopacki and Golebiewski, 1993). Moreover, these effects were suppressed by atropine sulphate, a muscarinic antagonist, and muscimol, a GABA<sub>A</sub> receptor agonist (Golebiewski et al., 1996).

### *5.2 Theta Suppression Model*

The work on the hippocampal theta model of anxiety was first initiated in experiments by McNaughton and Sedgwick (1978). Here, it was suggested that the suppression of evoked hippocampal theta rhythm through reticularly activated stimulation is a predictive neurophysiological index of anxiolytic drug action (Gray and McNaughton, 2000; McNaughton et al., 2007; McNaughton and Sedgwick, 1978). There is a plethora of pharmacological evidence suggesting that reductions in the frequency of the theta rhythm occur only after the administration of clinically effective anxiolytic drugs (e.g. benzodiazepines, 5HT<sub>1A</sub> agonists, SSRIs) (McNaughton et al., 2007). Furthermore, other drugs that do not have therapeutic use as anxiolytics (e.g antipsychotics, anticonvulsants, or precognitive drugs), do not have this effect on hippocampal theta rhythm.

Given the involvement of GABAergic mechanisms in the generation, pacing and maintenance of hippocampal frequency, it is not surprising that a benzodiazepine such as diazepam, a well established GABA<sub>A</sub> receptor agonist would have potent effects on theta frequency suppression. Surprisingly, anxiolytic drugs with no associated GABAergic mechanisms such as 5HT<sub>1A</sub> agonist Buspirone, and SSRI fluoxetine, also produced significant suppressions to theta frequency (reference). In view of this, delineating the similarities and



differences associated with theta suppression by all classes of anxiolytic drugs may be a useful biomarker and tool for studying the neurobiology of anxiety.

There has been evidence showing that agents with no prior anxiolytic profile could also depress the frequency of hippocampal theta. For example, a few studies have found that microinfusions of the cyclic neuropeptide somatostatin, and a ligand for the SSTR2 receptor not only depressed theta, but had robust anxiolytic and anti-depressant effects in the elevated plus maze and the forced swim tests (Engin et al., 2008; Engin and Treit, 2009). There has also been mounting evidence for the anxiolytic profile of anti-epileptic drugs in reducing the frequency of evoked theta (e.g. Pregabalin, see (Siok et al., 2009)). Recently, we tested the predictive validity of the McNaughton et al (2007) model. To do this, we selected two neuroactive compounds (phenytoin, and ZD7288) that we presumed would have a suppressive effect on theta but that were not known to have anxiolytic properties. The suppression of theta frequency by these drugs along with corresponding findings of anxiolysis in the elevated plus-maze would strengthen the predictive validity of this model. However, a false positive where only theta suppression occurred, or a false negative with anxiolysis in the elevated-plus maze in the absence of theta suppression would provide evidence against theta suppression as a biomarker of anxiolytic drug action..

We were also interested in examining the construct validity of this model system. In other words, we asked whether the direction of theta frequency modulation (upwards or downwards) could be used as an absolute measure of behavioral anxiety. We approached this problem by using three behaviorally confirmed anxiogenic (i.e., anxiety enhancing) agents: FG7142 and  $\beta$ CCE, which are partial inverse agonists at the benzodiazepine site on the GABA<sub>A</sub> receptor (Evans and Lowry, 2007; Taguchi and Kuriyama, 1990), and yohimbine, an antagonist at pre-

synaptic  $\alpha 2$  adrenergic receptor sites (Millan et al., 2000). We postulated that if anxiolytic drugs such as diazepam *decrease* hippocampal theta, then anxiogenic agents should *increase* hippocampal theta.

One important impediment for assessing the relationship between fear behavior and hippocampal theta oscillatory changes is that most of the experimental tests of the neural model have been established using systemic (i.p.) drug administration (McNaughton et al., 2007; Siok et al., 2009; Yeung et al., 2012), or central (intracranial) drug infusions into structures other than the hippocampus itself (e.g. lateral septum-(Chee et al., 2014)). In addition, brainstem-evoked theta frequency is almost exclusively measured in the dorsal hippocampus. However, multiple lines of evidence including selective lesion and local drug infusion studies have shown that the ventral hippocampus is importantly involved in the modulation of anxiety-related behaviors (Bannerman et al., 2004; Fanselow and Dong, 2010; McEown and Treit, 2009; McEown and Treit, 2010). To address these limitations, we assessed the effects of intra-hippocampal infusions of histamine in rats, while recording theta from both the dorsal and ventral hippocampus. The effects of these intra-hippocampal infusions on behavioral anxiety were also assessed in the elevated plus-maze.

## **6.) Novel pharmacological targets in anxiety research**

### *6.1 Somatostatin*

Somatostatin (SST) is a cyclic polypeptide, which exists in two biologically active isoforms: somatostatin 14 (SST 14) and somatostatin 28 (SST 28). SST 28 is synthesized from

precursor prosomatostatin, whereas SST 14 is synthesized from either prosomatostatin or by proteolytic conversion of SST 28. In the periphery, SST 14 and SST 28 inhibit the release of peptide hormones, including growth hormone (GH) from the pituitary, insulin and glucagon from the pancreas, and cholecystinin from the gastrointestinal tract (Moller et al., 2003). In addition to the paracrine functions of somatostatin in the peripheral tissues, numerous studies have shown that both SST 14 and SST 28 are distributed throughout the central nervous system, where they act as both neurotransmitters and neuromodulators (Cervia and Bagnoli, 2007; Moller et al., 2003; Selmer et al., 2000). To date, five different G-protein linked somatostatin receptors (sst1-sst5) have been cloned, all of which are expressed in the brain. It is these subtypes that presumably mediate the biological effects of SST 14 and SST 28 (Cervia and Bagnoli, 2007; Moller et al., 2003). At a behavioural level, SST 14 and SST 28 have been implicated in locomotion (Semenova et al., 2010), analgesia (Williams et al., 1987), epilepsy (Vecsei and Widerlov, 1990), spatial memory (Dutar et al., 2002), and emotion (Engin et al., 2008). Specifically, Engin et al. found that intracerebroventricular (ICV) microinfusions of either SST 14 or a selective sst2 agonist produced significant reductions in rat 'anxiety' in the elevated plus-maze (Engin et al., 2008; Engin and Treit, 2009). In contrast, no significant changes in affective behavior were found following microinfusions of selective agonists of sst1, 3, 4, and 5 (Engin and Treit, 2009). ICV SST 14 also suppressed hippocampal theta activity, an effect common to all known anxiolytic drugs (e.g., benzodiazepines, 5-HT<sub>1A</sub> agonists, and SSRIs; (McNaughton et al., 2007)). Additionally, it has also been well documented that somatostatin in some amygdalar neurons is both co-localized and co-released with GABA, an inhibitory neurotransmitter which has been long implicated in anxiety (Saha et al., 2002). GABA, through the GABA<sub>A</sub> receptor and an allosteric binding site for benzodiazepines, modulates both

experimental anxiety in animals and clinical anxiety in humans (Treit et al., 2010). Furthermore, the SST receptor gene expression in the amygdala is increased by predatory stress (Nanda et al., 2008). Finally, the septum also has high levels of both somatostatin and GABA, and has been independently implicated in anxiety (Degroot and Treit, 2004; Pesold and Treit, 1994; Pesold and Treit, 1996; Shin et al., 2009). In view of this, the diverse pharmacological and functional profile of somatostatin making it a promising target in the study of anxiolytic mechanisms.

## *6.2 Histamine*

The neural histaminergic system originates exclusively from hypothalamic tuberomammillary neurons and has diverse bidirectional connections throughout the brain, with projections to limbic system structures involved in emotional regulation, including the septal nuclei, amygdala, thalamus and hippocampus (Haas and Panula, 2003; Haas, 1981; Haas et al., 2008). Histamine has four G-protein coupled receptors (H1R-H4R) with distinct distribution densities throughout the brain and periphery (H4 receptors). H1 and H2 receptors are primarily located post-synaptically and have excitatory actions on neuronal membranes. Conversely, H3 receptors function as autoreceptors, regulating the release of histamine, and also as heteroreceptors inhibiting the release of various neurotransmitters including GABA, dopamine, serotonin and acetylcholine (Benarroch, 2010; Brown et al., 2001; Haas and Panula, 2003; Haas, 1981; Haas et al., 2008). Within the dorsal and ventral regions of the hippocampus, H3Rs show strong expression in the pyramidal cell layers of CA1 and CA3 suggesting that they may play an important modulatory role in the regulation of behavioral functions and the regulation of theta oscillatory activity (Pillot et al., 2002). Functionally, several behavioral studies using two well established rodent models of anxiety, the elevated plus-maze and novelty-induced suppression of

feeding test have shown that local infusions of histamine into the lateral septum and dorsal hippocampus have robust anxiolytic effects (Chee and Menard, 2013; Zarrindast et al., 2006). Conversely, ventral hippocampal and intra-amygdalar infusions of histamine have been reported to promote anxiety-like behavior (Rostami et al., 2006; Zarrindast et al., 2006). There is also evidence suggesting a dissociation for the functional role of H1 and H2 receptors vs. H3 receptors. H1 and H2 receptors modulate feeding behaviors (Chee and Menard, 2013), different aspects of memory including object recognition (da Silveira et al., 2013) and conditioned place preference (Zlomuzica et al., 2008), while H3 receptors may play an important role in mediating fear memory consolidation (da Silva et al., 2006) and unconditioned anxiety behaviors (Chee and Menard, 2013). This is consistent with immunolabelling studies which have shown varying densities of histaminergic fibers within the hippocampus and amygdala and differences in the functional regional distribution of histamine receptors along the septotemporal axis (Alvarez and Banzan, 1985; Alvarez and Banzan, 2001; Haas et al., 2008). The robust associations between the pharmacological profile, neuroanatomical distribution, and functional roles of histamine and HRs throughout the CNS make it an attractive target for studying behavioral anxiety.

## **7.) Thesis outline and objectives**

The general purpose of this thesis is to identify novel biomarkers, models and mechanisms in the neurobiology of anxiety. While classical behavioural studies have contributed considerably to our knowledge of the neurobiology of anxiety through lesion by behavior associations and classical agonist/antagonist studies, the absence of a common behavioral signature has hampered research within this domain. At the molecular and anatomical levels, multiple neurotransmitter systems and brain areas are involved in modulating the

neurobiology of anxiety. With regards to this, these systems can work in a synchronous, hierarchical and/or opposing fashion to mediate the physiological and behavioral responses associated with fear and anxiety. The studies in this thesis use behavioral and neurophysiological models to systematically evaluate the possibility that theta suppression is the common neural index, which consolidates the molecular, neuroanatomical, and behavioral correlates associated with a diverse class of anxiety disorders. Establishing a common neural signature for the index of anxiolytic potential would advance our understanding of the neural mechanisms of anxiety and would be particularly useful to identifying putative anxiolytic compounds. In this thesis, I describe and critique Neil McNaughton's hippocampal theta suppression model of anxiolytic drug action. I also assess the predictive validity of this model using a number of novel compounds. Additionally, I evaluate the construct validity of this model using well established anxiogenic agents. Finally, I delineate the neuroanatomical correlates of this model with a focus on the dorsal vs. ventral hippocampal functional dichotomy in relation to behavioral anxiety.

The first study investigates the possibility of somatostatin as a novel therapeutic target. We have previously demonstrated that both biologically active isoforms of somatostatin produced robust anxiolytic effects at the level of both the septum and amygdala (Yeung et al., 2011). Additionally, our laboratory has also shown that intra-cerebral infusions of a highly specific sst2 receptor agonist elicited anxiolytic and antidepressant properties (Engin and Treit, 2009). Nevertheless, the receptor specificity of these effects have not yet been confirmed with an antagonist. Additionally, the neuroanatomical specificity of these effects in the amygdala and septum, have not been clearly established. This classical agonist/antagonist interaction study characterizes the receptor specificity of the anxiolytic effects of somatostatin in the septum and amygdala using a highly selective sst2 receptor antagonist PRL2903. If the anxiolytic effects of

SST are predominantly mediated by the sst2 receptor, PRL2903 should reverse these effects. Conversely, an absence of anxiolytic antagonism would indicate that the sst2 receptor subtype may not be involved in modulating the anxiolytic properties of somatostatin.

The second set of studies in my thesis assesses the possibility that theta frequency suppression is an index for anxiolytic drug potential. Thus far, there is substantial pharmacological evidence to indicate that all clinically effective anxiolytic drugs suppress theta frequency (McNaughton et al., 2007). However, the predictive validity of this model still requires further investigation. If the suppression of theta frequency is indeed an index of anxiolytic drug action, then drugs that suppress theta should also show corresponding anxiolytic effects in behavioral models. Conversely, theta suppression in the absence of anxiolytic effects in behavioral models would be evidence against the predictive validity of the model.

Here, we test ZD7288, a bradycardic agent and hyperpolarization-activated inward current ( $I_h$ ) channel blocker, in both the theta suppression model and a well-established rodent model of anxiety, the elevated plus-maze. Given that ZD7288 has been shown to alter hippocampal theta oscillatory properties but has never been clinically used in the treatment of anxiety, converging evidence across our electrophysiological and behavioral tests would provide powerful support for the predictive validity of the theta suppression model.

My third study evaluates the construct validity of the theta suppression model using three well established anxiogenic agents FG7142, yohimbine, and  $\beta$ CCE. If the model is a true index of anxiety and not only anxiolytic drug action, then anxiogenic drugs should increase theta frequency while increasing behavioral anxiety in the elevated plus maze. Anxiogenic effects in

the absence of a theta frequency increase would suggest that the model lacks construct validity and is not a true assay of behavioral anxiety.

The final study in my thesis evaluates the neuroanatomical and functional differences of the dorsal and ventral hippocampus in relation to the theta suppression model. Recently, multiple lines of evidence including selective lesion and local drug infusion studies have shown that the ventral hippocampus is involved in the modulation of anxiety-related behaviors (Bannerman et al., 2004; Fanselow and Dong, 2010; McEown and Treit, 2009; McEown and Treit, 2010). Nevertheless, a major limitation of current studies evaluating the theta suppression model is the focus on recording dorsal hippocampal theta frequency while neglecting the role of the ventral hippocampus. Here, we address this limitation by histamine into the dorsal and ventral hippocampus and recording the changes to dorsal and ventral theta frequency. Given the complex pharmacological and functional profile of histamine in the dorsal and ventral hippocampus, we expect to see corresponding dissociations specific to hippocampal infusion site in our neurophysiological and behavioral assays.



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**CHAPTER 2: THE ANXIOLYTIC EFFECTS OF  
SOMATOSTATIN FOLLOWING INTRA-SEPTAL AND INTRA-  
AMYGDALAR MICROINFUSIONS ARE REVERSED BY THE  
SELECTIVE SST2 ANTAGONIST PRL2903**



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## **1.) Introduction**

Somatostatin (SST) is a cyclic polypeptide that inhibits an array of endocrine, exocrine, and gastrointestinal functions (e.g. inhibition of growth hormone, insulin, glucagon, secretin, gastrin, and cholecystokinin, Brazeau et al., 1972; Cervia and Bagnoli, 2007; Moller et al., 2003; Selmer et al., 2000). In addition, there is now compelling evidence of SST acting as both a neuromodulator and neurotransmitter in the brain (e.g., Wang et al., 1989; Karschin 1995; Kreienkamp et al. 1997; Yeung et al., 2011). These central effects of SST are thought to be mediated by two somatostatin isoforms [i.e., SST14 and SST28] acting on five, G-protein-coupled somatostatin receptors [i.e., sst1-5], all of which are found throughout the brain (Epelbaum, 1986; Moller et al., 2003; Weckbecker et al., 2003).

SST was recently implicated in affective responses such as anxiety. Engin et al., (2008) found that intracerebroventricular (ICV) microinfusions of somatostatin produced anxiolytic effects in both behavioural and neurophysiological models of anxiolytic drug action in rats. Anxiety-like behavior in the rat elevated plus-maze was also inhibited by ICV infusions of a selective agonist of the sst2 receptor subtype, L-779976, whereas infusions of sst1, and sst3-5 agonists were without effect (Engin and Treit, 2009). Finally, the two isoforms of somatostatin found in the brain (SST14 and SST28) each produced anxiolytic-like effects in the elevated plus-maze after intracerebral microinfusions into the amygdala and septum (Yeung et al., 2011). The absence of this anxiolytic effect after identical infusions into the striatum--which also contains

SST receptors--suggested some degree of site-specificity for the anxiolytic effects of intracerebral SST14 and SST28 (Yeung et al., 2011).

It seems likely that the anxiolytic-like effects of SST are mediated by sst2 receptor subtype, for three reasons. First, ICV infusion of the selective sst2 receptor agonist L-779976 produced anxiolytic effects in the plus-maze that were comparable to both SST and diazepam, a potent anxiolytic compound (Engin and Treit, 2009). Second, the sst2 receptor subtype is densely expressed in structures already implicated in anxiety, such as the amygdala and septum (Holloway et al., 1996; Selmer et al., 2000; Treit and Menard, 2000). Third, microinfusion of SST into either of these brain areas produced robust anxiolytic effects in the elevated plus-maze (Yeung et al., 2011). Despite this evidence, however, the receptor specificity of the anxiolytic effects of SST in general, and in the amygdala and septum specifically, has not been clearly established.

An obvious approach to establishing the receptor specificity of the anxiolytic effects of somatostatin is to determine whether such effects can be blocked by co-infusion of an sst2 antagonist. Although early attempts to establish an antagonist with selective affinity for the sst2 subtype were not particularly successful, systematic work by Coy and others eventually led to such a development. PRL2903 is a receptor antagonist with a high, *selective* affinity for the sst2 receptor subtype (e.g., Hocart et al., 1998; 1999; Rossowski et al., 1994; 1998). Functional studies have shown, for example, that PRL2903 completely blocks intracellular Ca<sup>2+</sup> influx produced by the selective sst2 agonist L-779976, with no intrinsic effect by itself (Cheng et al., 2002). Thus, PRL2903 seemed to be an ideal pharmacological tool for determining the receptor subtype mediating the anxiolytic-like effects of SST.

The present experiments were designed to assess the role of the sst2 receptor in the anxiolytic effects of SST. Classical agonist-antagonist interaction studies were conducted in both the amygdala and septum, using SST as agonist and PRL2903 as antagonist. Complete reversal of the anxiolytic effects of SST would indicate a predominant role of the sst2 receptor in anxiety-reduction. Partial or incomplete antagonism of SST-induced anxiolysis would indicate that other receptors in addition to sst2 may be involved in the anxiolytic effects of SST. The complete absence of anxiolytic antagonism by PRL2903 would indicate--but not prove--that the sst2 subtype is not involved in anxiety reduction.

## **Materials and Methods**

### *2.1. Subjects*

Subjects were 99 male, Sprague-Dawley rats, weighing 200-300g at the time of surgery. Rats were individually housed in 47x25x20.5 cm polycarbonate cages for the duration of the experiment and maintained on a 12:12 h light/dark cycle (lights on at 0600 h). Food and water were available ad libitum. The treatment of all animals was in compliance with the National Institute of Health Guide for Care and Use of Laboratory Animals, and the Canadian Council on Animal Care. All procedures were also approved locally by the Biosciences Animal Policy and Welfare Committee of the University of Alberta. Power analyses were carried out before the experiments to minimize the number of animals used, and all possible measures to minimize suffering and stress were taken during the experiments. Just prior to surgery, the rats were

randomly assigned to surgery conditions (bilateral amygdalar cannulation or bilateral septum cannulation).

## *2.2. Surgery*

Rats were anaesthetized with isoflurane (5% induction, 1.5% maintenance in N<sub>2</sub>O and O<sub>2</sub>, 67% N<sub>2</sub>O and 33% O<sub>2</sub>; Halocarbon Product Corp. River Edge NJ, USA), and injected with Marcaine (1.5 mg/0.3mL s.c.; Hospira, Quebec, Canada), and Rimadyl© (2.5 mg/0.5 mL s.c.: Pfizer, Quebec, Canada), just under the cranial cutaneous membranes, and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). Following hydration with 0.9% saline (4cc, i.p.), an incision was made to expose the skull. The subjects were then bilaterally implanted with Stainless-steel 22-gauge guide cannulae (Plastics One, Roanoke, VA, USA) targeting the amygdala (AP: -2.5, ML:-4.2, DV:-6.6), or the septum (AP: 0.7 mm, ML: -2.6, DV: -4.2, angled 22° towards the midline), using Paxinos and Watson's (1986) stereotaxic atlas of the rat brain. The cannulae were lowered to within 0.5 mm of their intended targets and secured to the skull with three jeweler's screws and cranioplastic cement. A dummy cannula was inserted into each guide cannula in order to keep the cannula tract clear. The surgical area was treated with Hibitain® (Pfizer, Quebec, Canada), upon completion of the procedure. Following surgery, the rats were placed in a warm environment until they regained consciousness. Rats were then allowed to recover for at least 4 days in their home cages, during which time they were briefly handled to assess their general health and to check for any obstruction in the cannulae.

### *2.3. Infusion procedure*

Just prior to behavioural testing, rats from the amygdalar and septal surgical groups were randomly assigned to one of four drug conditions: 1.) Somatostatin (SST) (AnaSpec, CA, USA), 3µg/ µL (1.5 µg per hemisphere), 2.) a selective sst2 antagonist PRL2903 (kindly donated by Dr. David Coy, Tulane Health Sciences Center, LA), 3µg/ µL (1.5 µg per hemisphere), 3.) the co-Infusion of SST and PRL2903 at the same doses and concentrations as above, or 4.) a 5% DMSO vehicle solution (0.5µl per hemisphere). Both SST and PRL2903 were dissolved in a 5% DMSO vehicle at final concentrations of 1µg/µL. The drugs were infused bilaterally (1.5µg/ hemisphere) via an infusion pump (Harvard Apparatus 22, MA, USA) at a rate of 1µL/min for 30 sec per hemisphere through 26-gauge stainless-steel internal cannulae attached to a 10-µl Hamilton syringe by polyethylene tubing. The internal infusion cannulae extended 0.5mm below the ventral tip of the guide cannulae. Drug flow was confirmed by displacement of a bubble inside the polyethylene tubing. The internal infusion cannulae were left in place for 30 seconds after the end of the infusion period to allow for diffusion.

### *2.4. Behavioral testing*

The behavioral procedures were the same as those described previously (e.g., Treit et al. 1993) and below. The experimenter handled each of the rats for 5 min, checking the cannulae tracts for blockage and habituating the rats to the infusion procedures, on each of the 4 consecutive days prior to testing. All behavioral testing occurred in a quiet testing room between 0900 h and 1800 h and was recorded on videotape. Testing started 10 min after the end of the infusion procedure. The experimenter always sat in a chair positioned in the same place in the

room during plus-maze testing. To ensure blind behavioral coding, only the animal number and not the group it belonged to was shown in the videotape.

#### *2.4.1. Elevated plus-maze*

The maze was a plus-shaped apparatus with an open roof, consisting of two 50×10 cm open-arms, and two 50×10×50 cm enclosed arms, and elevated at a height of 50 cm. All testing was conducted between 0900 and 1800 h in a quiet and dimly illuminated room. Each animal was tested for 5 min. Four variables were measured: (1) time spent in the open-arms; (2) time spent in the closed arms; (3) number of entries into the open-arms; and (4) number of entries into the closed arms. A rat was considered to have entered or spent time in an arm only when all four paws were in the respective arm. The time spent in the open-arms and the number of open-arm entries were expressed as a percentage of total arm activity ( $\text{open-arm time} / (\text{open-arm time} + \text{closed-arm time}) \times 100$ , and total arm entries ( $\text{open-arm entries} / (\text{open-arm entries} + \text{closed-arm entries}) \times 100$ , respectively. Increases in the percentage of open-arm time or open-arm entries are taken as measures of anxiety-reduction (anxiolysis). In addition, the total of all arms entered, as well as the total of closed arms entered were used as indexes of general activity (Hogg, 1996; Pellow and File, 1986).

#### *2.5. Histology*

Following behavioral testing, rats were euthanized in a gas chamber with 100% N<sub>2</sub>O and perfused intracardially with 0.9% (wt/vol) saline followed by 10% (vol/vol) formaldehyde. Post-

fixation, the brains were removed from the skull and placed in a 10% formaldehyde solution for at least 48 h. The brains were then frozen with dry ice and cut into 60- $\mu$ m coronal sections with a sliding microtome (Model 860, American Optical Company, Buffalo, New York). Every second section was collected and mounted onto a frosted microscope slide and later stained with thionin (for details see Gerfen, 2003). The behavioral data from animals with either one or both cannulae outside of the target area were excluded from the behavioral analysis.

## 2.6. Statistical Analyses

The results from the elevated plus-maze test and shock probe test were expressed as means and standard errors of the mean (S.E.M). Behavioural measures of anxiety in the elevated plus-maze (% time; % entries) were analyzed with planned pair-wise comparisons (Keppel and Zedeck 1989; ANOVA;  $\alpha = 0.05$ ). Control measures on the other hand were analyzed with omnibus ANOVAs,  $\alpha = 0.05$ , followed by post-hoc comparisons when appropriate.

## 3.) Results

*3.1 Experiments 1 and 2: Histology.* If rats had either one or both cannulae tips outside of the target areas, their data were excluded from the behavioral analyses. Figure 1 shows the approximate infusion sites of rats with cannulae aimed at the septum in Experiment 1. All 46 of these rats had cannulae tips bilaterally centered in the intermediate and dorsolateral regions of the septum. Figure 2 shows the infusion sites of rats with cannulae aimed at the amygdala in Experiment 2. Four out of forty-seven of these rats (SST n=1, PRL2903 n=2, vehicle n=1, SST+PRL2903 n=0), were excluded from the behavioral analyses as a result of misplaced cannulae (indicated by the red squares in Figure 2). The other 43 rats in this surgical group had

cannulae tips that were bilaterally clustered in the medial and lateral divisions of the central amygdala, as well as the basolateral amygdala (Figure 2). However, five of these remaining rats in experiment 2 (SST n=0, PRL2903 n=2, vehicle n=1, SST+PRL2903 n=2) fell off the plus-maze four or more times than the average of all groups [mean falls =1.08( $\pm$ 0.96)  $z=3.3$ ]. Hence, these outliers were also excluded from analysis of Experiment 2, leaving a final N of 38.

### *3.2 Experiment 1: Intra-septal infusions*

As can be seen in Figure 3, SST produced robust anxiolytic-like effects in the elevated plus-maze. Planned comparisons showed that SST-treated group had significantly greater percentages of open-arm entries compared to the vehicle- treated group ( $F_{(1,42)} = 4.07$ ;  $p=0.050$ ), the PRL2903 -treated group ( $F_{(1,42)} = 5.89$ ;  $p=0.020$ ), and the co-infusion- treated group ( $F_{(1,42)} = 15.72$ ;  $p=0.001$ ). Likewise, the SST group spent a significantly higher percentage of time in the open-arms than the vehicle ( $F_{(1,42)} = 4.81$ ;  $p=0.034$ ), PRL2903 ( $F_{(1,42)} = 7.58$ ;  $p=0.009$ ), and co-infusion ( $F_{(1,42)} = 7.72$ ;  $p=0.008$ ) groups. Neither the percentage of open-arm entries nor the percentage of time spent in the open-arms differed significantly between the vehicle, PRL2903, or co-infusion groups (% open-arm entries,  $F_{(2,33)} = 2.87$ ;  $p=0.07$ ; % open-arm time,  $F_{(2,33)} = 0.24$ ;  $p=0.79$ ). There were no differences between the groups on either measure of general activity (closed arm entries,  $F_{(3,42)} = 1.34$ ;  $p=0.27$ ; total arm entries,  $F_{(3,42)} = 0.11$ ;  $p=0.95$ ; see Table 1).



### 3.3 Experiment 2: Intra-amygdalar infusions

Similar to the findings of Experiment 1, figure 4 shows that SST produced anxiolytic effects in the elevated plus-maze. Planned comparisons showed that the SST group spent a significantly greater percentage of time in the open-arms than the vehicle-treated ( $F_{(1,34)} = 4.48$ ;  $p=0.042$ ), PRL2903-treated ( $F_{(1,34)} = 7.48$ ;  $p=0.010$ ), and co-infusion-treated ( $F_{(1,34)} = 5.80$ ;  $p=0.022$ ) groups. There were no significant differences between the vehicle, PRL2903, or co-infusion groups in percent time in the open arms (% open-arm time,  $F_{(2,25)} = 0.33$ ;  $p=0.72$ ). However, SST failed to increase the percentage of open-arm entries compared to vehicle-treated controls ( $F_{(1,34)} = 0.75$ ;  $p=0.53$ ). There were no significant differences between the groups on either measure of general activity (closed arm entries: ( $F_{(3,34)} = 1.00$ ;  $p=0.40$ ; total arm entries: ( $F_{(3,34)} = 1.16$ ;  $p=0.34$ ; see Table 2).

#### 4.) Discussion

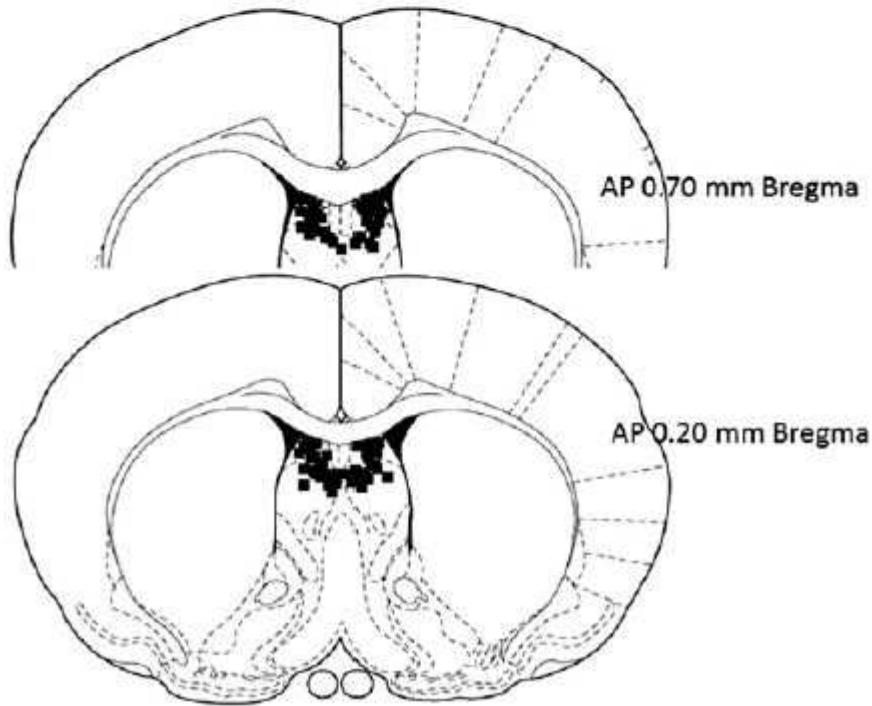
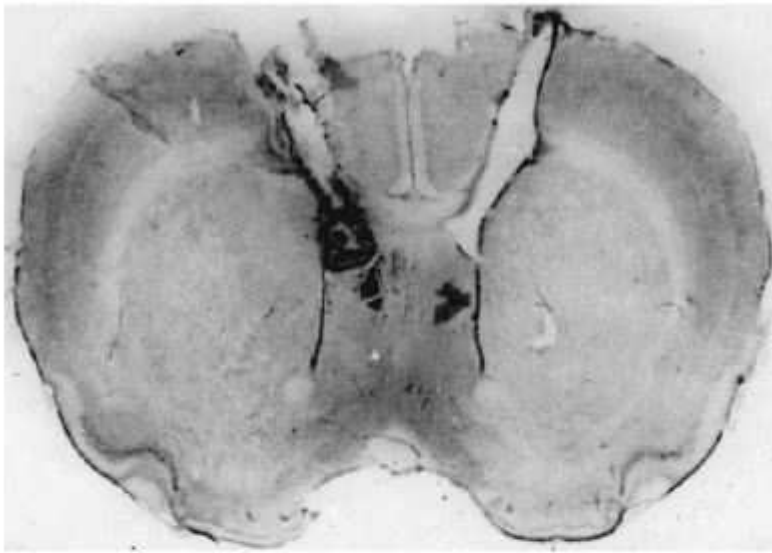
Both intra-septal and intra-amygdalar microinfusions of SST produced an increase in open-arm activity, replicating previous experiments (Yeung et al., 2011). The magnitude of these anxiolytic effects was comparable to that previously observed following intra-amygdalar or intra-septal infusions of SST14 and SST28, intracerebroventricular [ICV] infusion of L-779976, (the synthetic sst2 agonist), and ICV diazepam, a classical benzodiazepine anxiolytic (Engin et al., 2008; Engin and Treit, 2009; Yeung et al., 2011). It is noteworthy, therefore, that in these experiments, the anxiolytic effects of SST in the plus-maze were completely reversed by the highly selective sst2 antagonist PRL2903. Taken together, these results indicate that the sst2 receptor mediates the anxiolytic actions of somatostatin.

The absence of a significant anxiolytic effect on one, but not the other percentage measure of open-arm activity is not an exceptional occurrence in the experimental literature. Nevertheless, it detracts from the results of Experiment 2, where intra-amygdalar infusions of SST, while significantly increasing the percentage of open-arm time, did not increase the percentage of open-arm entries compared to control (see figure 4). Although a “ceiling effect” might have obscured a significant increase in percent open-arm entries (i.e., control mean =  $38.24 \pm 5.66$ ; SST mean =  $39.87 \pm 3.85$ ) the mean percent open-arm entries in Experiment 1 were equivalent to those in Experiment 2, and yet in Experiment 1 the percentage of open-arm entries was significantly increased by SST. It is also important to note in this regard that both measures of general activity in the plus maze-were non-significant in both experiments (see table 1). Thus, it is difficult to pinpoint a credible cause for this anomaly in percent entries in Experiment 2. Nevertheless, the absence of a significant effect here on the percent entries data is probably

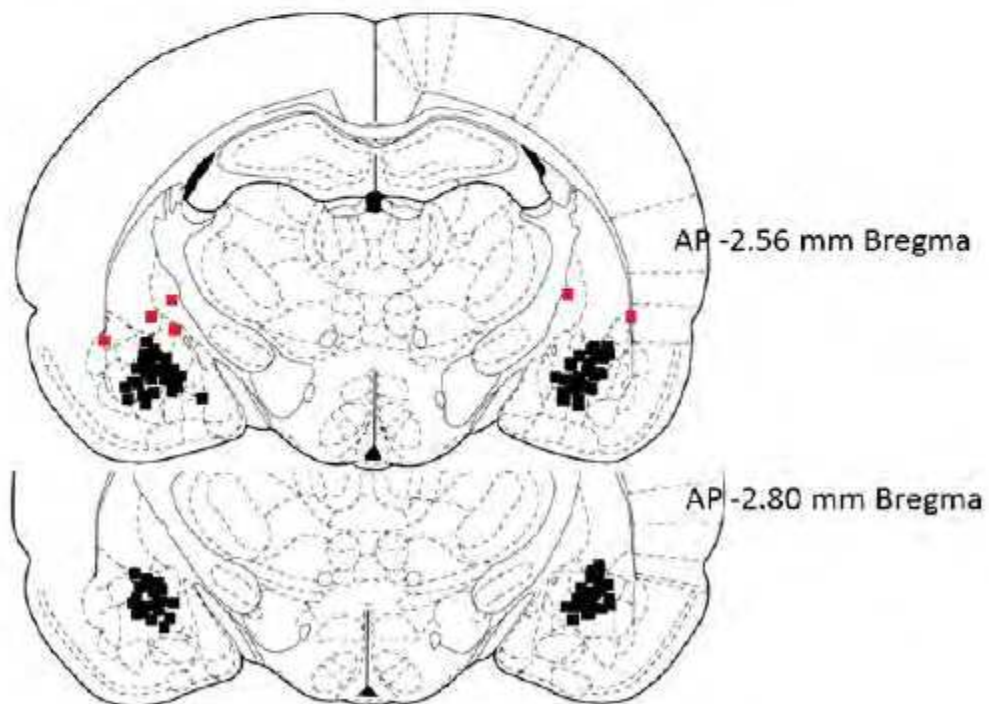
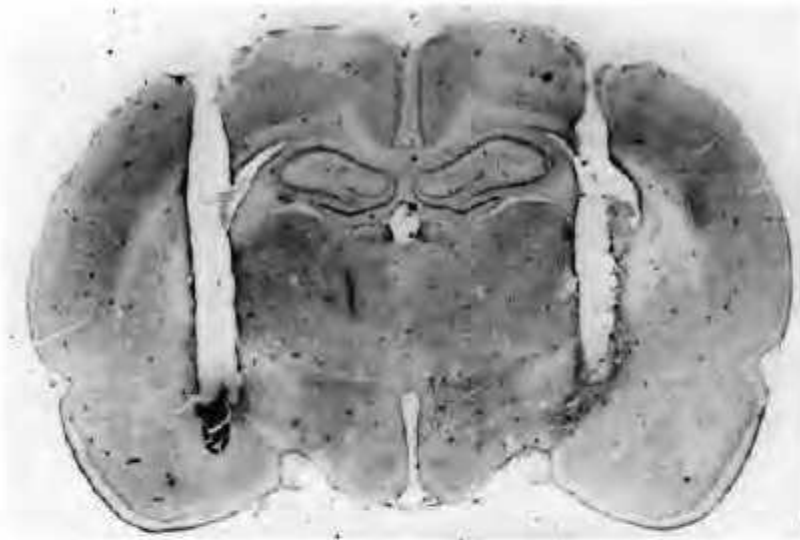
anomalous, since Yeung and Treit (2011) have previously demonstrated robust anxiolytic effects in the plus-maze on both % time and % entries when SST was microinfused into the amygdala.

Despite the strong evidence of receptor specificity presented here, we should keep in mind that this does not mean that somatostatin therefore acts *alone* in producing its anxiolytic effects. Within the amygdala somatostatin is co-localized in GABA interneurons (Epelbaum et al., 1994), and both somatostatin and GABA are co-released. (Batten et al., 2002; Esclapez and Houser, 1995; Momiyama and Zaborsky, 2006; Xie and Sastry, 1992). In addition, the sst2 receptor is expressed in nearly all hippocampal GABAergic neurons projecting to the medial septum (Bassant et al., 2005). Thus, neuropharmacological interactions between GABA, somatostatin, and sst2 may uniquely contribute to the behavioral expressions of anxiolysis. Evidence in favour of such interaction was provided by Engin et al (2008), who found that bicuculline, a competitive GABA<sub>A</sub> antagonist, reversed the anxiolytic effects of somatostatin in the elevated plus-maze. Conversely, there is evidence that the selective sst2 receptor antagonist PRL2903 modulates effects of selective GABA<sub>A</sub> receptor antagonists. For example, Macdonald et al., 2007 found that PRL2903 increased glucagon secretion in a manner similar to the selective GABA<sub>A</sub> receptor antagonist SR-95531. In a similar vein, Piqueras et al., 2004 found that gastric acid secretion was stimulated by the potent GABA<sub>B</sub> agonist SKF-97541 and inhibited by the sst2 antagonist PRL2903. This suggests that somatostatin and GABA systems interact in a dualistic fashion, sometimes cooperating in the mediation of certain functions, while opposing each other in mediating other functions. The apparent complexity of these interactions in the control of basic, physiological functions suggests that any mechanistic hypotheses of how somatostatin and GABA interact in the control of anxiety will almost certainly be incomplete.

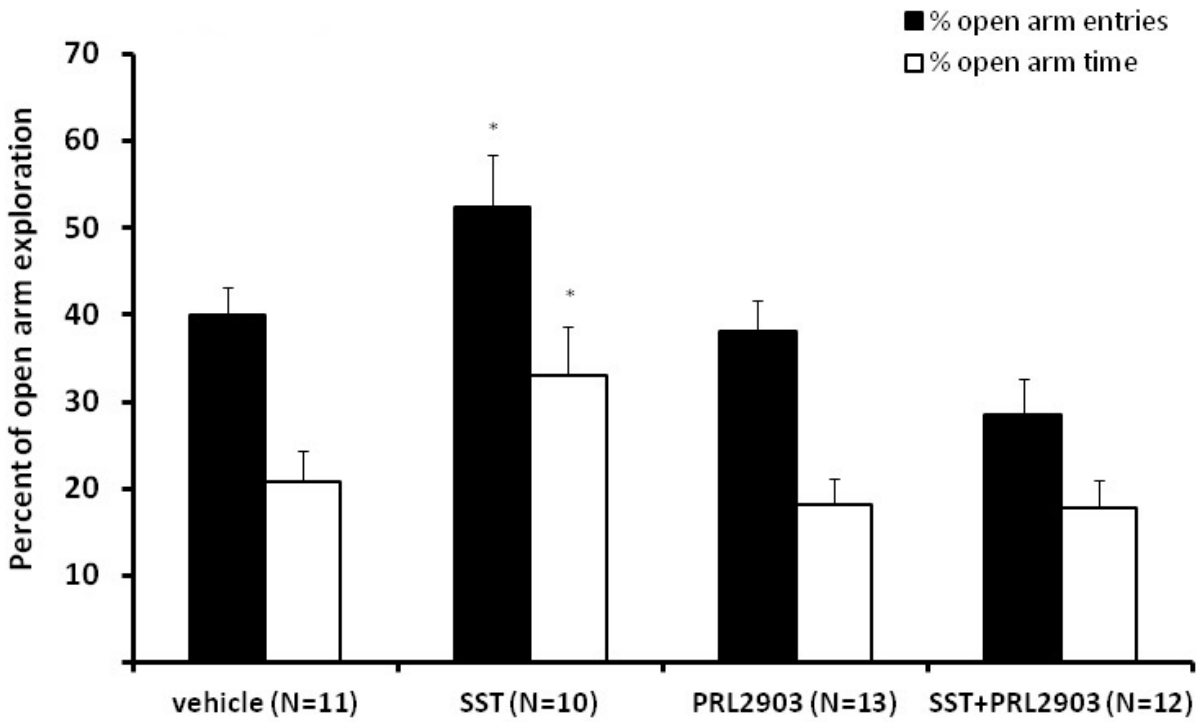
**Fig 2.1:** Schematic diagram of coronal sections of the rat brain illustrating the approximate locations (black squares) of septal infusion sites in Experiment 1. There were no misplaced cannulae in Experiment 2. The atlas plates are adapted from Paxinos and Watson (1986). Below is a representative photomicrograph of septal cannulae sites.



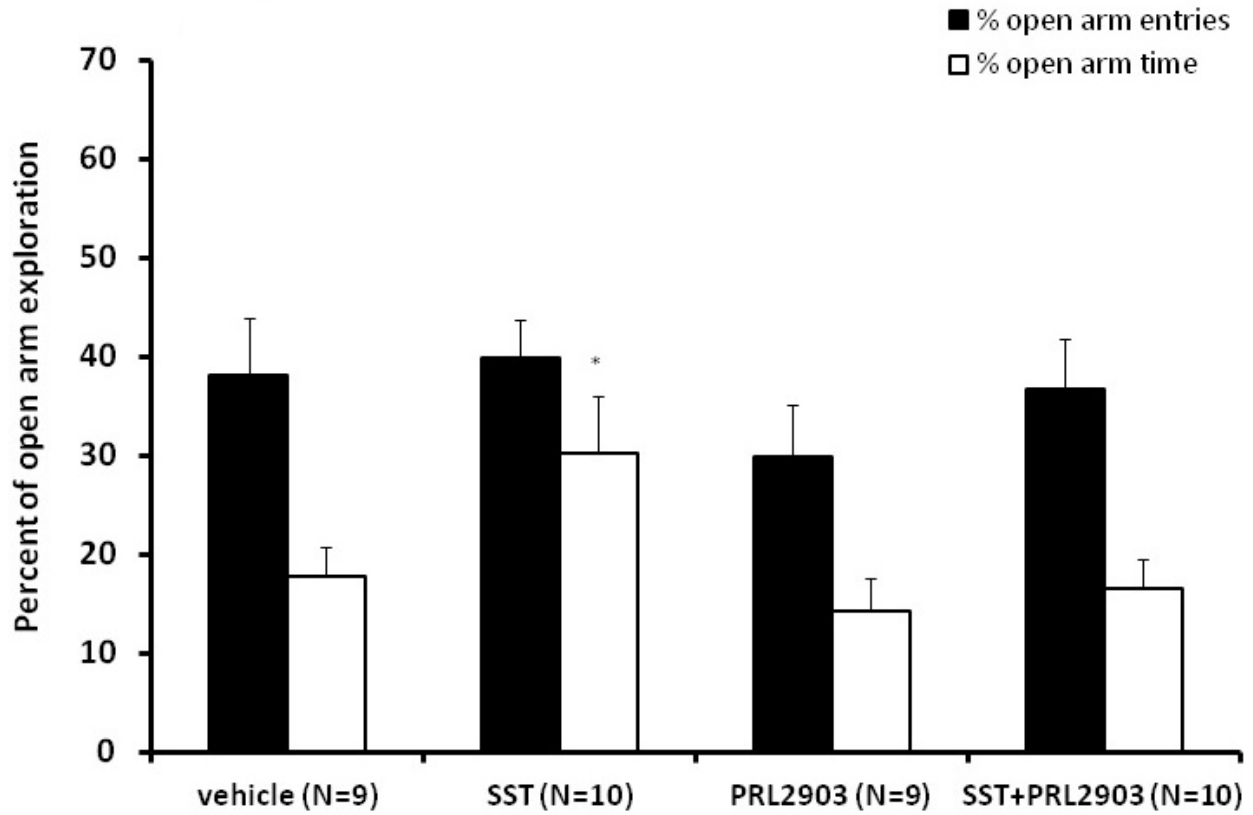
**Fig 2.2:** Schematic diagram of coronal sections of the rat brain illustrating the approximate locations (black squares) of amygdalar infusion sites in Experiment 2. The red squares illustrate misplaced cannulae in this experiment. The atlas plates are adapted from Paxinos and Watson (1986). Below is a representative photomicrograph of amygdalar cannulae sites.



**Fig 2.3:** Open-arm activity following intra-septal microinfusions of vehicle, 1.5 $\mu$ g bilateral of SST, 1.5 $\mu$ g bilateral of PRL2903, or 3 $\mu$ g coinfusion of SST and PRL2903 in Experiment 1. Black bars represent mean ( $\pm$ SEM) percentage of open-arm entries, white bars represent mean ( $\pm$ SEM) percentage of open-arm time. \*Significantly different from the all other groups ( $p < 0.05$ ).



**Fig 2.4:** Open-arm activity following intra-amygdalar microinfusions of vehicle, 1.5 $\mu$ g bilateral of SST, 1.5 $\mu$ g bilateral of PRL2903, or 3 $\mu$ g coinfusion of SST and PRL2903 in Experiment 2. Black bars represent mean ( $\pm$ SEM) percentage of open-arm entries, white bars represent mean ( $\pm$ SEM) percentage of open-arm time. \*Significantly different from the all other groups ( $p < 0.05$ ).



**Table 2.1:** General activity measures (Means $\pm$ SEM) in the elevated plus-maze test following intra-septal microinfusions in Experiment 1.

Drug	Number of closed arm entries	Number of total entries
Vehicle control	6.18 $\pm$ (0.67)	10.40 $\pm$ (1.08)
SST (3 $\mu$ g)	5.50 $\pm$ (0.96)	11.30 $\pm$ (1.24)
PRL2903 (3 $\mu$ g)	6.92 $\pm$ (0.86)	10.77 $\pm$ (0.89)
SST + PRL2903 (3 $\mu$ g each)	7.83 $\pm$ (0.88)	10.92 $\pm$ (1.06)



**Table 2.2:** General activity measures (Means±SEM) in the elevated plus-maze test following intra-amygdalar microinfusions in Experiment 2

Drug	% open arm entries	Number of closed arm entries	Number of total entries
Vehicle control	38.24±(5.66)	7.56±(1.25)	12.00±(1.44)
SST (3 µg)	39.87±(3.85)	6.60±(0.76)	11.20±(1.33)
PRL2903 (3 µg)	29.98±(5.23)	8.11±(1.03)	11.44±(1.08)
SST + PRL2903 (3 µg each)	36.83±(5.03)	5.90±(0.89)	9.10±(0.85)

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## **CHAPTER 3**

**INTRA-HIPPOCAMPAL INFUSION OF THE IH BLOCKER  
ZD7288 SLOWS EVOKED THETA RHYTHM  
AND PRODUCES ANXIOLYTIC-LIKE EFFECTS IN THE  
ELEVATED PLUS MAZE.**

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## 1.) Introduction

Hippocampal theta is a large-amplitude, near-sinusoidal rhythm having a fundamental peak frequency ranging from 4 to 14 Hz (Bland, 1986; Buzsaki, 2002; Green and Arduini, 1954; Vanderwolf, 1975). Functionally, hippocampal theta has been implicated in a myriad of brain and behavioural functions, including voluntary movement (Skaggs et al., 1996; Vanderwolf, 1969), sensorimotor integration (Bland and Oddie, 2001), cognition and memory (Cohen and Eichenbaum, 1991; Hasselmo, 2005; Hasselmo, 2009; O'Keefe and Conway, 1978), spatial navigation (O'Keefe, 1999) and affective responses. (Engin et al., 2008; Gray and McNaughton, 2000; McNaughton et al., 2007; Yeung et al., 2012). With regards to the latter, a long-standing hypothesis has specified that a strong correspondence exists between hippocampal theta and behavioral expressions of anxiety (Gray, 1982).

More recently, it has been suggested that the suppression of evoked hippocampal theta frequency is a reliable neurophysiological signature of anxiolytic drug action. Based upon data obtained from both freely moving and anesthetized rats, McNaughton, Kocsis, and Hajos (McNaughton et al., 2007) systematically determined that all clinically proven anxiolytic drugs (e.g. benzodiazepines like diazepam, 5-HT<sub>1A</sub> agonists like buspirone, and SSRIs like fluoxetine) suppress the frequency of reticularly-elicited hippocampal theta brain rhythm in urethane-anesthetized rats, while drugs that do not affect anxiety but facilitate cognitive enhancement (e.g. procognitive drugs) do not modulate theta frequency, but instead increase the power of reticularly-elicited hippocampal theta. Additionally, this model has been used to evaluate the anxiolytic potential of anticonvulsant and antipsychotic drugs that target GABAergic

mechanisms, e.g., pregabalin, (Siok et al., 2009). However, the effect of the latter compounds on the behavioral expression of anxiety in animal models was not assessed in the same studies.

A truly critical test of the theta reduction model of anxiolysis would be to assess a novel compound in both the electrophysiological and the behavioral models. If this compound produced an anxiolytic profile in *both* models, this would constitute strong, converging evidence for the predictive validity of the theta suppression model. Recently, we evaluated this possibility using the anticonvulsant drug phenytoin, which was known to affect theta rhythmicity at the level of cellular membranes but that had no known anxiolytic properties. We demonstrated that not only did phenytoin significantly suppress theta frequency *in vivo*, it also produced robust anxiolytic effects in a well-established behavioural model of anxiolytic drug action, the elevated plus maze (Yeung et al., 2012). We concluded that this provided strong evidence for the predictive validity of the theta-suppression model.

In the present study, we attempt to provide an even more stringent test of the theta suppression model by assessing the *intrahippocampal* effects of a drug belonging to another therapeutic category, in this case, the bradycardic agent ZD7288. ZD7288 is a relatively specific blocker of the hyperpolarization-activated current  $I_h$  (BoSmith et al., 1993) and it is through this action that it slows heart rate (Berger et al., 1994). It is also known to block subthreshold theta oscillations and theta resonance in retrohippocampal and neocortical neurons (Dickson et al., 2000; Hutcheon et al., 1996). ZD7288 also alters hippocampal theta when applied at the level of the medial septum, a source of rhythmic and neuromodulatory input to the hippocampus (Kocsis and Li, 2004). However, the effects on theta of  $I_h$  blockade directly at the level of the

hippocampus are unknown. Finally and more importantly, ZD7288 has never been recognized as a drug with clinical efficacy as an anxiolytic.

To summarize, our objective in this study is to test whether intra-hippocampal administration of ZD7288 will suppress evoked hippocampal theta *and* produce anxiolysis in the elevated plus-maze. If ZD7288 does indeed produce reliable anxiolytic effects in the elevated plus-maze, along with theta suppression in the neurophysiological model, this would further strengthen the predictive validity of McNaughton's model of anxiolytic drug action. Conversely, if discordant effects are observed across the behavioural and neurophysiological models, the predictive validity of the theta suppression model would be put in question.

## **2.) Materials and Methods**

### *2.1 Subjects*

Subjects were 56 male Sprague-Dawley rats, weighing 200-300g, 14 of which were randomly assigned to the theta suppression test, and 42 of which were assigned to the elevated plus-maze test. Rats were pair-housed in polycarbonate cages 47x25x20.5 cm for the duration of the experiment and maintained on a 12:12 h light/dark cycle (lights on at 0600 h). Food and water were available ad libitum. The treatment of all animals was in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals, and the Canadian Council on Animal Care. All procedures were also approved locally by the Biosciences Animal Policy and Welfare Committee of the University of Alberta. Power analyses were carried out

before experiments to minimize the number of animals used and all possible measures to minimize suffering and stress were taken during the experiments.

## *2.2 Drug Administration - for both neurophysiological and behavioral testing*

Just prior to surgery, rats were randomly assigned to one of four intra-hippocampal infusion conditions: 1) low dose ZD7288 (1 $\mu$ g/ $\mu$ L, at a final concentration of 3.42mM), 2) high dose ZD7288 (15 $\mu$ g/ $\mu$ L, at a final concentration of 51.22mM), and 3) diazepam (5 $\mu$ g/ $\mu$ L at a final concentration of 17.56mM). A fourth group received a vehicle solution. ZD7288 was obtained from Abcam Biochemicals (MA, USA), and was dissolved in 100% double distilled H<sub>2</sub>O. Diazepam was obtained from Sabex Sandoz (QC, Canada) and was suspended at a final concentration of 5mg/mL in (40% propylene glycol, 10% dehydrated alcohol, 4.25% benzoic acid, and 1.5% benzyl alcohol in H<sub>2</sub>O). Solutions were infused via an infusion pump (Harvard Apparatus 22, MA, USA) at a rate of 1  $\mu$ l/min (0.5 $\mu$ l per hemisphere) for 1 minute through 26-gauge stainless-steel internal cannulae attached to a 10- $\mu$ l Hamilton syringe by polyethylene tubing. Infusions for neurophysiological experiments were performed stereotaxically while infusions for behavioural experiments were performed in the testing room on a table where the elevated plus maze apparatus was situated. In both neurophysiological and behavioural experiments, for all tests, intra-hippocampal microinfusions were made 30 min prior to evaluation.

## *2.3 Neurophysiology Experiments and Statistical Analysis*

Animals were initially anesthetized with isoflurane in 100% O<sub>2</sub> at a minimal alveolar concentration (MAC) of 4 and were maintained following induction at a MAC of between 1.5



and 2.5 while implanted with a jugular catheter. Isoflurane was discontinued, and general anesthesia was achieved with *i.v.* administration of urethane (0.8 g/ml; final dose:  $1.79 \pm 0.05$  g/kg) via the jugular vein. Level of anesthesia was assessed throughout the experiment by monitoring the toe pinch withdrawal reflex and a supplemental dose of urethane (0.01 ml) was administered whenever a withdrawal was observed. Body temperature was maintained at 37°C using a heating pad and rectal probe (TR-100; Fine Science Tools, BC, Canada).

Each rat was implanted with monopolar recording electrodes bilaterally at the level of the hippocampal fissure in the dorsal hippocampus (AP -3.3 mm, ML  $\pm 2.2$  mm, DV -2.7 mm) and a bipolar stimulating electrode was lowered into the brain stem (AP -8.0 mm, ML  $\pm 1.6$  mm, DV -6.7-7.2 mm). The electrodes were secured to the skull with jeweller's screws and cranioplastic cement. Recordings were made by referencing to the stereotaxic apparatus, amplifying at a gain of 1000 and filtering between 0.1 and 20 kHz using a differential AC amplifier (Model, 1700, A-M Systems Inc., Carlsborg, WA, USA). Signals were digitized on-line using automatic antialiasing filtering (high pass at half the sample frequency = 25Hz) and sampling at 50 Hz with a Digidata 1322A A-D board connected to a Pentium PC running the AxoScope acquisition program (Axon Instruments, Union City, CA, USA). Our sampling frequency and high pass filter was reduced markedly to eliminate the 100Hz artefact from stimulation trials which enabled us to characterize brainstem influences online.

Brainstem sites eliciting theta were selected based on minimal threshold intensities to evoke theta as well as an observable increase in theta frequency as stimulation intensities were elevated. Stimulation consisted of 0.1 ms biphasic constant current pulses at 100 Hz for 5 s using an electrically isolated pulse generator (Model 2100; A-M Systems). As reported earlier

(McNaughton and Sedgwick, 1978) the threshold intensity of stimulation to elicit theta differed from animal to animal ( $42.5 \pm 17.4 \mu\text{A}$ ). Therefore, a threshold intensity of stimulation to elicit theta was established separately for each animal and the stimulation intensities used in the experiment were 1, 2, 3 and 4 times threshold level. Three counterbalanced trials consisting of a randomized set at each stimulation intensity was used to compute the average evoked theta frequency for each level. At the end of the experiment, rats were euthanized with an overdose of urethane (1-2mL) and perfused intracardially with 0.9% (w/v) saline followed by 10% v/v formaldehyde. Once removed, brains were post-fixed in a 10% formalin solution for 48 hours before histological analysis (see below for details).

Evoked theta activity following brainstem stimulation was analyzed during each stimulation epoch by spectral analysis (Fast Fourier Transform: FFT) in Clampfit (Axon Instruments). Each trial was windowed using a Hamming function and was then subjected to the FFT algorithm. The peak frequency and power were extracted from the resulting spectrum and plotted as a function of stimulation intensity. The average of the peak frequency and average power at the four different time points pre-infusion and post-infusion were calculated and used as raw data. Because the critical statistical comparisons were *a priori* directional tests derived from the theta suppression model, and Gray's (1982) neuropsychological theory of anxiety, the data were analyzed using one-tailed t-tests,  $\alpha=0.05$ . Differences in average peak frequencies and average power pre and post drug infusion were assessed using paired within-subjects t-tests (Keppel and Zedeck, 1989). These assessed whether the average peak frequency and average power at each intensity was significantly suppressed after drug administration. Pairwise between-subjects comparisons of the peak frequencies and power between each of the three drug conditions (i.e., ZD7288  $1\mu\text{g}/\mu\text{L}$ , ZD7288  $15\mu\text{g}/\mu\text{L}$ , diazepam  $5\mu\text{g}/\mu\text{L}$ , and vehicle) were

conducted using independent samples t-tests (Keppel and Zedeck, 1989). These evaluated the significance of differences between the average peak frequency and average power at each stimulus intensity as a function of the three different drug conditions.

#### *2.4 Elevated Plus Maze and Statistical Analysis*

Rats were anaesthetized with isoflurane (5% induction, 1.5% maintenance in 67% N<sub>2</sub>O and 33% O<sub>2</sub>; Halocarbon Product Corp. River Edge NJ, USA), and injected with Marcaine (1.5 mg/0.3mL s.c.; Hospira, Quebec, Canada), and Rimadyl© (2.5 mg/0.5 mL s.c: Pfizer, Quebec, Canada), just under the cranial cutaneous membranes, and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). Following hydration with 0.9% saline (4cc, i.p.), an incision was made to expose the skull. The subjects were then bilaterally implanted with Stainless-steel 22-gauge guide cannulae (Plastics One, Roanoke, VA, USA) targeting the dorsal hippocampus (AP -3.3 mm, ML  $\pm$ 2.2 mm, DV -2.7 mm, (Paxinos, 1986)). The cannulae were lowered to within 0.5 mm of their intended target and secured to the skull with three jeweler's screws and cranioplastic cement. A dummy cannula was inserted into each guide cannula in order to keep the cannula tract clear. The surgical area was treated with Hibitain® (Pfizer, Quebec, Canada), upon completion of the procedure. Following surgery, the rats were placed in a warm environment until they regained consciousness. Rats were then allowed to recover in their home cage for one week prior to behavioral testing, during which time they were briefly handled to assess their general health and to check for any obstruction in the cannulae.

The maze was a plus-shaped apparatus with an open roof, consisting of two 50×10 cm open arms, and two 50×10×50 cm enclosed arms, and was elevated to a height of 50 cm. All testing was conducted between 0900 and 1700 h in a quiet and dimly illuminated room. Each animal was tested for 5 min. Four variables were measured: (1) time spent in the open arms; (2) time spent in the closed arms; (3) number of entries into the open arms; and (4) number of entries into the closed arms. A rat was considered to have entered or spent time in an arm only when all four paws were in the respective arm. The time spent in the open arms and the number of open-arm entries were expressed as a percentage of total arm activity (open-arm time/[open-arm time + closed-arm time]) x 100, and total arm entries (open-arm entries/[open-arm entries + closed-arm entries]) x 100, respectively. The total number of arms entered was taken as an index of general activity (Hogg, 1996; Pellow and File, 1986).

Following experimentation, rats were euthanized in a gas chamber with 100% N<sub>2</sub>O and perfused intracardially with 0.9% (wt/vol) saline followed by 10% (vol/vol) formaldehyde. Post-fixation, the brains were removed from the skull and placed in a 10% formaldehyde solution for at least 48 h.

The results from the elevated plus maze test were expressed as means and standard errors of the mean (S.E.M). Behavioural measures were analyzed with independent samples t-tests,  $\alpha=0.05$ , one tailed (Keppel and Zedeck, 1989). Two rats were excluded from analyses in the elevated plus maze data because they had obstructed cannulae. In addition, two rats (n=1 ZD7288 1 $\mu$ g, and n=1 vehicle) were excluded from the analysis of the plus-maze data because the number of times they fell off the maze was greater than three standard deviations from the mean ( $z \geq 4.74$ ), leaving a final N of 37.

## 2.5 Histology

Fixed brains were frozen with dry ice and cut into 60- $\mu$ m coronal sections with a sliding microtome (Model 860, American Optical Company, New York, USA). Every second section was collected and mounted onto a frosted microscope slide and later stained with thionin - for details see (Gerfen, 2003). The neurophysiological or behavioral data from animals with either one or both cannulae outside of the target area were excluded from the behavioral analysis.

## 3.) Results

### 3.1 Histology: Neurophysiological and Behavioural experiments

Figure 1 shows the approximate infusion sites of rats with cannulae aimed at the hippocampus in both neurophysiological and behavioural experiments. In our neurophysiological experiments, all 14 rats had cannula tips clustered in the dorsal regions of the hippocampus (shown by positions of the X symbols). In our behavioural experiments, all 42 rats had cannulae tips that were bilaterally clustered in the dorsal regions of the hippocampus (shown by the position of circles). Given adequate cannulae placement in all rats, none were excluded from data analysis.

### 3.2 Neurophysiology.

As previously shown (McNaughton et al., 2007), the frequency of brainstem-evoked hippocampal theta during baseline conditions showed a positive linear relationship with increasing intensities of stimulation in all animals tested (see example in Figure 2). In contrast,

the power of brainstem-evoked hippocampal theta did not change with increasing intensities of stimulation (not shown), a finding which is also consistent with previous work (McNaughton et al., 2007). Following drug infusions, while no differences in power were observed across any of the drug or vehicle groups at any level of intensity (all probabilities  $> 0.05$ ), there were significant reductions of the average peak frequency across levels of stimulus intensity that depended upon the drug and dosage infused. No differences were observed at any stimulus intensity following intra-hippocampal microinfusions of the low ( $1\mu\text{g}$ ) dose of ZD7288 (See Figure 3, all probabilities  $p>0.05$ ). In contrast, there were substantial and significant frequency reductions at all intensities for the high ( $15\mu\text{g}$ ) dose of ZD7288, and the  $5\mu\text{g}$  dose of diazepam (Figure 2 & 3). Interestingly, these differences were observed bilaterally despite the unilateral application since the two sides showed cycle-to-cycle matching of theta rhythms and matching of spectral peaks both pre- and post-infusion (e.g. Figure 2). This was also the case for all other manipulations and thus we will report only statistics for the ipsilateral side although the same results were observed bilaterally. It has previously been reported that unilateral manipulations have bilateral influences on hippocampal theta which suggests that the two hippocampi are functionally integrated during theta (Bland et al., 2007; Heynen and Bilkey, 1994; Rowntree and Bland, 1986).

In our pre and post comparisons, we observed a significant reduction in evoked theta frequency following the administration of the high dose ( $15\mu\text{g}$ ) of ZD7288 at all stimulation intensities: 1x threshold:  $t_{(1,8)} = 2.254$ ;  $p=0.016$ ; 2x threshold:  $t_{(1,8)} = 2.495$ ;  $p=0.01$ ; 3x threshold:  $t_{(1,8)} = 2.663$ ;  $p=0.007$ ; and 4x threshold:  $t_{(1,8)} = 2.047$ ;  $p=0.025$ . Similarly, a reduction in theta frequency was also observed following intra-hippocampal infusions of

diazepam (5 $\mu$ g), although this was only statistically significant for the second (2x threshold stimulation intensity:  $t_{(1,8)} = 2.009$ ;  $p=0.027$ ) and highest level of stimulus intensity (4x threshold stimulation intensity:  $t_{(1,8)} = 2.072$ ;  $p=0.024$ ). There were no differences observed when comparing evoked theta frequency pre- and post-drug administration for the 1 $\mu$ g ZD7288 and vehicle control group at any level of threshold stimulation intensity (all probabilities  $p>0.05$ ).

Additionally, we compared the degree of suppression in evoked theta frequencies across all four drug conditions (Figure 3). Independent samples t-tests revealed that at all intensity levels, both the ZD7288 15 $\mu$ g and diazepam 5 $\mu$ g groups showed a greater suppression of theta frequency in comparison to the vehicle control group: ZD7288 15 $\mu$ g group (1x threshold:  $t_{(1,8)} = 4.055$ ;  $p<0.001$ ; 2x threshold:  $t_{(1,8)} = 3.162$ ;  $p=0.002$ ; 3x threshold:  $t_{(1,8)} = 3.646$ ;  $p<0.001$ ; and 4x threshold:  $t_{(1,8)} = 3.232$ ;  $p<0.001$ ); diazepam 5 $\mu$ g groups (1x threshold stimulation intensity:  $t_{(1,8)} = 2.097$ ;  $p=0.045$ ); 2x threshold stimulation intensity:  $t_{(1,8)} = 2.061$ ;  $p=0.049$ ); 3x threshold stimulation intensity:  $t_{(1,8)} = 2.291$ ;  $p=0.030$ ; and (4x threshold stimulation intensity:  $t_{(1,8)} = 2.000$ ;  $p=0.055$ ). Likewise, the ZD7288 15 $\mu$ g group showed a significantly attenuated evoked theta frequency at all levels of intensity in comparison to the 1 $\mu$ g ZD7288 group: 1x threshold:  $t_{(1,7)} = 3.595$ ;  $p<0.001$ ; 2x threshold:  $t_{(1,7)} = 2.180$ ;  $p=0.020$ ; 3x threshold:  $t_{(1,7)} = 2.662$ ;  $p=0.007$ ; and 4x threshold:  $t_{(1,7)} = 3.633$ ;  $p<0.001$ . Finally, there were also no differences observed in evoked theta frequency between the 1 $\mu$ g ZD7288 and vehicle control groups at any level of threshold stimulation intensity (all probabilities  $p>0.05$ ).

### 3.3 Elevated Plus-Maze.

As can be seen in Figure 4, intra-hippocampal microinfusion of 15 $\mu$ g of ZD7288 produced an anxiolytic effect in the plus-maze. Independent samples t-tests revealed that

ZD7288 15 $\mu$ g significantly increased the percentage of open- arm entries compared to the vehicle control group ( $t_{(1,23)} = 2.95$ ;  $p=0.004$ ). A significant difference between the 15 and 1 $\mu$ g dose of ZD7288 was also found for percent open-arm entries ( $t_{(1,23)} = 2.212$ ;  $p=0.02$ ) - suggesting dose dependency. Despite this increase in open arm entries, ZD7288 15 $\mu$ g did not produce a significant increase in the percentage of time spent in the open arms of the plus-maze, compared to the vehicle control group ( $t_{(1,23)} = 0.562$ ;  $p=0.29$ ) or the 1 $\mu$ g group ( $t_{(1,23)} = 0.952$ ;  $p=0.18$ ). For the most part, the effects of diazepam 5 $\mu$ g in the plus-maze mirrored those of ZD7288, suggesting an equivalent anxiolytic potency. Thus, while diazepam produced a significant increase in percentage of open arm entries compared to the vehicle control group ( $t_{(1,21)} = 1.719$ ;  $p=0.04$ ) it did not produce a significant increase in the percentage of time spent in the open arms of the plus maze, compared to the vehicle control group ( $t_{(1,21)} = 1.028$ ;  $p=0.16$ ) or the 1 $\mu$ g group ( $t_{(1,21)} = 1.562$ ;  $p=0.07$ ). While the ZD7288 15 $\mu$ g group showed decreased levels of closed arm entries in comparison to vehicle control (Table 1), an analysis of covariance (ANCOVA) revealed that the significant increase in the proportion of open arm entries was still significant when closed arm entries were statistically controlled ( $F_{(2, 36)} = 5.52$ ,  $p=0.003$ ). Finally, neither the percentage of open arm entries nor percentage of open-arm time differed significantly between the ZD7288 1 $\mu$ g group and the vehicle control group ( $p>0.05$ ).

#### **4.)Discussion**

In this study, we directly compared the effects of ZD7288 in the theta suppression model with its effects in a well-established behavioural model of anxiolytic drug action, the elevated plus-maze. We found that ZD7288, at the level of the hippocampus, produced consistent



anxiolytic-like effects in both neurophysiological and behavioural models of anxiolytic drug action. Given our *prima facie* approach, these findings provide converging evidence of the predictive validity of the theta suppression model. It is worth mentioning that our present test is more stringent than our previous work with systemic administrations (Yeung et al., 2012) since the level at which our manipulation is acting is limited to the hippocampus itself.

Specifically, intra-hippocampal ZD7288 at a high (15 $\mu$ g), but not low (1 $\mu$ g) dose produced a significant suppression of both evoked theta frequency and an index of anxiolysis in the elevated plus maze that was comparable to that of a clinically proven anxiolytic drug, diazepam. Moreover, the increase in open-arm activity in the elevated plus maze was not explained by differences in general activity or closed arm entries. Although ZD7288 was used based on the presumption that it would have a suppressive effect on hippocampal population theta due to its blocking action on the pacemaker current  $I_h$ , there was no previous evidence to suggest that it would either 1) suppress theta or that it would 2) have anxiolytic-like effects when administered at the level of the hippocampus. Indeed, when applied to the basolateral amygdala, the action of ZD7288 has been suggested to be anxiogenic (Park et al., 2011). In this light, the anxiolytic-like effects of ZD7288 in the theta suppression model and the elevated plus-maze is even more convincing evidence for the predictive validity of the theta suppression test.

We selected doses of ZD7288 by extrapolating the effective dose from prior studies (Kocsis and Li, 2004; Park et al., 2011). Since the volume of the dorsal hippocampus is at least an order of magnitude larger than the medial septum and basolateral amygdala (Yossuck et al., 2006) we predicted that a dose of 15  $\mu$ g ZD7288 would produce observable effects on theta rhythm and behaviour. Given that all infusion sites were located in dorsal hippocampus, we

assume that most to all subregions within that area (CA1, CA3 and dentate gyrus) were affected. Given the infusion volume (1  $\mu$ l) and size of infusion cannulae (26 gauge) used in the present study, the estimated spread of the infused substances was approximately 1 mm (Myers, 1966; Myers et al., 1971; Routtenberg, 1972). Therefore, it can be assumed that the spread of the drug was well within the dorsal regions of the hippocampus and that any differences between vehicle and drug-infused rats reflected a pharmacological alteration of dorsal hippocampal function.

The mechanism by which ZD7288 acts to slow hippocampal theta was not specifically addressed in this study but it is likely that its blocking role on  $I_h$  plays an important role. The effective concentration to block  $I_h$  *in vitro* is between 25-100 $\mu$ M (Dickson et al., 2000; Hutcheon et al., 1996) and we assumed that the final diluted concentration of infusate should be within this range for the upper end of our dilution factor. Both hippocampal pyramidal cells and interneurons express  $I_h$ , which plays an essential role in terms of intrinsic membrane theta oscillations and resonance (Chapman and Lacaille, 1999b; Griguoli et al., 2010; Hu et al., 2002; Zemankovics et al., 2010). Insofar as synchronizing influences are concerned, the effects of ZD7288 upon specific interneuronal populations like those in stratum oriens or lacunosum moleculare may be the most relevant since these cells can prominently pace activity in both pyramidal cells and other non-theta oscillatory interneurons (Chapman and Lacaille, 1999a; Cobb et al., 1995; Pike et al., 2000). Given that hippocampal theta is an interplay of the interaction between principal neurons and interneurons (Bland and Colom, 1993; Buzsaki, 2002; Klausberger et al., 2003), any manipulation that disrupts theta oscillations or resonance at a membrane level should be exhibited at the level of network activity. In this respect, targeting other ionic conductances such as  $I_m$ , which is thought to be uniquely involved in theta resonant

responses in hippocampal pyramids (Hu et al., 2002), may be useful in teasing out the unique influence of principal cells in theta field generation and moreover, anxiolytic behaviour.

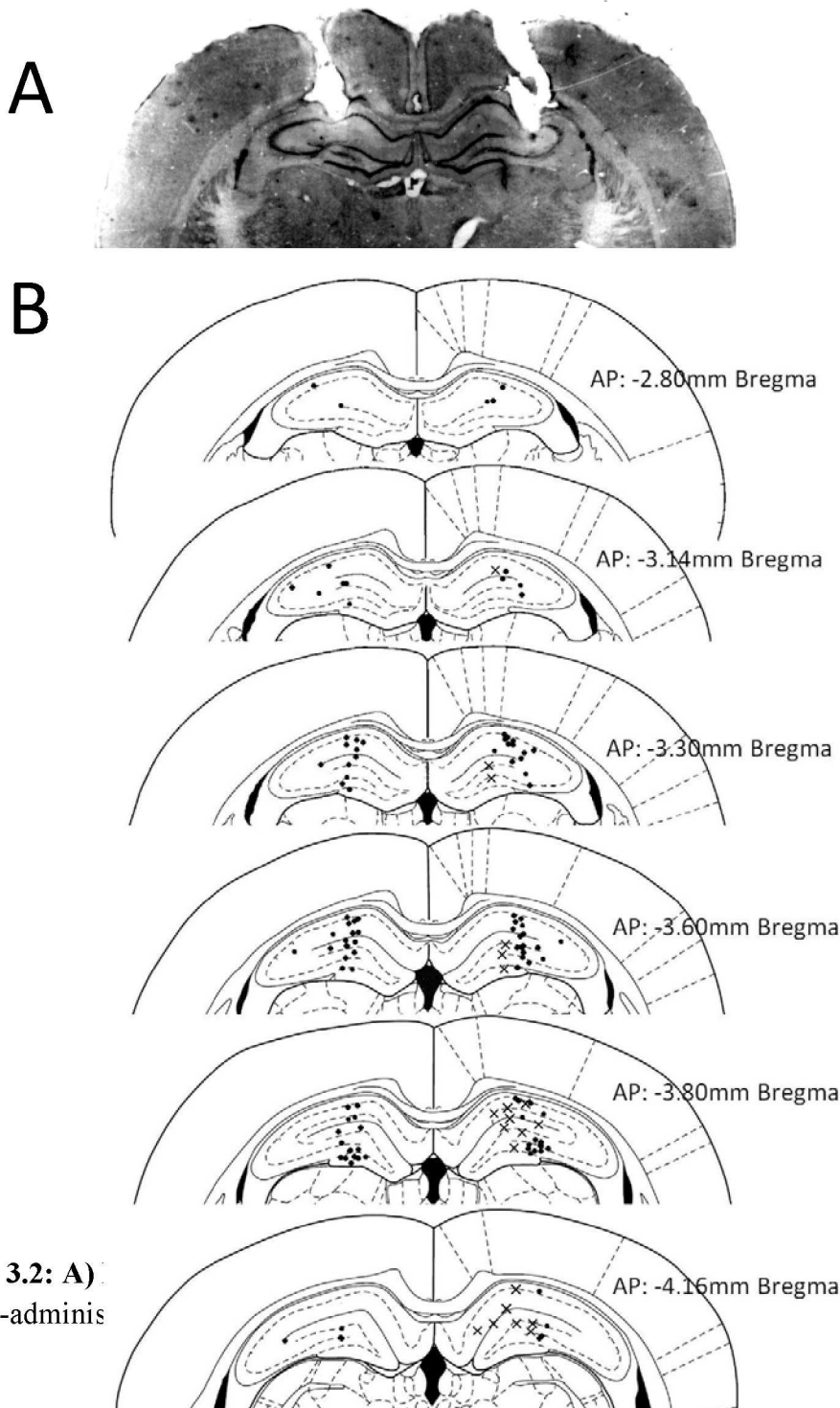
Although ZD7288 (at 50 $\mu$ M in final bath concentration) has been shown to have  $I_h$ -independent depressive effects on excitatory neurotransmission following continuous and long (30-minute) perfusions *in vitro*, (Chevaleyre and Castillo, 2002) our conditions *in situ* are substantially different that this effect might not occur in our experiments. For example, we would not expect - following intracranial applications – that the concentration of some infused substance would attain a continuous and steady state equilibrium at any point within the localized sphere affected due to diffusion, uptake and active removal (Nicholson and Sykova, 1998; Sykova and Nicholson, 2008). Regardless, our purpose in using ZD7288 was to test a predicted effect on hippocampal theta against a specific effect on behaviour. In effect, the mechanism of the theta frequency reduction *in vivo* is moot given the ultimate test of the predictive validity of this effect as an anxiolytic assay.

With the exception of the present findings, there is no direct evidence that ZD7288 has anxiolytic potential either in animal models or clinical trials. Nevertheless, ZD7288 has been shown to modulate affective responses. For example, (Cao et al., 2010) found that local infusions of ZD7288 in the ventral tegmental area produced anti-depressant effects in a social defeat stress paradigm. Similarly, (Giesbrecht et al., 2010) found that in the basolateral amygdala (BLA), a limbic system structure commonly implicated in anxiety (Davis et al., 1994; LeDoux, 2000), ZD7288 mimics the action of NPY by suppressing post-synaptic  $I_h$  and also abolishes the increases in  $I_h$  amplitude following administration of anxiogenic CRF. Since NPY is well known for its anxiolytic efficacy in reducing the expression of fear responses (Gutman et al., 2008; Karlsson et al., 2005; Trent and Menard, 2011), the ability of ZD7288 to suppress  $I_h$  in a similar

and even more powerful fashion than NPY strengthens its potential for anxiolysis. In direct contrast, however, microinfusion of ZD7288 by itself into the basolateral amygdala *in vivo* has been shown to yield an anxiogenic behavioural profile in the elevated plus-maze (Park et al., 2011). Regardless, the effects on anxiety of ZD7288 would be expected to vary depending upon the specific sub-regions of the brain into which it is infused, similar to the effects of clinically proven benzodiazepine anxiolytics (Pesold and Treit, 1995).

When evaluated together, our neurophysiological and behavioral findings not only strengthen the predictive validity of the theta suppression model, but bring attention to the possibility that this model is a useful screen for drugs that have therapeutic potential for anxiety but are primarily used in treating other medical disorders (Ettinger and Argoff, 2007; Yeung et al., 2012). However, there remain unanswered questions regarding the validity of this model. For example, can this model be applied (in a reversed or opposing fashion) to drugs having anxiogenic actions? That is, one would expect that a drug that promotes (rather than reducing) anxiety (e.g. FG7142) (Cole et al., 1995) might enhance (rather than depress) evoked theta frequency. Since the theta suppression model suggests that all clinically effective anxiolytic drugs suppress theta, the inverse demonstration as above would provide potential evidence of the *construct* validity of this model. That is to say, theta suppression may be more than simply a correlate of anxiolytic drug action, theta itself may reflect the neural underpinnings of anxiety (Gray and McNaughton, 2000; Gray, 1982). Of course, this remains to be demonstrated. In summary, it is hoped that the combination of neurophysiological and behavioural techniques as used here will further extend our understanding of the neural mechanisms of anxiety.

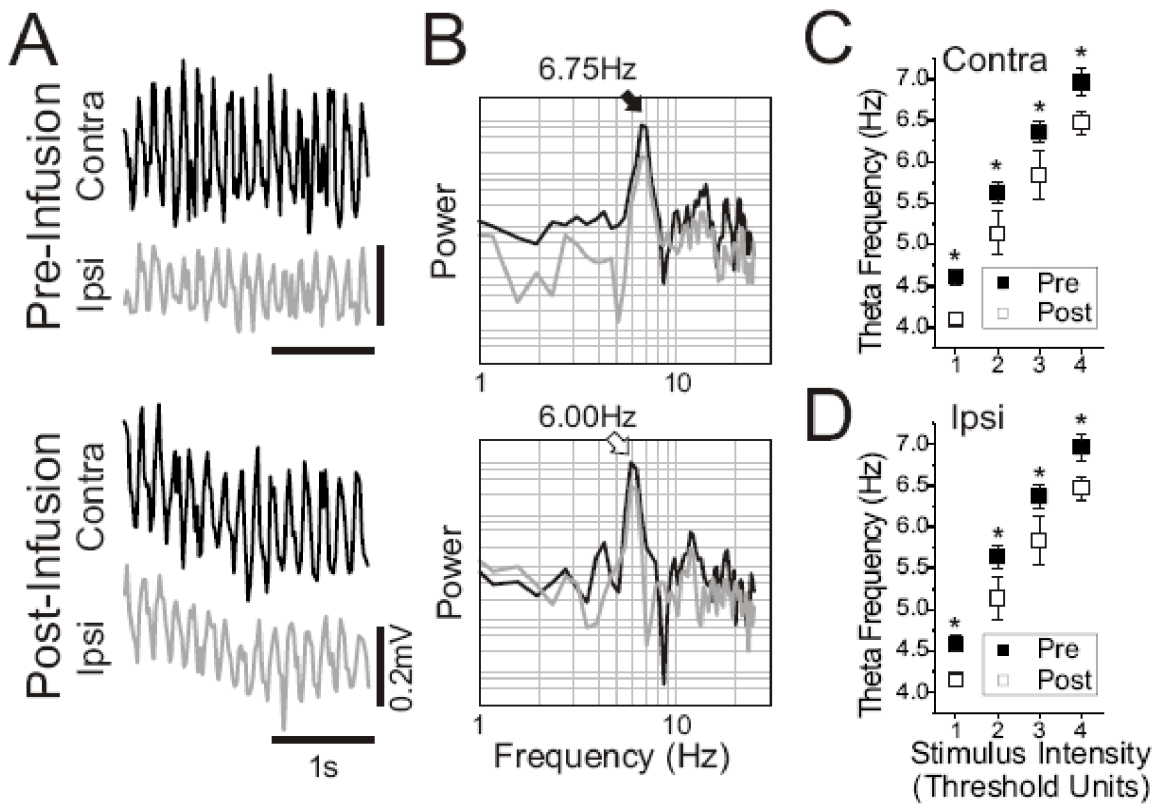
**Fig 3.1: Cannulae Placements. A.)** Representative photomicrograph of hippocampal cannulae sites. **B.)** Schematic diagram of coronal sections of the rat brain illustrating the approximate locations of hippocampal infusion sites in neurophysiological experiments (black x's) and behavioural experiments (black circles). There were no misplaced cannulae in either neurophysiological or behavioural experiments. The atlas plates are adapted from Paxinos and Watson (1986).



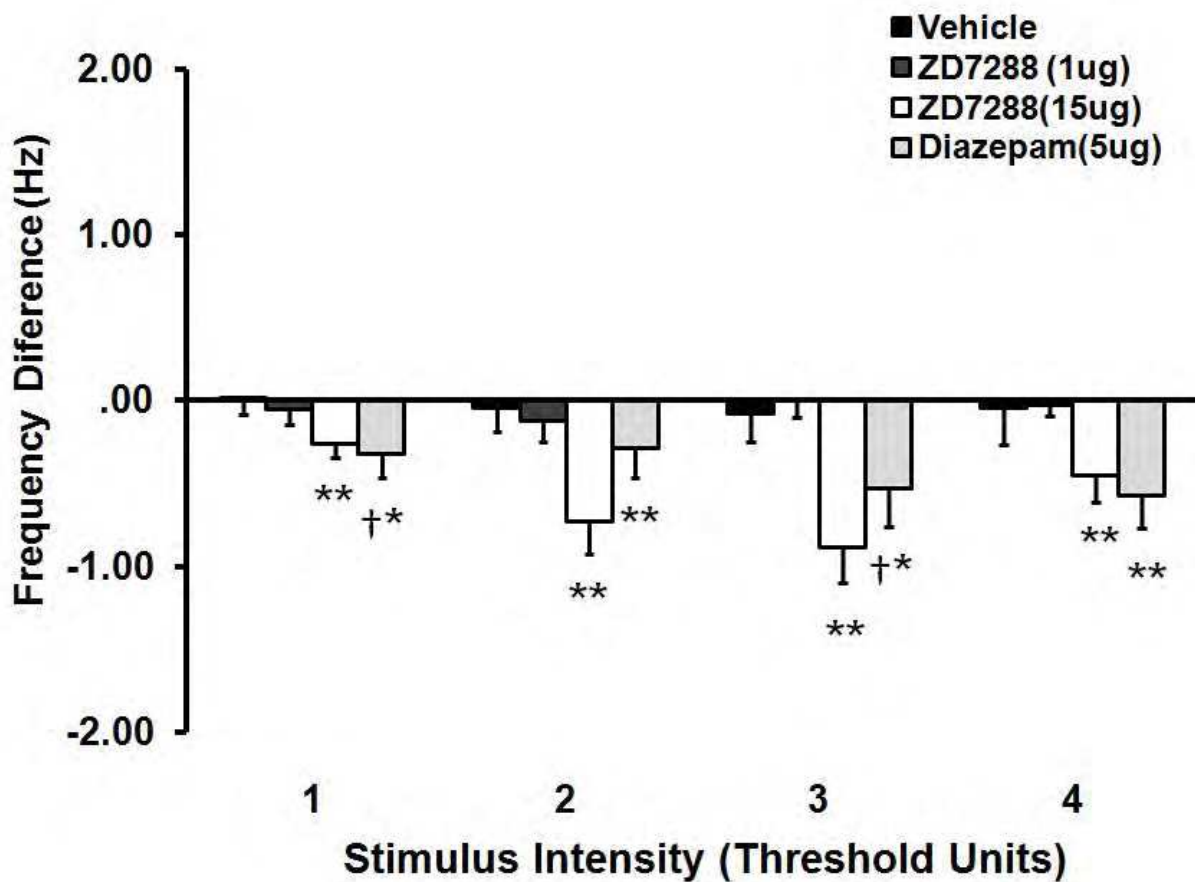
**Fig. 3.2: A)** post-adminis

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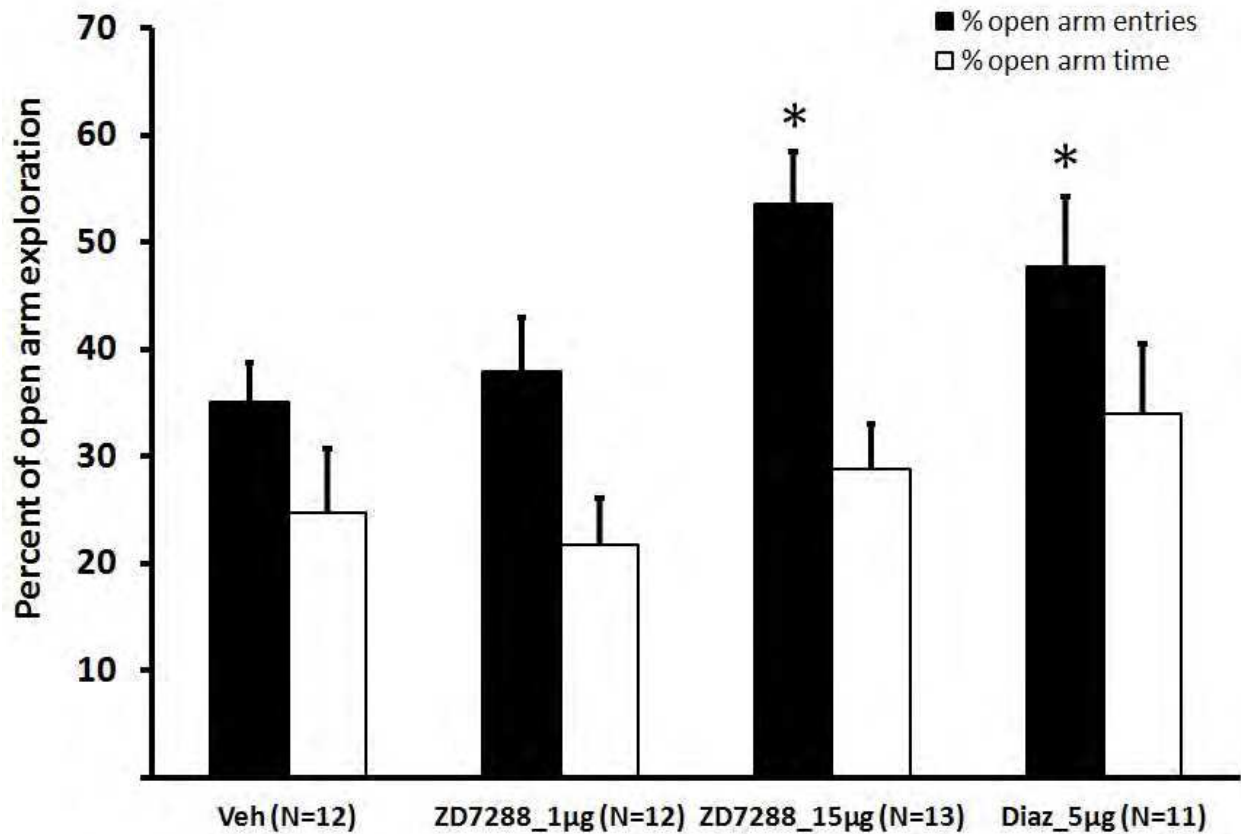
trace indicates the response ipsilateral to the side of infusion, and the black trace shows the response contralateral to the side of drug infusion. **B)** Spectra components of the respective pre- and post-drug treatment LFP traces shown in panel **A** are overlaid. Suppression of evoked frequency can be seen post administration of 15 $\mu$ g of ZD7288 both ipsilaterally and contralaterally to the sides of drug infusion. **C and D)** Average data for this experiment showing the frequency of evoked theta at each level of stimulus intensity as measured in threshold units (1x, 2x, 3x, 4x threshold value) as a function of drug treatment. The top panel and bottom panels show a significant suppression of evoked theta frequency contralaterally and ipsilaterally respectively following a 15 $\mu$ g ZD7288 infusion. Black squares represent means ( $\pm$ SEM) of evoked theta frequency pre administration of ZD7288. White squares represent means ( $\pm$ SEM) of evoked theta frequency post administration of ZD7288. All pre and post comparisons are significantly different (\*  $p < 0.05$ ).



**Fig. 3.3** Difference in evoked theta frequency (Hz) ipsilateral to hemisphere of infusion at 1x, 2x, 3x and 4x threshold units of stimulus intensity pre and post intra-hippocampal infusions of 1µg ZD7288, 15µg ZD7288, diazepam 5µg, or vehicle control in neurophysiological experiments. Black bars represent mean differences ( $\pm$ SEM) of evoked theta frequency pre and post administration of vehicle control. Dark gray bars represent mean differences ( $\pm$ SEM) of evoked theta frequency pre and post administration of 1µg ZD7288. White bars represent mean differences ( $\pm$ SEM) of evoked theta frequency pre and post administration of 15µg ZD7288. Light gray bars represent mean differences ( $\pm$ SEM) of evoked theta frequency pre and post administration of Diazepam 5µg. \*\*Significantly different pre and post infusion and from vehicle control group ( $p < 0.05$ ). †\*Marginally different pre and post infusion and significantly different from vehicle control group ( $p < 0.05$ ).



**Fig. 3.4:** Open-arm activity following intra-hippocampal infusions of 1 $\mu$ g ZD7288, 15 $\mu$ g ZD7288, diazepam 5 $\mu$ g, or vehicle control in the elevated plus-maze test. Black bars represent means ( $\pm$ SEM) percentage of open arm entries; white bars represent means ( $\pm$ SEM) percentage of open arm time. \*Significantly different from the vehicle control group ( $p < 0.05$ ).





**Table 3.1:** Percentages of Time Spent in the Open Arms and General Activity Measures (Means  $\pm$ SEM) in the Elevated Plus-Maze test. \* Significantly different from the vehicle control group  $p < 0.05$ .

Drug	% Open Time	Number of Closed Arm Entries	Number of Total Entries
Vehicle Control (N=12)	24.69 $\pm$ (6.07)	8.50 $\pm$ (0.86)	13.67 $\pm$ (1.58)
ZD7288 1 $\mu$ g (N=12)	21.78 $\pm$ (4.31)	6.91 $\pm$ (0.85)	11.25 $\pm$ (1.12)
ZD7288 15 $\mu$ g (N=13)	28.79 $\pm$ (4.21)	*4.77 $\pm$ (0.69)	10.38 $\pm$ (1.11)
ZD7288 1 $\mu$ g (N=12)	21.78 $\pm$ (4.31)	6.91 $\pm$ (0.85)	11.25 $\pm$ (1.12)
Diazepam 5 $\mu$ g (N=11)	33.94 $\pm$ (6.61)	8.18 $\pm$ (1.11)	16.00 $\pm$ (1.76)

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## **CHAPTER 4**

**FG-7142, YOHIMBINE, AND BCCE PRODUCE ANXIOTIC-LIKE EFFECTS IN THE ELEVATED PLUS-MAZE BUT DO NOT AFFECT BRAINSTEM ACTIVATED HIPPOCAMPAL THETA.**

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## 1.) Introduction

Anxiety disorders continue to inflict personal, societal and economic hardships and have spurred an enormous amount of neuropsychiatric research. At the forefront of this effort is the identification of neurobiological systems (e.g., GABA<sub>A</sub> receptor currents in limbic structures) that mediate fearful behaviour, both adaptive and maladaptive. One relatively novel target system is rhythmical slow (~3-12Hz) local field activity (theta) generated by the septo-hippocampal complex (Gray and McNaughton, 2000; McNaughton et al., 2007). The most compelling evidence for this neural focus, outside of actual behavioural correlates, is the demonstration that all clinically proven anxiolytic drugs (e.g. benzodiazepines like diazepam, 5-HT<sub>1A</sub> agonists like buspirone, and selective serotonin reuptake inhibitors (SSRIs) like fluoxetine) suppress the frequency of reticularly-elicited hippocampal theta brain rhythm in urethane-anesthetized rats (McNaughton et al., 2007).

Recently, our group has assessed the predictive validity of this neurophysiological model by testing novel compounds that were not known to have anxiolytic action but showed theta-slowng effects (Engin et al., 2008; Yeung et al., 2012a; Yeung et al., 2012b). In all cases thus far – including the persistent sodium current ( $I_{NaP}$ ) channel blocker and antiepileptic agent phenytoin, as well as the hyperpolarization-activated inward current ( $I_h$ ) channel blocker and bradycardic agent ZD7288 – agents that slow evoked hippocampal theta also produced anxiolytic-like effects in rodent behavioural models such as the elevated plus maze (Yeung et al., 2012a; Yeung et al., 2012b). Although this is powerful support for the predictive validity of the theta suppression model, it is not proof that theta frequency in this assay directly indexes anxiety

*per se*, and as such does not support its construct validity (Cronbach and Meehl, 1955; Treit et al., 2010; Westen and Rosenthal, 2003). Although the construct validity of this neurophysiological model is impossible to address behaviorally (since animals are fully anesthetized), a pharmacological approach is feasible: We reasoned that if anxiolytic agents slow theta frequency, then anxiogenic agents should accelerate it.

The present study tested this critical prediction using three well established anxiogenic agents (FG7142,  $\beta$ CCE, and yohimbine). All three have produced anxiogenic effects in a variety of mammalian species, including humans (Cole et al., 1995; Dorow et al., 1987; Dorow et al., 1983; Handley and Mithani, 1984; Johnston and File, 1989; Rodgers et al., 1995). In addition, while FG7142 and  $\beta$ CCE target similar neural substrates to elicit their anxiogenic effects, yohimbine exerts its effects at a completely separate target. This could provide clues about the possible contributions of GABAergic and noradrenergic mechanisms to the results. FG7142 and  $\beta$ CCE are partial inverse agonists at the benzodiazepine site on the GABA<sub>A</sub> receptor (Evans and Lowry, 2007; Taguchi and Kuriyama, 1990). In direct opposition to benzodiazepine anxiolytic drugs such as diazepam, they *decrease* inhibitory chloride ion flux normally mediated by GABA at the GABA<sub>A</sub> receptor, thus increasing neuronal excitability. In contrast, yohimbine is an antagonist at pre-synaptic  $\alpha$ 2 adrenergic receptor sites (Millan et al., 2000). Antagonists at these sites increase the release of norepinephrine, which can in turn increase post-synaptic neuronal excitability (Goddard et al., 2010; Goldberg et al., 1983; Starke et al., 1975).

If these three anxiogenic drugs increase anxiety as measured in both behaviour (e.g., a decrease in open arm exploration in the elevated plus-maze), and neurophysiology (e.g. an increase in the frequency of brainstem-evoked theta in the urethane anesthetized rat) this would provide powerful evidence for the construct validity of the theta suppression model. If, however,



there are discontinuities between the behavioral and electrophysiological effects of these drugs, the construct validity of the theta suppression model would be questionable.

## **2.) Materials and Methods**

### *2.1 Subjects*

Subjects were 76 male Sprague-Dawley rats, weighing 200-300g, 28 of which were randomly assigned for neurophysiology (evoked theta frequency analysis), and 48 of which were assigned for behavioural analysis (the elevated plus-maze). Rats were pair-housed in polycarbonate cages 47x25x20.5 cm for the duration of the experiment and maintained on a 12:12 h light/dark cycle (lights on at 0600 h). Food and water were available ad libitum. The treatment of all animals was in compliance with the Canadian Council on Animal Care, and the National Institutes of Health Guide for Care and Use of Laboratory Animals. All procedures were also approved locally by the Biosciences Animal Policy and Welfare Committee of the University of Alberta. Power analyses were carried out before experiments to minimize the number of animals used and all possible measures to minimize suffering and stress were taken during the experiments.

### *2.2 Elevated Plus-Maze and Analysis*

Just prior to experimentation, rats in the behavioural experiment were randomly assigned to one of four drug conditions: 1) FG7142 (10mg/kg) obtained from Tocris Bioscience (Bristol, UK), 2.)  $\beta$ CCE (10mg/kg) obtained from Sigma-Aldrich (ON, Canada), or 3) yohimbine (5mg/kg) obtained from Abcam Biochemicals (MA, U.S.A). A fourth group received a vehicle solution. FG7142,  $\beta$ CCE and yohimbine were dissolved in a 10% DMSO vehicle solution at a

final concentration of 10mg/mL or 5mg/mL respectively. The final injection volumes ranged from 0.2mL – 0.3mL, depending on the weight of the animal. These drugs and doses reliably produce anxiogenic effects in rat models of anxiety such as the elevated plus-maze (Cole et al., 1995; Johnston and File, 1989; Mason et al., 1998). All injections were *i.p.* and made 30 minutes prior to testing.

The maze was a plus-shaped apparatus with an open roof, consisting of two 50×10 cm open arms, and two 50×10×50 cm enclosed arms, elevated at a height of 50 cm. All testing was conducted between 0900 and 1700 h in a quiet, dimly illuminated room and was recorded on videotape. The experimenter always sat in a chair positioned in the same position in the room during plus-maze testing. To ensure blind behavioral coding, only the animal number and not the group it belonged to was shown in the videotape. Each animal was tested for 5 min. Four variables were measured: (1) time spent in the open arms; (2) time spent in the closed arms; (3) number of entries into the open arms; and (4) number of entries into the closed arms. A rat was considered to have entered or spent time in an arm only when all four paws were in the respective arm. The time spent in the open arms and the number of open-arm entries were expressed as a percentage of total time spent in the open arms ( $\text{open-arm time} / (\text{open-arm time} + \text{closed-arm time}) \times 100$ ), and total arm entries ( $\text{open-arm entries} / (\text{open-arm entries} + \text{closed-arm entries}) \times 100$ ), respectively. A lower percentage of open-arm time or open-arm entries compared to vehicle control indicated increased anxiety (anxiogenesis). In addition, the number of closed arms entered and the total number of arms of either kind entered were taken as indices of general activity (Hogg, 1996; Pellow and File, 1986).

The results from the elevated plus- maze test were expressed as means and standard errors of the mean (S.E.M). Behavioural measures were analyzed with planned comparisons ANOVA;  $\alpha = 0.05$  (Keppel and Zedeck, 1989). One rat (n=1 vehicle) was excluded from the analysis of the plus-maze data because the number of times it fell off the maze was greater than three standard deviations from the mean ( $z \geq 3.08$ ), leaving a final N of 47. Following experimentation, rats were euthanized in a gas chamber with 100% N<sub>2</sub>O.

### *2.3 Neurophysiological Experiments and Analysis*

The same four drug conditions from the behavioural experiments were tested in the neurophysiological experiments. In addition, three other drug conditions were tested in the neurophysiological experiments: 1) high dose of FG7142 (100mg/kg) and 2) high dose of  $\beta$ CCE (100mg/kg) were added to establish a high-dose comparison, and 3) an anxiolytic dose of diazepam (5mg/kg), was added as a positive control condition. The final injection volumes ranged from 2.0-3.0 mL depending on the weight of the animal. In the neurophysiological experiments, *i.p.* injections were made following baseline recordings of evoked theta and 30 minutes prior to post-infusion recordings.

Animals were initially anesthetized with isoflurane in 100% O<sub>2</sub> at a minimal alveolar concentration (MAC) of 4 and were maintained following induction at a MAC of between 1.5 and 2.5 while implanted with a jugular catheter. Isoflurane was discontinued, and switching of general anesthesia was achieved with gradual *i.v.* administration of urethane (0.8 g/ml; final dose:  $1.27 \pm 0.01$  g/kg) via the jugular vein. Level of anesthesia was assessed throughout the experiment by monitoring the toe pinch withdrawal reflex and a supplemental dose of urethane (0.01 ml) was administered whenever a toe withdrawal was observed. Once anesthetized to a surgical plane, rats were transferred and positioned in a stereotaxic apparatus (Model 900, David

Kopf, Tujunga, CA, USA) where they remained for the duration of the experiment. Body temperature was maintained at 37 °C using a servo-controlled heating pad that was coupled to rectal temperature (TR-100; Fine Science Tools, Vancouver, BC, Canada).

Each rat was implanted with a monopolar recording electrode at the level of the hippocampal fissure in the dorsal hippocampus (AP -3.3 mm, ML  $\pm$ 2.2 mm, DV -2.7 mm) and a bipolar stimulating electrode was lowered into the brain stem (AP -8.0 mm, ML  $\pm$ 1.6 mm, DV -6.8-7.5 mm) (Paxinos, 1986). The electrodes were secured to the skull with jeweller's screws and cranioplastic cement. Recordings began following successful implantation of all electrodes. The entire experimental protocol was subsequently conducted without supplemental injections of urethane. Recordings were made by referencing to the stereotaxic apparatus, amplifying at a gain of 1000 and filtering between 0.1 and 20kHz using a differential AC amplifier (Model, 1700, A-M Systems Inc., Carlsborg, WA, USA). Signals were digitized on-line using automatic antialiasing filtering (low pass at half the sample frequency) and sampled at 50 Hz with a Digidata 1322A A-D board connected to a Pentium PC running the AxoScope acquisition program (Axon Instruments, Union City, CA,). Our sampling frequency and low pass filter were reduced markedly to eliminate the 100 Hz artifact from stimulation trials which enabled us to characterize brainstem influences online and also reduced artifacts during spectral analysis. Stability of the brainstem-evoked responses was assured based on the low variance of theta frequencies evoked by multiple stimulation trials at the same supra-threshold level.

Brainstem sites eliciting theta were selected based on 1) minimal threshold intensities to evoke theta (conducted during deactivated states (Wolansky et al., 2006) when theta was not apparent spontaneously) as well as 2) an observable increase in theta frequency as stimulation intensities were elevated. Stimulation consisted of 0.1 ms biphasic constant current pulses at 100

Hz for 5 s with an inter-pulse period of 30 s using an electrically isolated pulse generator in constant current mode (Model 2100; A-M Systems). As reported earlier (McNaughton and Sedgwick, 1978) the mean threshold intensity of stimulation to elicit theta differed from animal to animal (overall mean threshold:  $30.4 \pm 8.8 \mu\text{A}$ ). Therefore, a threshold intensity to elicit theta was established separately for each animal and the stimulation intensities used in the experiment were 1, 2, 3 and 4 times this threshold level. Three counterbalanced trials consisting of a randomized set of each stimulation intensity were used to compute the average evoked theta frequency for each level. Following such a baseline (no drug) recording session, animals were administered *i.p.* drugs according to their assignments (above). Following a 30 minute period after administration, the above stimulation trials were repeated using the same series of intensities, again randomized. At the end of the experiment, rats were euthanized with an *i.v.* overdose of urethane (1-2mL).

Evoked theta activity during the 5s interval of brainstem stimulation was analyzed by spectral analysis (Fast Fourier Transform: FFT) in Clampfit (Axon Instruments). In some cases, signals were high pass filtered at 1Hz using a 8-pole Bessel function in order to eliminate slow near-DC artifacts caused by the stimulation. Each trial was windowed using a Hamming function and was then subjected to the FFT algorithm using a window size of 128 samples without overlap. The peak frequency and power were extracted from the resulting spectrum and plotted as a function of stimulation intensity. The average of the peak frequency and power at the three different time points pre-infusion and post-infusion were calculated and used as raw data. All analytical data were graphically expressed as means and standard deviation of the means. Differences in average peak frequencies and average power pre and post drug infusion were assessed using paired within-subjects t-tests,  $\alpha=0.05$ , two tailed (Keppel and Zedeck, 1989).

These assessed whether the average peak frequency and average power at each intensity was significantly increased after drug administration. Pairwise between-subjects comparisons of the peak frequencies and power between each of the five experimental conditions (i.e. FG7142 (10 and 100mg/kg),  $\beta$ CCE (10 and 100mg/kg), and yohimbine (5mg/kg) groups were conducted using independent sample t-tests,  $\alpha=0.05$ , two tailed (Keppel and Zedeck, 1989). These evaluated the significance of differences between the average peak frequency and average power at each threshold stimulus intensity as a function of the five different experimental conditions. Animals were only excluded (n=5) from data analysis if evoked theta frequencies had a non-linear relationship with stimulus intensity (e.g., with each increase in stimulation intensity, there was no increase in peak frequency of theta).

### **3.) Results**

#### *3.1 Elevated Plus-Maze.*

As can be seen in Figure 1, FG7142 (10 mg/kg)  $\beta$ CCE (10 mg/kg) and yohimbine (5mg/kg) all produced clear anxiogenic-like effects in the elevated plus-maze. Planned comparisons revealed that the FG7142, ( $F_{(1,44)} = 5.38$ ;  $p < 0.05$ ),  $\beta$ CCE ( $F_{(1,44)} = 4.74$ ;  $p < 0.05$ ) and yohimbine groups ( $F_{(1,44)} = 5.73$ ;  $p < 0.05$ ) spent proportionally *less* time in the open arms of the plus-maze in comparison to the vehicle control group. There were no differences in the degree to which each drug suppressed the proportion of open-arm time (all probabilities  $< 0.05$ ). Although all three agents also tended to suppress open-arm entries (Table 1), neither FG7142 ( $F_{(1,44)} = 0.72$ ;  $p > 0.05$ ),  $\beta$ CCE ( $F_{(1,44)} = 1.73$ ;  $p > 0.05$ ), nor yohimbine ( $F_{(1,44)} = 2.60$ ;  $p > 0.05$ ) produced a significant reduction in this measure. Finally, measures of general activity (closed and total arm entries) did not differ significantly between any of the groups ( $p > 0.05$ ; Table 1).

### 3.2 Neurophysiology.

As previously shown (McNaughton et al., 2007), the frequency of brainstem-evoked hippocampal theta during baseline conditions showed a positive linear relationship with increasing intensities of stimulation during baseline conditions in all animals tested (see example in Figure 2). In contrast, the power of brainstem-evoked hippocampal theta did not change with increasing intensities of stimulation (not shown), a finding which is also consistent with previous work (McNaughton et al., 2007). We confirmed in our pre-post comparisons that the anxiolytic drug diazepam (5 mg/kg) significantly reduced the frequency of evoked theta at all stimulation intensities except for the lowest one (1x threshold stimulation intensity:  $F_{(1,2)} = 0.35$ ;  $p > 0.05$ ; 2x threshold stimulation intensity:  $F_{(1,2)} = 4.59$ ;  $p < 0.05$ ; 3x threshold stimulation intensity:  $F_{(1,2)} = 4.82$ ;  $p < 0.05$ ); and 4x threshold stimulation intensity  $F_{(1,2)} = 14.44$ ;  $p < 0.05$ ) (Figure 3). In contrast to the prominent effects of diazepam, *none* of the anxiogenic drugs (i.e. FG7142 (10 and 100mg/kg),  $\beta$ CCE (10 and 100mg/kg), yohimbine (5mg/kg) produced any changes in theta frequency in pre-post drug comparisons (and certainly no increases), at any stimulation intensity (all probabilities  $p > 0.05$ , Figure 3).

We also compared evoked theta frequencies across all five experimental drug conditions (Figure 3). Consistent with our pre-post comparisons, no differences in theta frequency were observed between the FG7142 (10 and 100mg/kg),  $\beta$ CCE (10 and 100mg/kg), and yohimbine (5mg/kg) groups in comparison to the vehicle control group at any stimulation intensity (all probabilities  $p > 0.05$ , Figure 3). Similarly, there were no differences in evoked theta frequency between any of these experimental groups at any level of threshold stimulation intensity (all probabilities  $p > 0.05$ ). Finally, diazepam (5 mg/kg) significantly reduced the frequency of evoked

theta at all stimulation intensities in comparison to the vehicle control group (all probabilities  $p < 0.05$ )

#### 4.) Discussion

Although the reduction of evoked hippocampal theta frequency in the urethane anesthetized rat has been shown to be remarkably predictive of anxiolytic action, even for completely novel agents, it is clear from our present results that anxiogenic drug action is not indexed by changes in theta frequency in this same assay. All three confirmed anxiogenic agents (FG7142,  $\beta$ CCE and yohimbine) promoted clear anxious-like behaviour in the most widely validated behavioral model of anxiety, the elevated plus-maze; however, in our neurophysiological assessment, we found no enhancement of evoked theta frequency following the administration of any of the three anxiogenic drugs in rats under urethane anaesthesia. Taken together, these findings suggest that while the reduction of theta frequency in the neurophysiological assay is still useful for detecting anxiolytic drug action, it lacks construct validity as a measure of anxiety *per se*.

The dose parameters and route of administration chosen for FG7142,  $\beta$ CCE, and yohimbine were based on those shown to be anxiogenic in behavioral tests of anxiety such as the elevated plus-maze (Braun et al., 2011; Cole et al., 1995; Handley and Mithani, 1984; Johnston and File, 1989; Jones et al., 2002). Indeed, the efficacy of these parameters was confirmed in every case in the current study using the elevated plus- maze. However, the same doses that produced significant anxiogenesis in the plus-maze had no significant effect on evoked hippocampal theta across stimulation intensity. Even when we increased the dosage of FG-7142 and  $\beta$ CCE by an order of magnitude (100mg/kg), evoked hippocampal theta frequency did not



increase at any stimulation intensity. At the same time, we confirmed the significant suppressive effect of the prototypical anxiolytic diazepam on hippocampal theta frequency at all stimulation intensities. Finally, none of the three anxiogenic drug effects in the plus-maze were confounded by non-specific effects on general activity (see Table 1).

One possible limitation of the present study could be that the stimulation parameters we used to detect drug effects were at a ceiling for anxiogenic effects. In other words, the brainstem stimulation intensities we used to elicit hippocampal theta were too high for detecting drug-induced increases. This is highly unlikely for several reasons. First of all, and most convincingly, agonists of cholinergic/muscarinic neurotransmission have been directly shown to increase theta frequencies in the urethane anesthetized preparation, either during spontaneous (Olpe et al., 1987; Rowntree and Bland, 1986) or brainstem-evoked conditions (Kinney et al., 1999). Secondly, the maximum frequency elicited by brainstem stimulation using similar stimulation parameters has been reported at around 15Hz (McNaughton et al., 2007). Although we obtained clear intensity by frequency effects, suggesting that we were stimulating relevant brainstem regions, we did not observe stimulation-induced frequencies beyond 8.18Hz (average peak frequency:  $6.48 \pm 0.87$ Hz). Regardless, if theta frequency was indexed directionally to a pharmacological modulation of anxiety we should have observed a drug effect at threshold and sub maximal intensities, which we did not.

Our results clearly show that none of FG7142, yohimbine or  $\beta$ CCE had any positive modulatory effect on evoked hippocampal theta frequency. Interestingly, Hajos and colleagues (2004) found that FG7142 (1mg/kg) enhanced the firing rate and theta rhythmicity of medial septal/diagonal band neurons and induced an increase in the power of spontaneous theta in

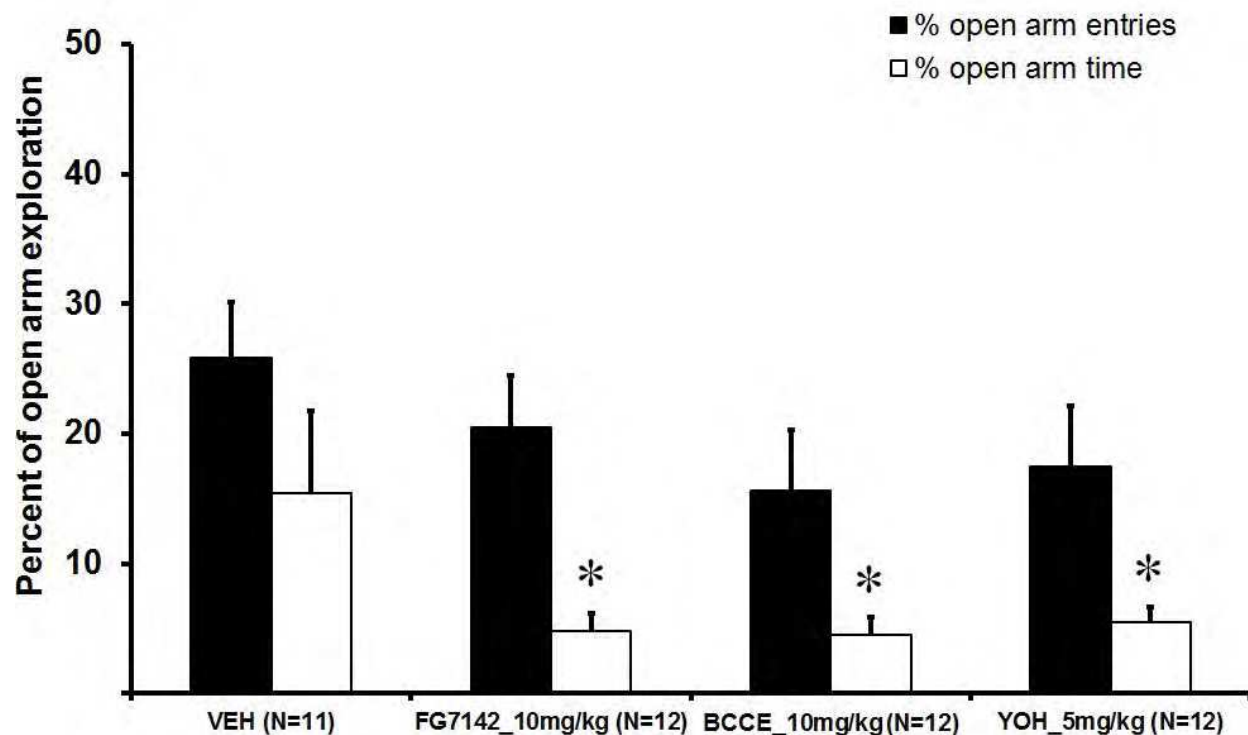
chloral hydrate anesthetized rats. In addition, they also found that FG7142 could reverse the inhibition of the above measures induced by diazepam . While these data suggest that an anxiogenic agent acting at the benzodiazepine receptor can affect the detection of theta, it does not provide any evidence for the specific modulation of theta frequency that is critical for the neurophysiological assay we tested here. This is also true of other agents or manipulations that alter GABA<sub>A</sub> receptor mediated neurotransmission or kinetics which have effects on either the power or rhythmicity (or both) of theta (Hentschke et al., 2009; Steffensen, 1995; White et al., 2000; Ylinen et al., 1992).

The “online” relationship of hippocampal theta and its modulation by anxiety related drugs in behaving preparations for the moment to moment expression of anxiety is also unclear. In those cases in which this has been assessed, there is inconclusive evidence about the directionality of theta modulation e.g. whether theta frequency is increased (Yabase et al., 1988; Yamamoto, 1998) or decreased (Chilingarian and Bogdanov, 1998). Additionally, a serious and often overlooked confound in freely behaving experiments is the potential for a concomitant drug-induced modulation of motor behaviour, the vigour of which has been shown to correlate with increases in theta frequency (Vanderwolf, 1969, 1975). This is in contrast with the idea that theta, independent of movement *per se*, might correlate with the expression of fear (Sainsbury, 1985). Nevertheless, it has been shown that theta power - as well as coherence to frontal sites - was significantly increased in freely moving rats in the open field and elevated plus-maze (Adhikari et al., 2010; Gordon et al., 2005), suggesting that specific properties of theta can be associated with situations designed to promote anxiety. In order to further delineate the role of anxiogenic agents on theta and behavioral anxiety, future studies will require simultaneous intra-

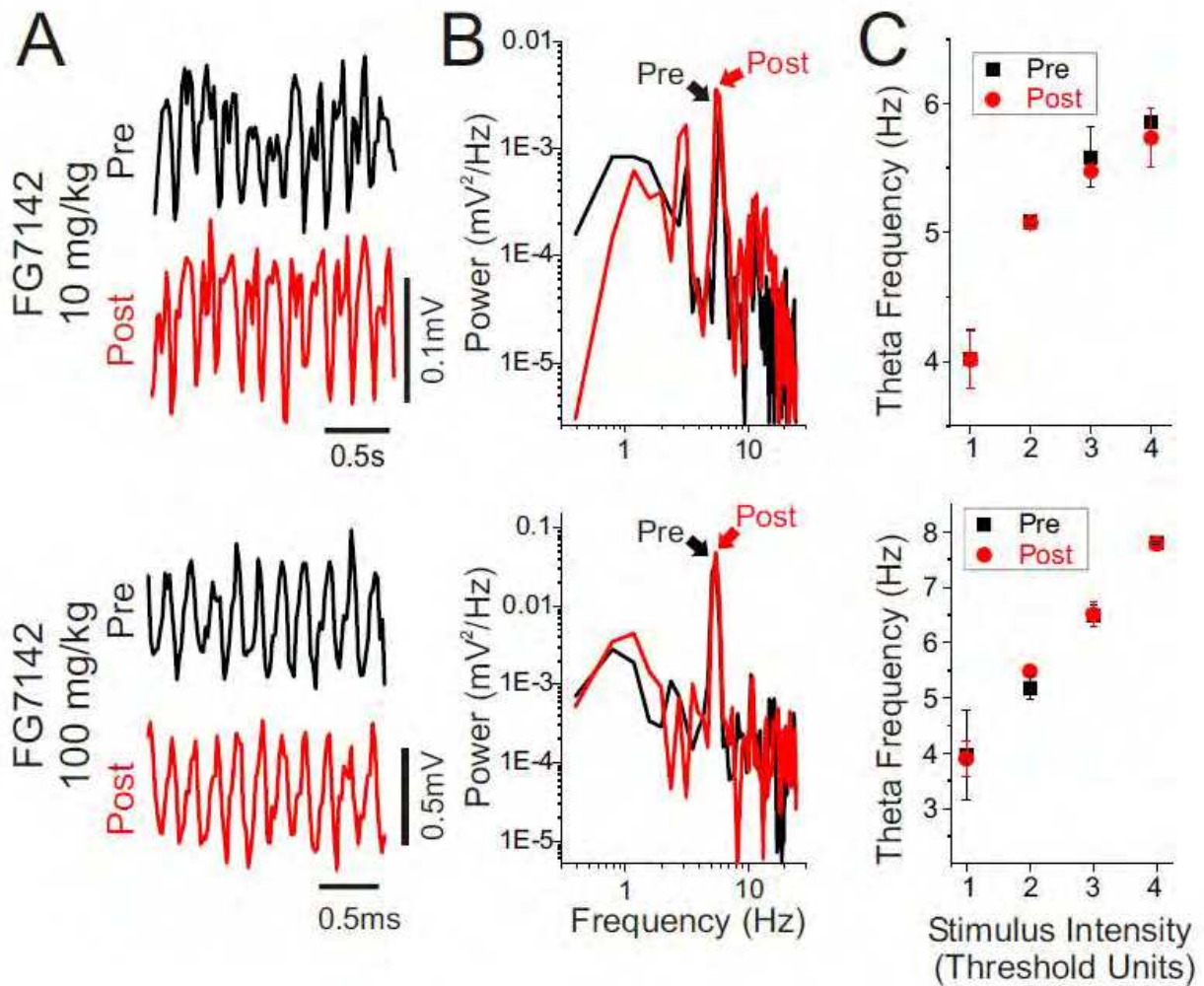
hippocampal ligand micro-infusions and in situ neural recordings in awake animals under strict behavioural monitoring.

Given the clear lack of an anxiogenic-induced increase in evoked theta frequencies in the present study, the question remains as to why anxiolytic drugs all appear to depress evoked theta frequencies in the urethane-anesthetized rat. An explanation that is parsimoniously consistent with the present findings would be that the neurobiological mechanisms of anxiolytic drug action, while perhaps independent, may overlap with those that might mediate theta frequency slowing. At the level of the hippocampus, a likely target is GABAergic neurotransmission which, when agonized, reduces neuronal (and perhaps behavioural) excitability and would slow synaptic currents contributing to local field potentials such as theta. In this regard, many clinically-validated anxiolytics, including benzodiazepines and monoamine reuptake inhibitors such as SSRIs are thought to act directly or indirectly on GABAergic neurotransmission (Engin et al., 2012; Luscher et al., 2011; Mendez et al., 2012; Mendez, 2012). Taken together, while theta frequency suppression in the urethane anesthetized preparation is a powerful and predictive correlate of anxiolytic drug action, this measure is not bidirectionally indexed to anxiety *per se*, as tested in behavioural assays.

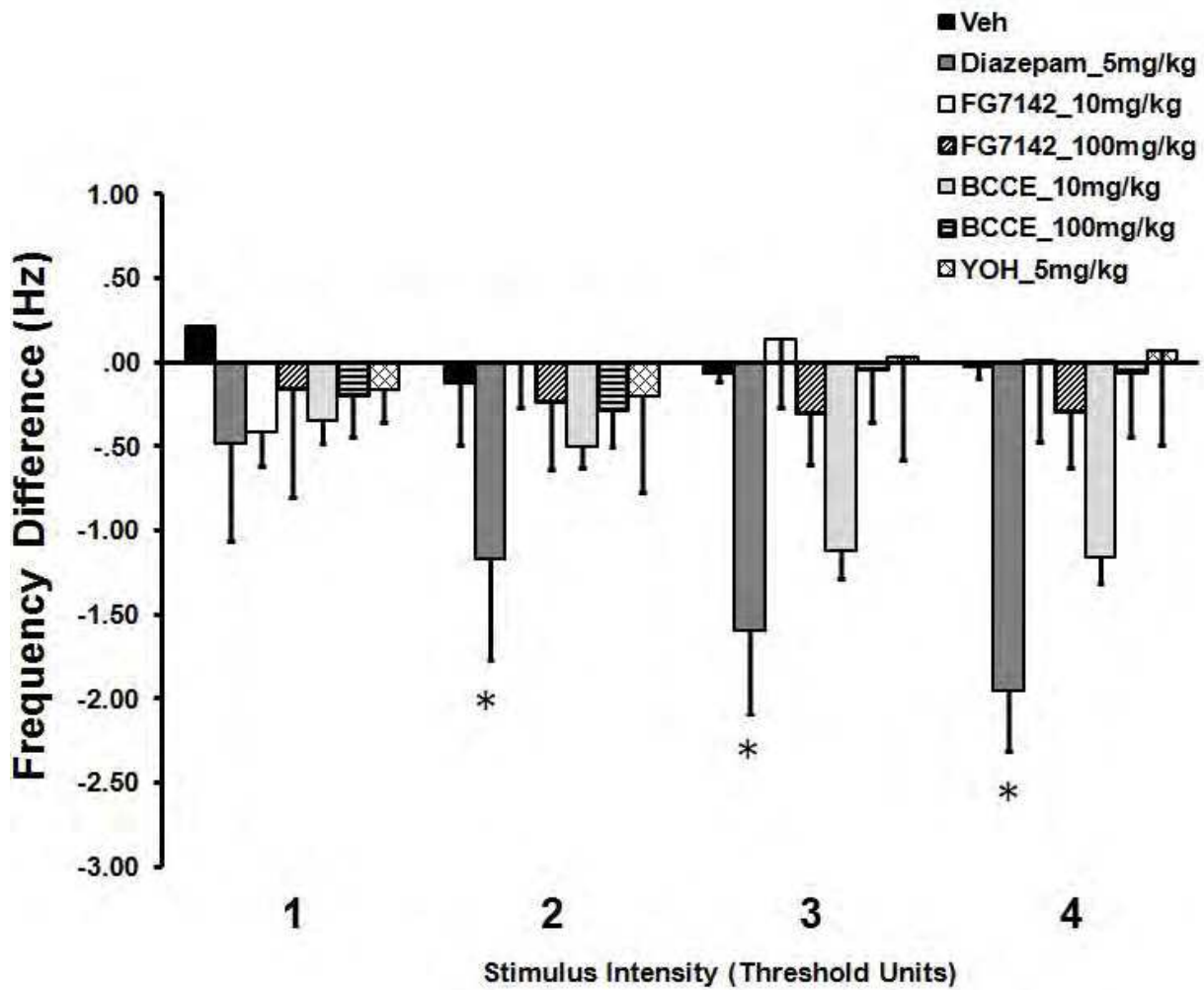
**Fig. 4.1:** Open-arm activity following intra-peritoneal (i.p) injections of FG7142 (10mg/kg), BCCE (10mg/kg), yohimbine (5mg/kg), or vehicle control in the elevated plus-maze test. Black bars represent means ( $\pm$ SEM) percentage of open arm entries; white bars represent means ( $\pm$ SEM) percentage of open arm time. \*Significantly different from the vehicle control group ( $p < 0.05$ ).



**Fig. 4.2:** A) Representative raw LFP traces of brainstem-evoked theta pre- and post-administration of FG7142 are shown in both upper and lower panels (the top panel shows the response to 120 $\mu$ A -4X threshold- stimulation and 10 mg/kg drug and the bottom to 40 $\mu$ A -2X threshold- stimulation and 100 mg/kg drug). B) Spectra of the respective pre and post-drug treatment traces shown in panel A are overlaid. Neither the low or high dose of FG7142 produced any notable changes in either peak frequency or amplitude of evoked theta. C) Average data for the experiments shown in A and B showing the frequency of evoked theta at each level of stimulus intensity as measured in threshold units (1X, 2X, 3X, and 4X threshold value) as a function of drug treatment. Both panels show a lack of effect of the 10 and 100 mg/kg of FG7142. Black squares represent means ( $\pm$ SD) of evoked theta frequency pre-administration of FG7142. Red circles represent means ( $\pm$ SD) of evoked theta frequency post-administration of FG7142.



**Fig. 4.3:** Difference in evoked theta frequency (Hz) at 1x, 2x, 3x, and 4x threshold units of stimulus intensity pre and post intra-peritoneal (i.p) administration of FG7142 (10 or 100mg/kg), BCCE (10 or 100mg/kg), yohimbine (5mg/kg), diazepam (5mg/kg), or vehicle control in neurophysiological experiments. Black bars represent mean differences ( $\pm$ SEM) of evoked theta frequency pre and post administration of vehicle control. Dark grey bars represent mean differences ( $\pm$ SEM) of evoked theta frequency pre and post administration of diazepam. White bars represent mean differences ( $\pm$ SEM) of evoked theta frequency pre and post administration of FG10. Diagonally striped bars represent mean differences ( $\pm$ SEM) of evoked theta frequency pre and post administration of FG100. Light grey bars represent mean differences ( $\pm$ SEM) of evoked theta frequency pre and post administration of BCCE10. Horizontally striped bars represent mean differences ( $\pm$ SEM) of evoked theta frequency pre and post administration of BCCE100. Checkered bars represent mean differences ( $\pm$ SEM) of evoked theta frequency pre and post administration of yohimbine. \*Pre and post comparisons significantly different ( $p < 0.05$ ).



**Table 4.1:** Percentages of Time Spent in the Open Arms and General Activity Measures (Means  $\pm$ SEM) in the Elevated Plus-Maze test.

Drug	% Open Entries	Number of Closed Arm Entries	Number of Total Entries
Vehicle Control (N=11)	25.85 $\pm$ (4.28)	6.45 $\pm$ (0.93)	8.64 $\pm$ (1.12)
FG7142 10mg/kg (N=12)	20.45 $\pm$ (4.07)	6.17 $\pm$ (0.94)	7.83 $\pm$ (1.29)
BCCE 10mg/kg (N=12)	15.59 $\pm$ (4.73)	6.67 $\pm$ (0.76)	7.75 $\pm$ (0.81)
yohimbine 5mg/kg (N=12)	17.47 $\pm$ (4.65)	6.66 $\pm$ (1.21)	7.92 $\pm$ (1.34)

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## **CHAPTER 5**

**VENTRAL HIPPOCAMPAL HISTAMINE INCREASES THE  
FREQUENCY OF EVOKED THETA RHYTHM BUT PRODUCES  
ANXIOLYTIC-LIKE EFFECTS IN THE ELEVATED PLUS  
MAZE.**

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## 1.) Introduction

Despite an extensive and ongoing research effort directed towards characterizing the neurobiology of anxiety, current animal models focus mainly on behavioural effects, while neglecting other biomarkers. One relatively novel target system is rhythmical slow (~3-12Hz) local field activity (theta) generated by the septo-hippocampal complex (Gray and McNaughton, 2000; McNaughton et al., 2007). The most compelling evidence for this anxiolytic neurophysiological assay, outside of actual behavioural correlates, is the demonstration that all clinically proven anxiolytic drugs (e.g. benzodiazepines like diazepam, 5-HT<sub>1A</sub> agonists like buspirone, and selective serotonin reuptake inhibitors (SSRIs) like fluoxetine) suppress the frequency of brainstem-elicited hippocampal theta rhythm in urethane-anesthetized rats (McNaughton et al., 2007). Recently, we tested the predictive validity of this model by using two neuroactive compounds that we presumed would have a suppressive effect on theta but that were not known to have anxiolytic properties: 1) the persistent sodium current ( $I_{Nap}$ ) channel blocker and antiepileptic agent phenytoin, as well as 2) the hyperpolarization-activated inward current ( $I_h$ ) channel blocker and bradycardic agent ZD7288. As predicted, each of these drugs slowed evoked hippocampal theta, and in support of the McNaughton et al (2007) model, also produced anxiolytic-like behaviour in the elevated plus maze (Yeung et al., 2013; Yeung et al., 2012). Altogether, these findings point towards the potential usefulness of this neurophysiological assay as a reliable index of anxiolytic drug action.

More recently, however, this anxiolytic assay has been challenged. By infusing histamine at the level of the lateral septum, which is a target of hippocampal afferents (Haas and Panula, 2003; Haas et al., 2008), a potential modulator of theta activity via its connections to the medial septum (Panula et al., 1989), and a brain region known to be involved in emotional responding (Chozick, 1985; Menard and Treit, 2000; Sheehan et al., 2004; Chee et al., 2014) demonstrated a strong reduction of behavioural anxiety which corresponded to prominent *increases* as opposed to decreases in theta frequency in the neurophysiological assay. While these findings generally dispute the relevance of theta suppression to anxiety reduction, there are questions remaining. Given that theta is ultimately generated by hippocampal circuits (Goutagny et al., 2009) it is important to evaluate the effects of histamine directly at the level of the hippocampus itself.

The neural histaminergic system originates exclusively from hypothalamic tuberomammillary neurons and has diverse bidirectional connections to limbic system structures involved in emotional regulation, including the septal nuclei, amygdala, thalamus, as well as both dorsal and ventral hippocampus (Haas and Panula, 2003; Haas et al., 2008). Related to its modulatory influence on anxious-like behaviour, it has been shown that histamine infused into the lateral septum and dorsal hippocampus produces robust anxiolytic effects while ventral hippocampal infusions of histamine promotes the opposite, namely anxiogenesis (Chee and Menard, 2013; Chee et al., 2014; Piri et al., 2013; Rostami et al., 2006; Zarrindast et al., 2006). Given the seeming diversity of behavioural effects of histamine on dorsal versus ventral regions of the hippocampus, we sought to dissociate potential opposing influences on anxiety-like behaviours with influences on theta frequency modulation across these same two regions using targeted infusions of histamine. Here, we show that while dorsal hippocampal infusions of

histamine produced no changes in either anxiety-like behaviour in the elevated plus maze or in the frequency of evoked theta, ventral hippocampal infusions of histamine produced potent behavioural anxiolysis but contrastingly, robust *increases* in evoked theta frequency. These results further challenge the theta frequency suppression model as a measure of anxiolytic drug action *per se*.

## **2.) Materials and Methods**

### *2.1 Subjects*

Subjects were 129 male Sprague-Dawley rats, weighing 200-400g, 36 of which were randomly assigned to the theta suppression test, and 93 of which were assigned to the elevated plus-maze test. Rats were pair-housed in polycarbonate cages 47x25x20.5 cm for the duration of the experiment and maintained on a 12:12 h light/dark cycle (lights on at 0600 h). Food and water were available ad libitum. The treatment of all animals was in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals, and the Canadian Council on Animal Care. All procedures were also approved locally by the Biosciences Animal Policy and Welfare Committee of the University of Alberta. Power analyses were carried out before experiments to minimize the number of animals used and all possible measures to minimize suffering and stress were taken during the experiments.

### *2.2 Elevated Plus-Maze and Analysis*

Just prior to experimentation, rats in the behavioural experiment were randomly assigned to one of four drug conditions: 1) low dose histamine (1µg/µL). 2.) medium dose histamine

(10 $\mu$ g/ $\mu$ L), or 3) high dose histamine (100 $\mu$ g/ $\mu$ L). A fourth group received a vehicle solution. Histamine was obtained from Sigma-Aldrich (ON, Canada) and dissolved in 100% double distilled H<sub>2</sub>O. Solutions were infused via an infusion pump (Harvard Apparatus 22, MA, USA) at a rate of 1  $\mu$ l/min (0.5 $\mu$ l per hemisphere) for 1 minute through 26-gauge stainless-steel internal cannulae attached to a 10- $\mu$ l Hamilton syringe by polyethylene tubing. Infusions for neurophysiological experiments were performed stereotaxically while infusions for behavioural experiments were performed in the testing room on a table where the elevated plus maze apparatus was situated. Intra-hippocampal microinfusions of histamine and vehicle solution were made 5 min prior to evaluation.

The maze was a plus-shaped apparatus with an open roof, consisting of two 50 $\times$ 10 cm open arms, and two 50 $\times$ 10 $\times$ 50 cm enclosed arms, elevated at a height of 50 cm. All testing was conducted between 0900 and 1700 h in a quiet, dimly illuminated room and was recorded on videotape. The experimenter always sat in a chair positioned in the same position in the room during plus-maze testing. To ensure blind behavioral coding, only the animal number and not the group it belonged to was shown in the videotape. Each animal was tested for 5 min. Four variables were measured: (1) time spent in the open arms; (2) time spent in the closed arms; (3) number of entries into the open arms; and (4) number of entries into the closed arms. A rat was considered to have entered or spent time in an arm only when all four paws were in the respective arm. The time spent in the open arms and the number of open-arm entries were expressed as a percentage of total time spent in the open arms (open-arm time/open-arm time + closed-arm time)  $\times$  100, and total arm entries (open-arm entries/open-arm entries + closed-arm entries)  $\times$  100, respectively. A higher percentage of open-arm time or open-arm entries compared to vehicle control indicated increased anxiety (anxiolysis). In addition, the number of closed

arms entered and the total number of arms of either kind entered were taken as indices of general activity (Hogg, 1996; Pellow and File, 1986).

The results from the elevated plus- maze test were expressed as means and standard errors of the mean (S.E.M). Behavioural measures were analyzed with planned comparisons ANOVA;  $\alpha = 0.05$  (Keppel and Zedeck, 1989). Following experimentation, rats were euthanized in a gas chamber with 100% N<sub>2</sub>O. Three rats were excluded from analyses in the elevated plus maze data because they had obstructed cannulae.

### *2.3 Neurophysiology Experiments and Statistical Analysis*

The low (1 $\mu$ g/ $\mu$ L) and high dose histamine (100 $\mu$ / $\mu$ L) drug conditions from the behavioural experiments were tested in the neurophysiological experiments. In addition, a vehicle group and an anxiolytic dose of diazepam (5 $\mu$ g/ $\mu$ L at a final concentration of 17.56mM) was added as a positive control condition. Diazepam was obtained from Sabex Sandoz (QC, Canada) and was suspended in 40% propylene glycol, 10% dehydrated alcohol, 4.25% benzoic acid, and 1.5% benzyl alcohol in H<sub>2</sub>O. Intra-hippocampal microinfusions (procedure as detailed above) were made following baseline recordings of evoked theta. Histamine and vehicle microinfusions were made 5 min prior to evaluation, while diazepam microinfusions were made 30 min prior to evaluation.

Animals were initially anesthetized with isoflurane in 100% O<sub>2</sub> at a minimal alveolar concentration (MAC) of 4 and were maintained following induction at a MAC of between 1.5 and 2.5 while implanted with a jugular catheter. Isoflurane was discontinued, and general anesthesia was achieved with *i.v.* administration of urethane (0.8 g/ml; final dose: (1.87 $\pm$ 0.04g/kg) via the jugular vein. Level of anesthesia was assessed throughout the



experiment by monitoring the toe pinch withdrawal reflex and a supplemental dose of urethane (0.01 ml) was administered whenever a withdrawal was observed. Body temperature was maintained at 37°C using a heating pad and rectal probe (TR-100; Fine Science Tools, BC, Canada).

Each rat was implanted with monopolar recording electrodes at the level of the hippocampal fissure in the dorsal hippocampus (AP -3.3 mm, ML  $\pm$ 2.2 mm, DV -2.7 mm) and the ventral hippocampus (AP -5.3 mm, ML  $\pm$ 5.0 mm, DV -6.0-7.0 mm) and a bipolar stimulating electrode was lowered into the brain stem (AP -8.0 mm, ML  $\pm$ 1.6 mm, DV -6.7-7.2 mm). The electrodes were secured to the skull with jeweller's screws and cranioplastic cement. Recordings were made by referencing to the stereotaxic apparatus, amplifying at a gain of 1000 and filtering between 0.1 and 20 kHz using a differential AC amplifier (Model, 1700, A-M Systems Inc., Carlsborg, WA, USA). Signals were digitized on-line using automatic antialiasing filtering (high pass at half the sample frequency = 25Hz) and sampling at 50 Hz with a Digidata 1322A A-D board connected to a Pentium PC running the AxoScope acquisition program (Axon Instruments, Union City, CA, USA). Our sampling frequency and high pass filter was reduced markedly to eliminate the 100Hz artefact from stimulation trials which enabled us to characterize brainstem influences online.

Brainstem sites eliciting theta were selected based on minimal threshold intensities to evoke theta as well as an observable increase in theta frequency as stimulation intensities were elevated. Stimulation consisted of 0.1 ms biphasic constant current pulses at 100 Hz for 5 s using an electrically isolated pulse generator (Model 2100; A-M Systems). As reported earlier (McNaughton and Sedgwick, 1978) the threshold intensity of stimulation to elicit theta differed from animal to animal ( $28.63 \pm 5.06 \mu\text{A}$ ). Therefore, a threshold intensity of stimulation to elicit

theta was established separately for each animal and the stimulation intensities used in the experiment were 1, 2, 3 and 4 times threshold level. Three counterbalanced trials consisting of a randomized set at each stimulation intensity was used to compute the average evoked theta frequency for each level. At the end of the experiment, rats were euthanized with an overdose of urethane (1-2mL) and perfused intracardially with 0.9% (w/v) saline followed by 10% v/v formaldehyde. Once removed, brains were post-fixed in a 10% formalin solution for 48 hours before histological analysis (see below for details).

Evoked theta activity following brainstem stimulation was analyzed during each stimulation epoch by spectral analysis (Fast Fourier Transform: FFT) in Clampfit (Axon Instruments). Each trial was windowed using a Hamming function and was then subjected to the FFT algorithm. The peak frequency and power were extracted from the resulting spectrum and plotted as a function of stimulation intensity. The average of the peak frequency and average power at the four different time points pre-infusion and post-infusion were calculated and used as raw data. Because the statistical comparisons for the diazepam group ( $5\mu\text{g}/\mu\text{L}$ ) were *a priori* directional tests derived from the theta suppression model (Gray and McNaughton, 2000; McNaughton et al., 2007) and a replication of our previous experiments (Yeung et al., 2013; Yeung et al., 2012), these data were analyzed using one-tailed t-tests,  $\alpha=0.05$ . The data for our other experimental groups, histamine  $1\mu\text{g}$  and  $100\mu\text{g}/\mu\text{L}$  were analyzed using two-tailed t-tests,  $\alpha=0.05$ . Differences in average peak frequencies and average power pre and post drug infusion were assessed using paired within-subjects t-tests (Keppel and Zedeck, 1989). These assessed whether the average peak frequency and average power at each intensity was significantly suppressed after drug administration. Pairwise between-subjects comparisons of the peak frequencies and power between the histamine and vehicle conditions (i.e., histamine  $1\mu\text{g}/\mu\text{L}$ ,

histamine 100 $\mu\text{g}/\mu\text{L}$ , and vehicle) were conducted using two-tailed t-tests (Keppel and Zedeck, 1989). These evaluated the significance of differences between the average peak frequency and average power at each stimulus intensity as a function of histamine application.

#### *2.4 Histology*

Fixed brains were frozen with dry ice and cut into 60- $\mu\text{m}$  coronal sections with a sliding microtome (Model 860, American Optical Company, New York, USA). Every second section was collected and mounted onto a frosted microscope slide and later stained with thionin - for details see (Gerfen, 2003). The neurophysiological or behavioral data from animals with either one or both cannulae outside of the target area were excluded from the behavioral analysis.

### **3.) Results**

#### *3.1 Histology: Neurophysiological and Behavioural experiments*

Figures 1a and 1b shows the approximate infusion sites of rats with cannulae aimed at the hippocampus in both neurophysiological and behavioural experiments. In our neurophysiological experiments, 15 rats had cannula tips clustered in the ventral regions of the hippocampus (shown by positions of the x symbols), and 20 rats had cannula tips clustered in the dorsal regions of the hippocampus (shown by positions of the x symbols). One rat with cannula aimed at the dorsal hippocampus (n=1, diazepam 5 $\mu\text{g}/\mu\text{L}$ ) missed the target site and was excluded from data analysis. In our behavioural experiments, 43 rats had cannulae tips that were bilaterally clustered in the ventral regions of the hippocampus (shown by the position of the circles), and 45 rats had cannulae tips that were bilaterally clustered in the dorsal regions of the hippocampus (shown by the position of circles). Two rats with cannulae aimed at the dorsal hippocampus (n=2, histamine

1 $\mu$ g/ $\mu$ L) (shown by positions of star symbols) had placements that missed the target site and were excluded from data analysis.

### *3.2 Neurophysiology.*

As previously shown (McNaughton et al., 2007), the frequency of brainstem-evoked hippocampal theta prior to drug administration showed a positive linear relationship with increasing intensities of stimulation in all animals tested (see examples in Figure 2). In contrast, the power of brainstem-evoked hippocampal theta did not change with increasing intensities of stimulation (not shown), a finding which is also consistent with previous work (McNaughton et al., 2007). Prior to drug administration we also compared the differences in evoked theta frequency between our dorsal and ventral recording sites across all stimulation intensities. We found strong coupling of peak theta frequencies at both dorsal and ventral recording sites with no significant pairwise differences (see Figure 3a and 3b,  $p > 0.05$ ). This observation was also consistent across all experimental infusion conditions, regardless of site (dorsal or ventral). Given these findings, we collapsed our results across the two recording sites and report a single measure of evoked theta frequency across all conditions (means  $\pm$  SEM of dorsal/ventral theta frequency).

In our pre and post comparisons, we confirmed that anxiolytic drug diazepam (5  $\mu$ g/ $\mu$ L) administered following dorsal hippocampal infusions significantly reduced the frequency of evoked theta at all stimulation intensities except the lowest one (1x threshold stimulation intensity:  $t_{(14)} = 0.72$ ;  $p = 0.24$ ; 2x threshold stimulation intensity:  $t_{(14)} = 2.25$ ;  $p = 0.016$ ; 3x threshold stimulation intensity:  $t_{(14)} = 1.86$ ;  $p = 0.036$ ); and 4x threshold stimulation intensity  $t_{(14)}$

= 1.63;  $p=0.049$ ) (Figure 4a). We also demonstrated, for the first time, that ventral hippocampal infusions of diazepam significantly reduced the frequency of evoked theta at all stimulation intensities (1x threshold stimulation intensity:  $t_{(7)} = 1.78$ ;  $p=0.046$ ; 2x threshold stimulation intensity:  $t_{(7)} = 3.36$ ;  $p=0.002$ ; 3x threshold stimulation intensity:  $t_{(7)} = 2.63$ ;  $p=0.01$ ); and 4x threshold stimulation intensity  $t_{(7)} = 2.13$ ;  $p=0.026$ .

In contrast, a robust *increase* in theta frequency was observed at all stimulation intensities following ventral hippocampal infusions of the high dose of histamine (100 $\mu$ g) (1x threshold stimulation intensity:  $t_{(7)} = 7.65$ ;  $p<0.001$ ; 2x threshold stimulation intensity:  $t_{(1,7)} = 3.59$ ;  $p=0.001$ ; 3x threshold stimulation intensity:  $t_{(7)} = 2.88$ ;  $p=0.012$ ); and 4x threshold stimulation intensity  $t_{(7)} = 2.65$ ;  $p=0.019$ ) (Figure 4a). Additionally, we compared the level of increase in evoked theta frequencies following ventral histamine infusions (Figure 4a). Independent samples t-tests between groups also showed that ventral hippocampal infusions of 100 $\mu$ g histamine increased evoked theta frequency in comparison to the vehicle control group (1x threshold:  $t_{(7)} = 4.38$ ;  $p=0.001$ ; 2x threshold:  $t_{(7)} = 6.87$ ;  $p<0.001$ ; 3x threshold:  $t_{(7)} = 7.12$ ;  $p<0.001$ ; and 4x threshold:  $t_{(7)} = 4.86$ ;  $p<0.001$ ) and the 1 $\mu$ g histamine group (1x threshold stimulation intensity:  $t_{(7)} = 6.19$ ;  $p<0.001$ ); 2x threshold stimulation intensity:  $t_{(7)} = 5.91$ ;  $p<0.001$ ); 3x threshold stimulation intensity:  $t_{(7)} = 3.77$ ;  $p=0.001$ ; and (4x threshold stimulation intensity:  $t_{(7)} = 2.67$ ;  $p=0.018$ ). None of the lower doses of histamine infused into the ventral hippocampus had a significant effect on the frequency of evoked hippocampal theta (all probabilities  $p>0.05$ ). Finally, dorsal hippocampal infusions of histamine for all doses produced no significant differences on evoked theta frequencies, across all levels of stimulation intensity (all probabilities  $p>0.05$ ) (Figure 4b).

### 3.3 Elevated Plus-Maze.

Surprisingly (based on the electrophysiological results), as can be seen in Figure 5a, ventral hippocampal microinfusions of 100 $\mu$ g of histamine produced an anxiolytic effect in the plus-maze. Independent samples t-tests revealed that histamine at both 100 $\mu$ g ( $t_{(20)} = 2.99$ ;  $p=0.007$ ) and 10 $\mu$ g ( $t_{(21)} = 1.74$ ;  $p=0.048$ ) significantly increased the percentage of open-arm entries compared to the vehicle control group. A significant difference between the 100 and 1 $\mu$ g dose of histamine was also found for percent open-arm entries ( $t_{(18)} = 1.96$ ;  $p=0.033$ ) - suggesting a dose dependency of this action. Despite the increase in open arm entries, histamine did not produce a significant increase in the percentage of time spent in the open arms of the plus-maze at any dose, in comparison to the vehicle control group (histamine 100 $\mu$ g  $t_{(20)} = 1.01$ ;  $p=0.16$ ; histamine 10 $\mu$ g  $t_{(21)} = 0.46$ ;  $p=0.32$ ) or the 1 $\mu$ g group (histamine 100 $\mu$ g  $t_{(18)} = 0.85$ ;  $p=0.20$ ). Neither the percentage of open arm entries nor percentage of open-arm time differed significantly between the histamine 1 $\mu$ g group and the vehicle control group ( $p>0.05$ ). Finally, measures of general activity (closed and total arm entries) did not differ significantly between any of the groups ( $p>0.05$ ; Table 1).

In contrast with the significant effects of ventral hippocampal histamine infusions, dorsal hippocampal microinfusions of histamine did not produce a significant increase in the percentage of open arm entries or percentage of open-arm time in comparison to the vehicle control group ( $p>0.05$ ). In addition, measures of general activity (closed and total arm entries) did not differ significantly between any of the groups ( $p>0.05$ ).

#### 4.) Discussion

In stark contrast to both our and other's results demonstrating strong predictive validity of the theta frequency suppression model of anxiolytic drug action (McNaughton et al., 2007; Yeung et al., 2013; Yeung et al., 2012) we show here that ventral hippocampal infusions of histamine that produced anxiolytic-like effects on behaviour as assessed in the elevated plus-maze produced robust *increases* in brainstem-evoked theta frequencies. Interestingly, dorsal hippocampal infusions of histamine produced no significant changes in either the behavioral or neurophysiological assay. At a minimum, these results present a further challenge to the theta frequency suppression model of anxiolysis.

Histamine neurons originate from a single source, the tuberomammillary nucleus of the posterior hypothalamus, and have widespread innervations throughout the central nervous system including bidirectional projections to the septum, amygdala, and hippocampus. Neural histaminergic actions are mediated by three G-protein coupled receptors (H1-H3) and have typically been associated with the maintenance of wakefulness, behavioural arousal, and forebrain activation as well as a number of other physiological functions including feeding and weight homeostasis, learning and memory, inflammatory responses and affective and emotional processes including anxiety (Brown et al., 2001; Chee and Menard, 2013; Chee et al., 2014; Haas et al., 2008; Kumar et al., 2007). Histaminergic innervation of the cholinergic and GABAergic cells within the medial diagonal band of Broca (MDSB) could play an important role in hippocampal theta regulation given the known role of these MS projection cells in organizing rhythmic activity in the hippocampus (Green and Arduini, 1954; Kiss et al., 1997; Smythe et al., 1991; Yoder and Pang, 2005). Cholinergic mechanisms are themselves both necessary and important for theta (Kramis et al., 1975; Vanderwolf et al., 1988), and the co-localization and

release of histamine and acetylcholine into the MDSB may initiate a hyperactive cholinergic-histaminergic interaction, which in turn may modulate the enhancement of hippocampal theta properties. This explanation is also supported by radioimmunoassay studies which demonstrate that histamine acting via H1 and H3 receptors enhances acetylcholine secretion in the entorhinal cortex and basolateral amygdala (Bacciottini et al., 2002; Clapham and Kilpatrick, 1992; Passani et al., 2001).

The neuroanatomically distinct effects of histamine on both behaviour and theta properties highlight the pharmacological complexity and functional heterogeneity of histamine as a neurotransmitter in the hippocampus. Within the hippocampus, it has been observed that histamine injected into different cell layers can induce both depolarization and hyperpolarization of neuronal membrane potentials (Haas, 1981; Segal, 1981). With regards to this, it has been suggested that the inhibitory and behaviorally regulating actions of histamine may be localized to the ventral hippocampus. Indeed, it has been observed that ventral hippocampal application of histamine reduces behavioral anxiety by suppressing locomotion and exploratory behavior in the hole-board test, while dorsal hippocampal application produces no such effects (Alvarez and Banzan, 1985). The differences in both the behavioural and neurophysiological effects of dorsal versus ventral hippocampal infusions of histamine may be mediated by the differences in the density and distribution of H1, H2 and H3 receptors across the dorsal and ventral regions of the hippocampus and their potential interactions with cholinergic afferents (Alvarez and Banzan, 2001; Haas et al., 2008). H1 receptors and H2 receptors are primarily located post-synaptically and have excitatory actions on neuronal membranes. Conversely, H3 receptors function as an autoreceptors, regulating the release of histamine, and also as a heteroreceptors inhibiting the release of various neurotransmitters including GABA, dopamine, serotonin and acetylcholine



(Benarroch, 2010; Brown et al., 2001; Haas and Panula, 2003; Passani and Blandina, 2011; Passani et al., 2001). Within the dorsal and ventral regions of the hippocampus, H3 receptors show strong expression in the pyramidal cell layers of CA1 and CA3 suggesting that they may play an important modulatory role in the regulation of behavioral functions and the regulation of theta oscillatory activity (Pillot et al., 2002)

There is also substantial evidence suggesting a dissociation for the functional role of H1 and H2 receptors vs. H3 receptors. H1 receptors and H2 receptors have been shown to modulate feeding behaviors (Chee and Menard, 2013) and different aspects of memory including object recognition (da Silveira et al., 2013) and conditioned place preference (Zlomuzica et al., 2008) while H3 receptors may play an important role in mediating fear memory consolidation (da Silva et al., 2006) and unconditioned anxiety behaviors (Chee and Menard, 2013). This is consistent with immunolabelling studies, which have shown varying densities of histaminergic fibers within the hippocampus and amygdala and differences in the functional regional distribution of histamine receptors along the septotemporal axis. Specifically, there is evidence that ventral hippocampal histamine may modulate fear memory and avoidance responses while the dorsal anterior portions of the have no effect (Alvarez and Banzan, 2001; Haas and Panula, 2003; Haas et al., 2008)

Despite the discordant directionality between our behavioral and neurophysiological assays, our results of behavioral anxiolysis following histamine microinfusions into the brain directly parallel the findings from other groups using similar methodologies (Chee and Menard, 2013; Chee et al., 2014; Piri et al., 2013; Rostami et al., 2006; Zarrindast et al., 2006). Here, we observed a robust anxiolytic effect of histamine in the elevated plus-maze following ventral hippocampal infusions but not dorsal hippocampal infusions. Moreover, the observed increase in

open arm exploration following ventral hippocampal histamine application was dose-dependent, and independent from changes in locomotor activity. Our findings are consistent with recent studies which have demonstrated reliable anxiolytic effects of histamine following lateral septal injections (Chee and Menard, 2013; Chee et al., 2014), but in conflict with other studies which report opposing results to ours following dorsal and ventral hippocampal applications of histamine (Rostami et al., 2006; Zarrindast et al., 2006).

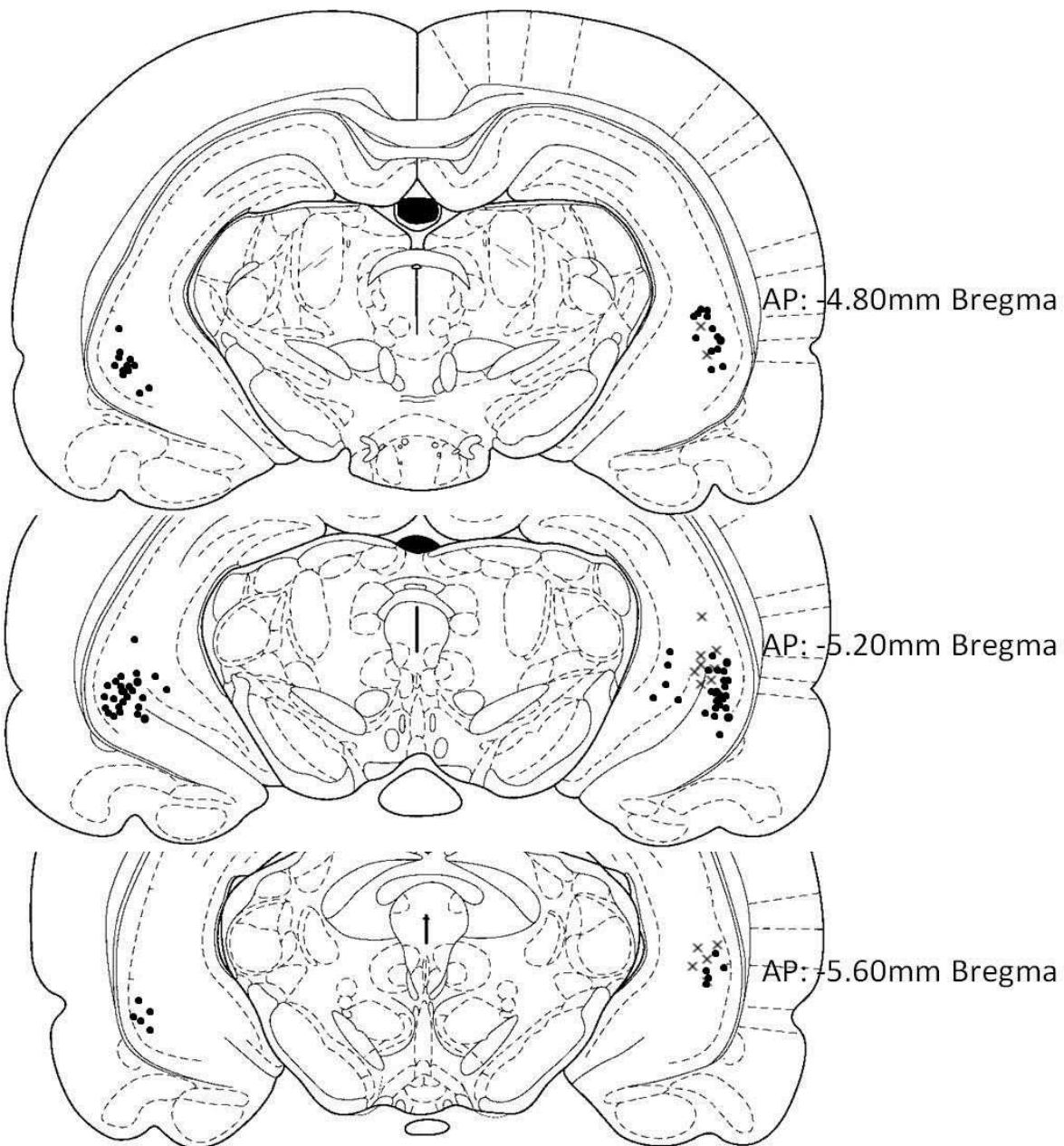
The discrepancies between our findings and the Zarrindast group may be attributed to a number of methodological differences, e.g. type (mouse (Piri et al., 2013) versus rat – present study) and strain of rodent (Wistar (Rostami et al., 2006) vs. Sprague Dawley – present study) as well as microinfusion parameters (unilateral microinfusion (Rostami et al., 2006) versus bilateral microinfusion – present study). Notably, our findings of anxiolysis following ventral histamine application are consistent with other experiments from this group evaluating the co-infusion of histamine along with an H1 (pyrilamine) and H2 receptor antagonist (ranitidine). The authors reported that high doses of pyrilamine and ranitidine alone produced anxiogenic-like responses when infused into the ventral hippocampus and histamine was able to reverse these effects- thereby suggesting an anxiolytic effect of histamine. Obviously, future studies are needed to delineate the interactions between histamine and other neurotransmitter systems at different doses.

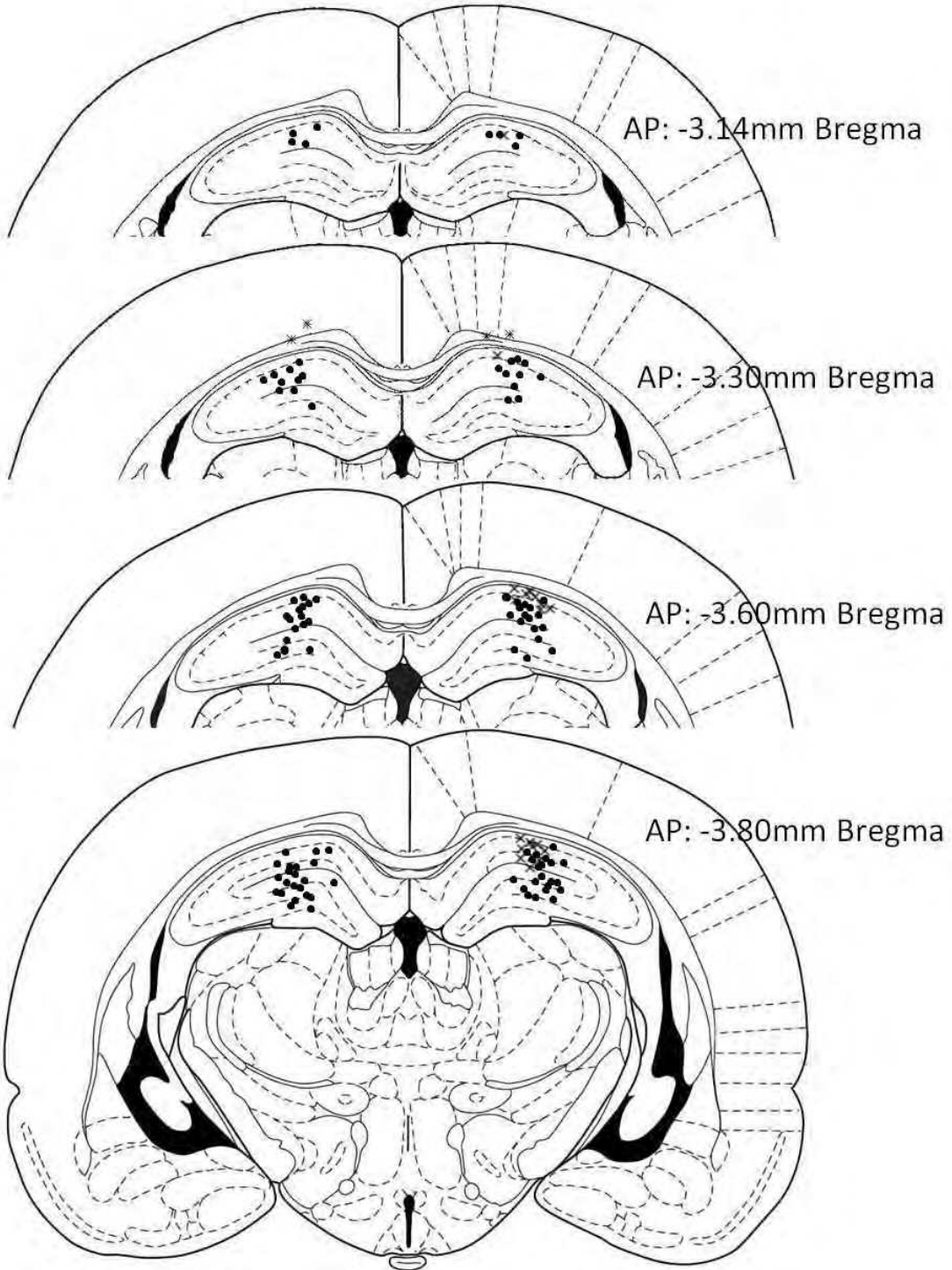
In our neurophysiology experiments, we clearly demonstrate findings that are consistent with our previous studies. Notably, we obtained clear intensity by frequency effects both prior to, and following application of histamine, suggesting that we were stimulating relevant brainstem regions. More convincingly, we also observed the robust suppression of evoked theta frequency

following administration of diazepam into both the dorsal and ventral hippocampus. This finding demonstrates, for the first time, that theta suppression following application of a well-established anxiolytic diazepam directly into different regions of the hippocampus, produces consistent theta slowing throughout the hippocampus.

While theta frequency suppression in the urethane anesthetized preparation has been a powerful and predictive correlate of anxiolytic drug action, our findings that histamine is anxiolytic and increases evoked frequency specifically following ventral hippocampal infusions directly challenges the theta suppression model as an index of anxiolytic drug action *per se*. Taken together, the robust associations between the pharmacological profile, neuroanatomical distribution, and functional roles of histamine, its receptors, and other neurotransmitters throughout the CNS make it an attractive target for further exploration of the relationship between behavioral anxiety and theta oscillatory properties.

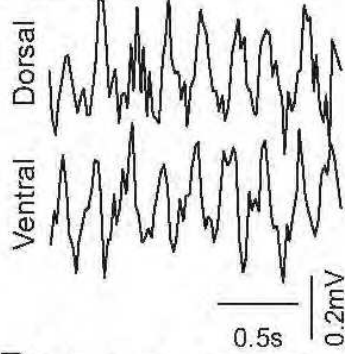
**Fig 5.1A,B:** **A)** Schematic diagram of coronal sections of the rat brain illustrating the approximate locations of ventral hippocampal infusion sites in behavioral experiments (black circles) and neurophysiological experiments (black x symbols). There were no misplaced cannulae in either neurophysiological or behavioural experiments. **B)** Schematic diagram of coronal sections of the rat brain illustrating the approximate locations of dorsal hippocampal infusion sites in behavioral experiments (black circles) and neurophysiological experiments (black x symbols). N=2 animals had misplaced cannulae in behavioral experiments (black asterisk symbols). There were no misplaced cannulae in neurophysiological experiments. The atlas plates are adapted from Paxinos and Watson (1986).



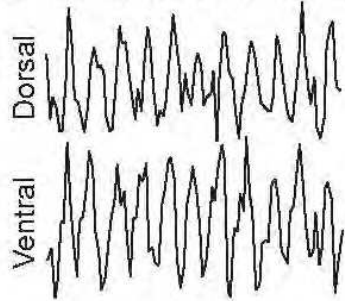


**Fig 5.2:** Ventral, but not dorsal, hippocampal infusions of 100 $\mu$ g histamine enhance evoked theta frequencies. **A)** Baseline dorsal and ventral hippocampal LFP traces during 3X threshold stimulation. **B)** The frequency of evoked theta at both sites at the same intensity is increased post-ventral hippocampal infusion of histamine. **C)** Spectral traces from the full 5s of stimulation for traces shown in panels A and B. Black traces are baseline, while red traces are post-histamine. Note the overlap of theta frequencies at both dorsal and ventral sites within each condition but the increase in frequency following histamine. **D)** Relationship of evoked theta frequency across stimulation intensity and condition. Note the prominent and parallel increase of theta frequency across dorsal (filled circles) and ventral (open circles) sites when comparing baseline measures (black) to those following histamine application (red). **E)** Baseline dorsal and ventral hippocampal LFP traces during 3X threshold stimulation in another experiment. **F)** Following dorsal hippocampal infusion of histamine there is no change in evoked frequency at the same level of stimulus intensity. **G)** Spectral traces from the full 5s of stimulation for traces shown in panels E and F. Black traces are baseline, while red traces are post-histamine. Note the overlap of theta frequencies at both dorsal and ventral sites within and across conditions. **H)** Relationship of evoked theta frequency across stimulation intensity and condition. There is no change of evoked theta frequencies following dorsal infusions of histamine.

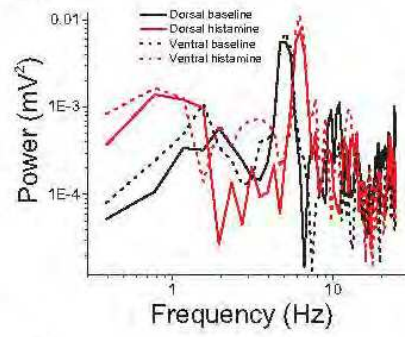
### A Baseline



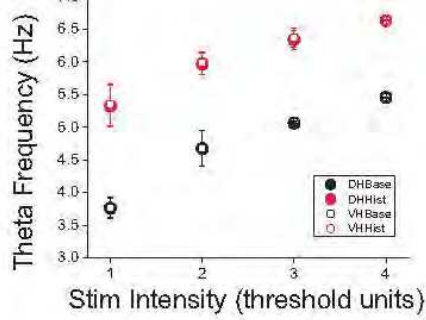
### B Ventral Histamine



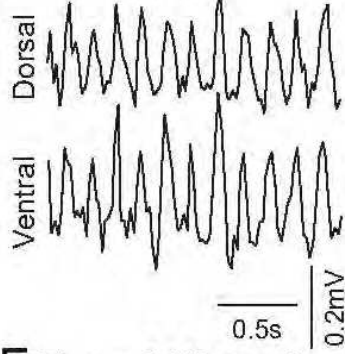
### C



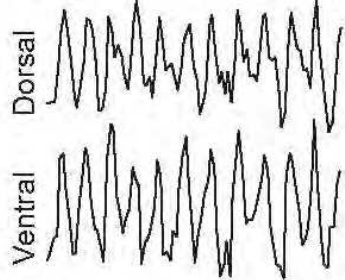
### D



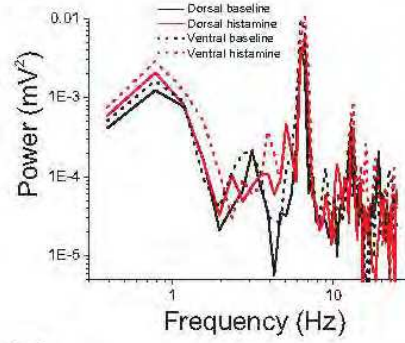
### E Baseline



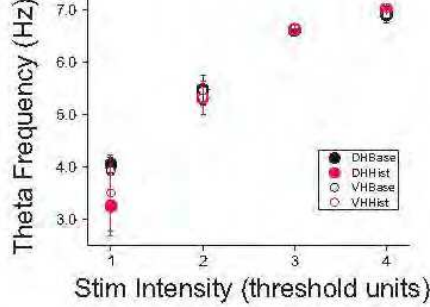
### F Dorsal Histamine



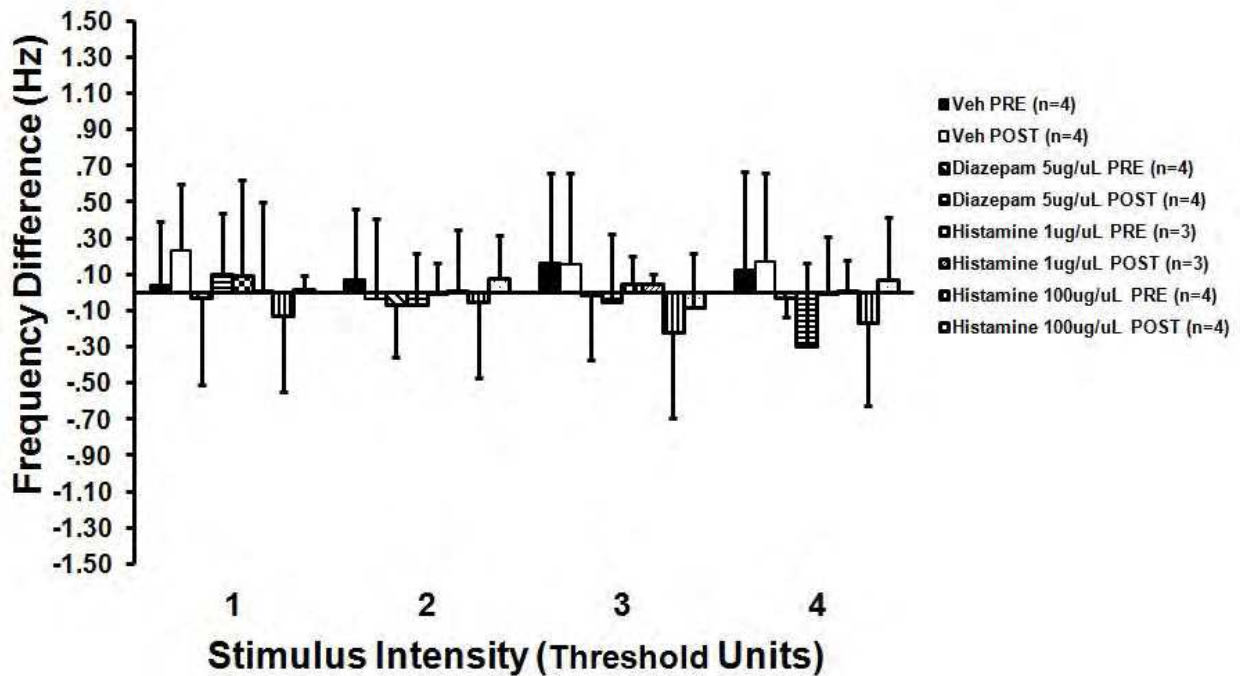
### G



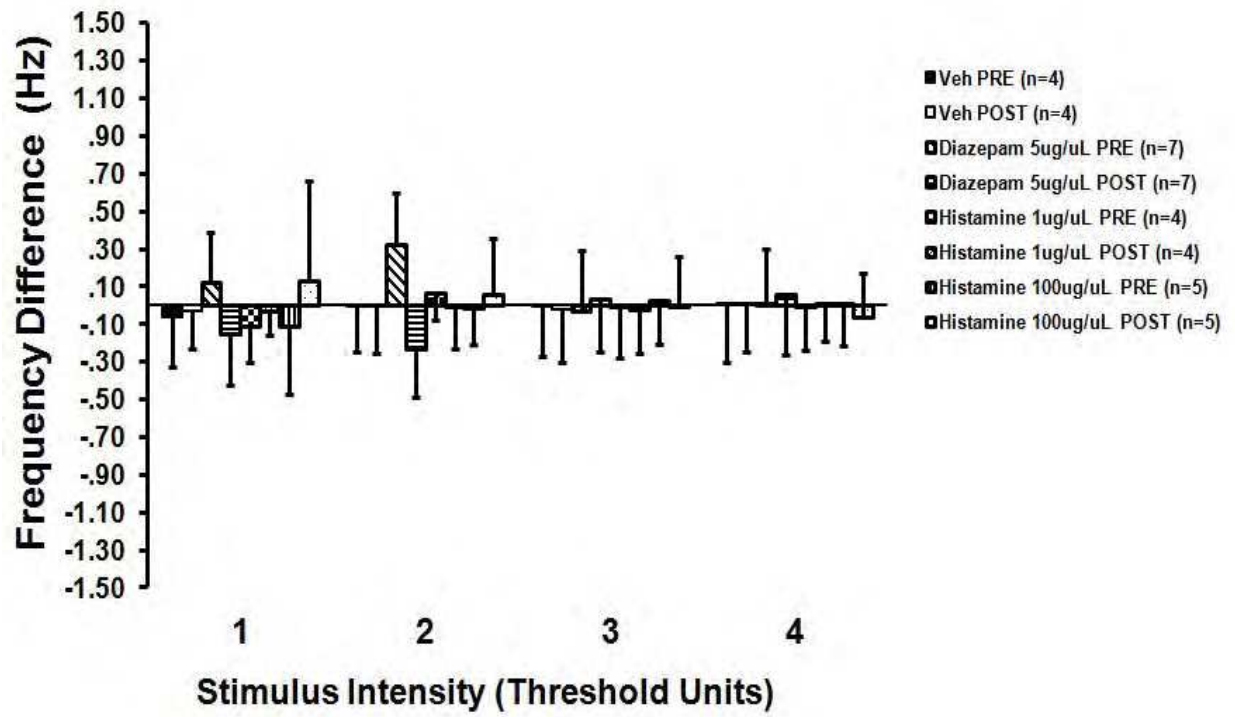
### H



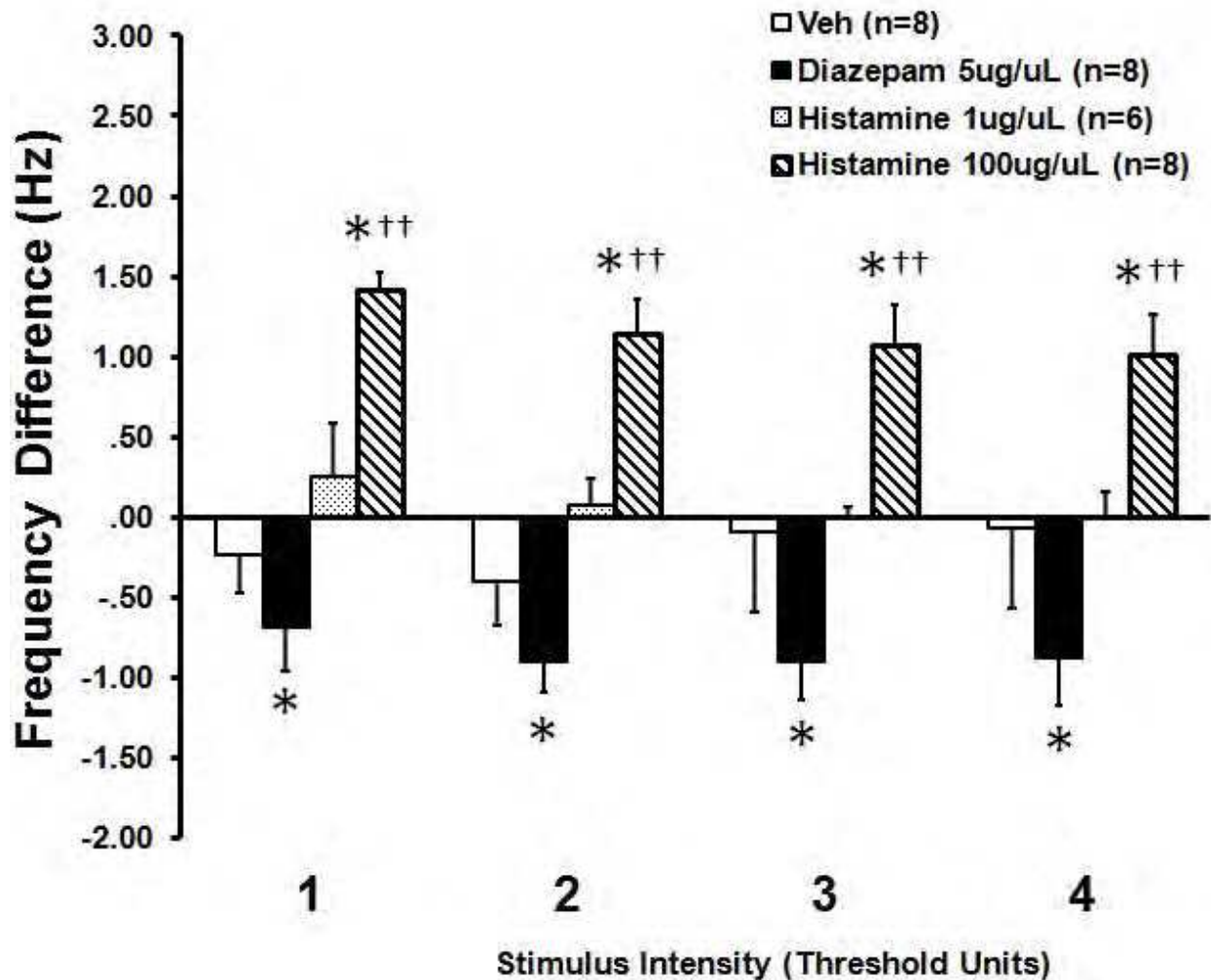
**Fig 5.3A,B:** Comparisons of evoked dorsal vs. ventral theta frequency (Hz) at 1x, 2x, 3x, and 4x threshold units of stimulus intensity pre and post ventral and dorsal intra-hippocampal microinfusions of histamine (1 or 100 $\mu$ g/ $\mu$ L diazepam (5 $\mu$ g/ $\mu$ L), or vehicle control in neurophysiological experiments. Black and white bars represent mean differences ( $\pm$ SEM) of evoked theta frequency pre and post administration of vehicle control. Diagonally and horizontally striped bars represent mean differences ( $\pm$ SEM) of evoked theta frequency pre and post administration of diazepam. Checkered and diagonally striped bars represent mean differences ( $\pm$ SEM) of evoked theta frequency pre and post administration of histamine 1 $\mu$ g. Vertically striped and dotted bars represent mean differences ( $\pm$ SEM) of evoked theta frequency pre and post administration of histamine 100 $\mu$ g.

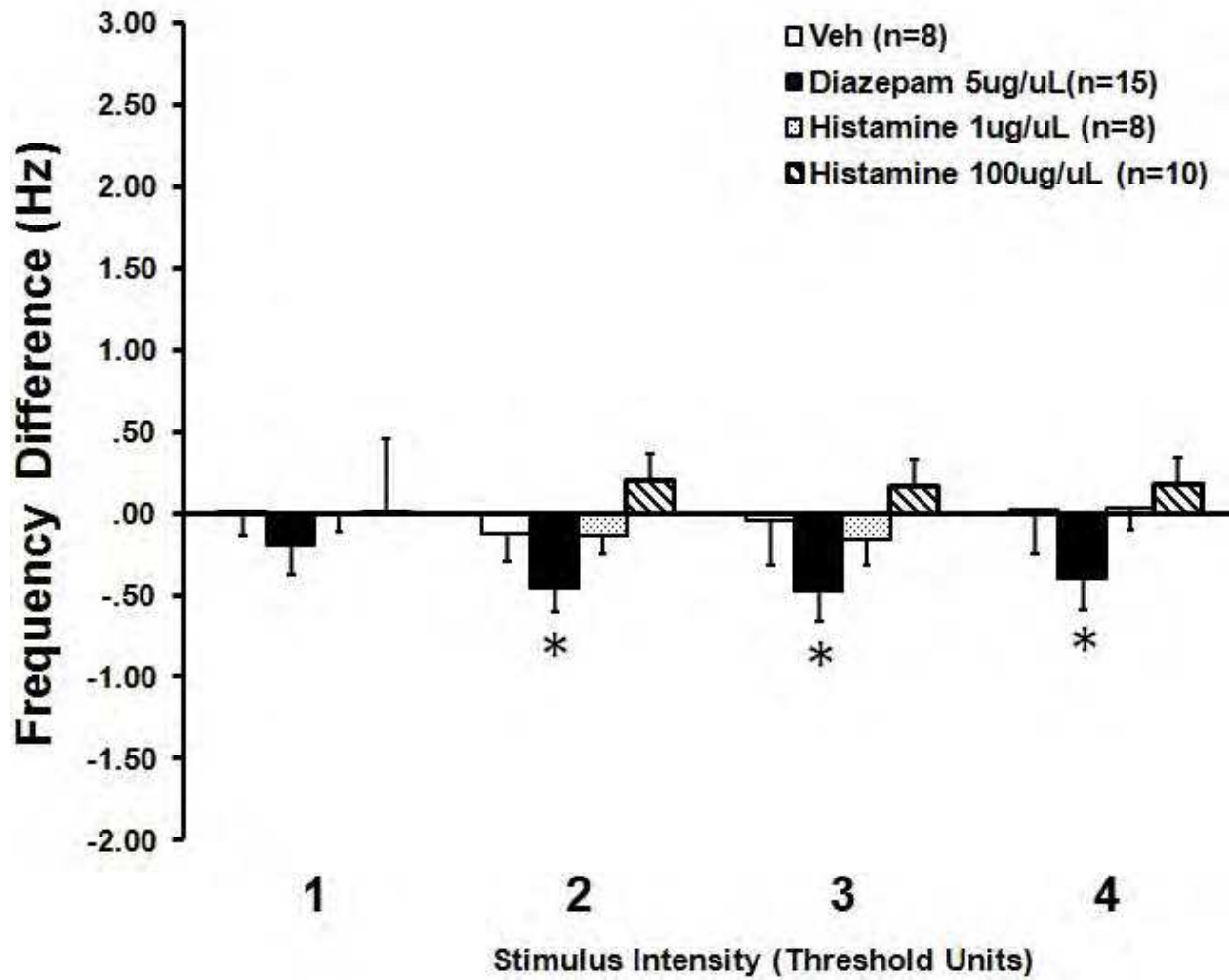




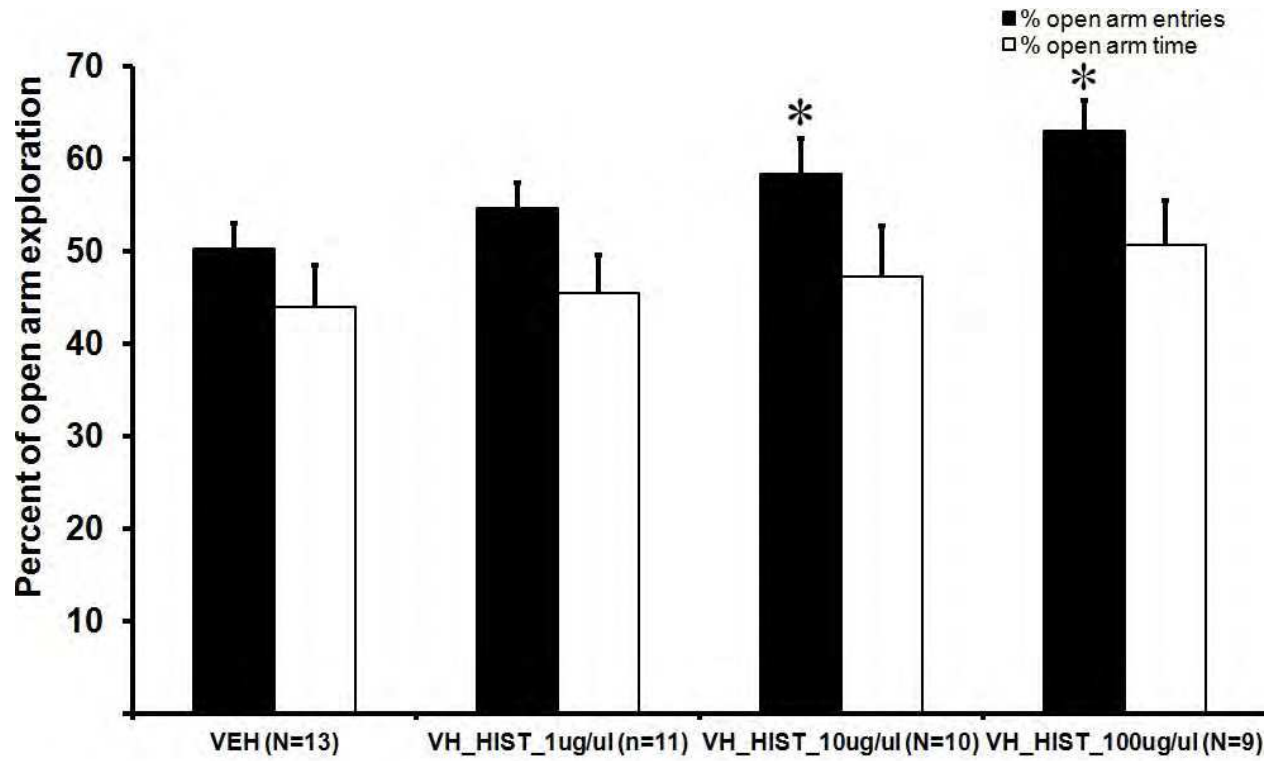


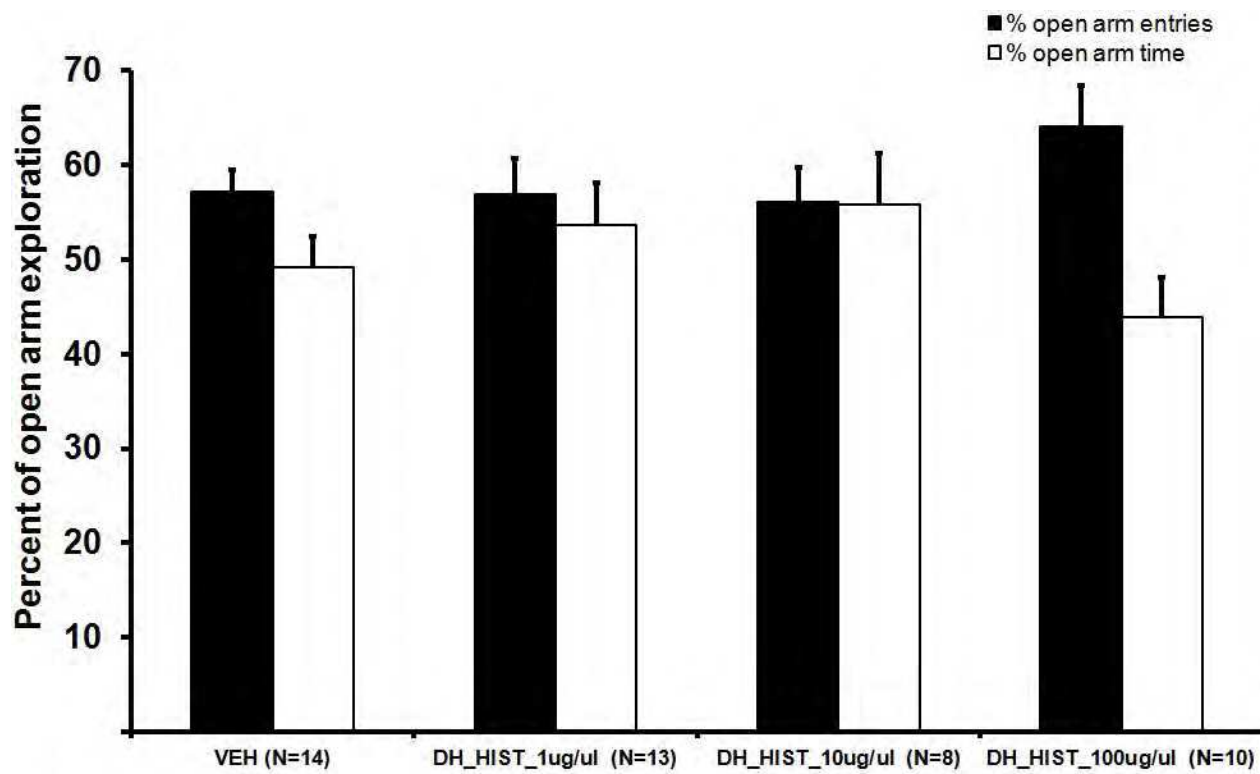
**Fig. 5.4A,B:** Difference in evoked theta frequency (Hz) at 1x, 2x, 3x, and 4x threshold units of stimulus intensity pre and post ventral and dorsal intra-hippocampal microinfusions of histamine (1 or 100 $\mu$ g/ $\mu$ L diazepam (5 $\mu$ g/ $\mu$ L), or vehicle control in neurophysiological experiments. White bars represent mean differences ( $\pm$ SEM) of evoked theta frequency pre and post administration of vehicle control. Black bars represent mean differences ( $\pm$ SEM) of evoked theta frequency pre and post administration of diazepam. White dotted bars represent mean differences ( $\pm$ SEM) of evoked theta frequency pre and post administration of histamine 1 $\mu$ g. Diagonally striped bars represent mean differences ( $\pm$ SEM) of evoked theta frequency pre and post administration of histamine 100 $\mu$ g. \*Pre and post comparisons significantly different ( $p < 0.05$ ). † Between groups comparisons histamine 100 $\mu$ g vs histamine 1 $\mu$ g and vehicle significantly different ( $p < 0.05$ ).





**Fig. 5.5A,B:** Open-arm activity following ventral and dorsal intra-hippocampal infusions of 1 $\mu$ g histamine, 10 $\mu$ g histamine, 100 $\mu$ g histamine, or vehicle control in the elevated plus-maze test. Black bars represent means ( $\pm$ SEM) percentage of open arm entries; white bars represent means ( $\pm$ SEM) percentage of open arm time. \*Significantly different from the vehicle control group ( $p < 0.05$ ).





**Table 5.1:** Percentages of Time Spent in the Open Arms and General Activity Measures (Means  $\pm$ SEM) in the Elevated Plus-Maze test following ventral hippocampal infusions.

Drug	% Open Time	Number of Closed Arm Entries	Number of Total Entries
Vehicle Control (N=13)	43.87 $\pm$ (4.88)	7.46 $\pm$ (0.69)	15.00 $\pm$ (1.25)
Histamine 1 $\mu$ g (N=11)	45.52 $\pm$ (4.01)	7.09 $\pm$ (0.76)	15.36 $\pm$ (1.28)
Histamine 10 $\mu$ g (N=10)	47.02 $\pm$ (5.53)	6.10 $\pm$ (0.62)	14.71 $\pm$ (0.68)
Histamine 100 $\mu$ g (N=9)	50.70 $\pm$ (4.68)	6.00 $\pm$ (0.90)	15.44 $\pm$ (1.68)

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## **CHAPTER 6**

### **GENERAL DISCUSSION AND CONCLUSIONS**

## **1.) Overview of the current findings**

The neurobiological manifestations of anxiety are set throughout a widespread parallel, hierarchical, and multi-dimensional neuroanatomical and neurochemical circuitry that requires systematic investigation at multiple levels of analysis. The complexity of this circuitry corresponds directly to the complexities of physiological and behavioral responses associated with the demonstration of fear and anxiety. With regards to this, classical psychopharmacology studies have contributed considerably to our knowledge of the neural mechanisms of anxiety through behavior-drug associations. Nevertheless, a universal neuro-behavioral index of anxiety has not yet been discovered. The studies in this thesis identify putative anxiolytic compounds and systematically evaluate a novel neurophysiological assay of anxiolytic drug action in an attempt to establish a correspondence to well established behavioral techniques.

Chapter 1 of this thesis provides a systematic overview of current behavioral animal models of anxiety, summarizes the biochemical basis and neuroanatomical correlates of anxiety, and characterizes the role of hippocampal theta in the neurobiology of anxiety. Chapter 2 of this thesis characterized the receptor specificity of the anxiolytic effects of somatostatin and highlighted the possibility of somatostatin receptors as emerging pharmacological targets. Chapters 3, 4, and 5 of this thesis tested the predictive and construct validity of the theta suppression model and characterized the role of hippocampal theta as a biomarker of anxiety. Establishing a common neural signature of anxiolytic potential would advance our understanding of the neural mechanisms of anxiety and would be particularly useful for the identification of novel anxiolytic compounds. By using a combination of behavioral and neurophysiological techniques we attempt to consolidate the pharmacological, neuroanatomical, and behavioral correlates associated with a diverse class of anxiety disorders.

## **2.) Somatostatin: a novel neuropeptide with anxiolytic potential**

The cyclic polypeptide somatostatin has emerged as a promising pharmacological target to study the neurobiological underpinnings of anxiety. SST and its receptors (sst1-5) are widely distributed throughout the brain and are thought to mediate a number of different physiological and behavioral functions (Cervia and Bagnoli, 2007; Moller et al., 2003). More importantly, SST is often co-localized with  $\gamma$ -amino butyric acid (Saha et al., 2002), an inhibitory neurotransmitter that has long been implicated in anxiety (Tallman and Gallager, 1985). Nevertheless, research into the precise neuroanatomical and receptor-specific role of somatostatin is still in its infancy.

Recently, Engin et al. found that intracerebroventricular (ICV) microinfusions of either somatostatin or a selective sst2 receptor agonist significantly reduced behavioral anxiety in the elevated plus-maze and suppressed the frequency of hippocampal theta (Engin et al., 2008; Engin and Treit, 2009). Subsequently, Yeung et al., 2011 demonstrated that both biologically active isoforms of somatostatin, SST14 and SST28, elicit robust anxiolysis when microinfused into two limbic system structures, the lateral septum and the amygdala. Conversely, no significant changes in affective behavior were found following microinfusions of selective agonists of sst1, 3, 4, and 5 (Engin and Treit, 2009) or somatostatin in the striatum (Yeung et al., 2011). This direct convergence between behavioral and neurophysiological assays highlighted 1.) the potential of somatostatin as an anxiolytic compound and 2.) the involvement of the sst2 receptor in mediating these anxiolytic properties in different parts of the brain. In chapter 2 of this thesis, we replicated the anxiolytic effects of somatostatin and directly characterized the role of the sst2 receptor and its involvement in the anxiolytic actions of somatostatin in a classical agonist-antagonist study using two well established animal models of anxiety, the elevated plus-

maze and shock probe burying test. Here, we found that both intra-septal and intra-amygdalar microinfusions of SST produced robust anxiolytic effects in the elevated plus-maze, which were consistent with those found previously (see (Engin et al., 2008; Engin and Treit, 2009; Yeung et al., 2011). More importantly, infusions of PRL2903, a selective sst2 antagonist, reversed the effects of SST in both the amygdala and the septum, further demonstrating that somatostatin's central anxiolytic effects are mediated by the sst2 receptor (Yeung and Treit, 2012). Taken together, these findings suggest that selective sst2 receptor agonists might be promising pharmacological targets in the development of novel therapeutics for anxiety disorders.

### **3.) Evaluating the predictive validity of the theta suppression model using ZD7288**

Recently, a neurophysiological model of anxiolytic drug action has been proposed and tested using a number of pharmacological agents (McNaughton et al., 2007). The findings from this neurophysiological model indicate that all clinically proven anxiolytic drugs (e.g. benzodiazepines, 5-HT<sub>1A</sub> agonists, SSRIs) reduce the frequency of reticularly-elicited hippocampal theta brain rhythm, while drugs that do not selectively affect anxiety (e.g. antipsychotic drugs, and pro-cognitive drugs) do not modulate theta frequency.

More recently, we systematically evaluated the predictive validity of the theta suppression model by using two, non-anxiolytic drugs that we presumed would have suppressive effects on theta frequency. Given that neither drug had documented anxiolytic effects, theta suppression with corroborating findings of anxiolysis in a well-established behavioral model of anxiety, the elevated plus-maze, would provide powerful evidence for the predictive validity of this model, as well as a new avenue for therapeutic discoveries. In the first set of studies we systemically (i.p.) injected the persistent sodium current ( $I_{Nap}$ ) channel blocker and antiepileptic

agent phenytoin. Since  $I_{\text{Nap}}$  is vital to maintaining theta rhythmical responses such as burst and resonance, a drug that blocks  $I_{\text{Nap}}$  would likely suppress theta frequency (Alonso and Llinas, 1989; D'Angelo et al., 2001; Hutcheon et al., 1996). Here, we demonstrated for the first time, that phenytoin significantly suppressed theta frequency and produced robust anxiolytic effects in the elevated plus-maze (Yeung et al., 2012).

In chapter 3, we extended this line of research using the hyperpolarization-activated inward current ( $I_h$ ) channel blocker and bradycardic agent ZD7288. Here, we were interested in 1.) testing a drug that targeted another intrinsic membrane current to alter theta rhythmicity and 2.) infusing ZD7288 directly at the level of the hippocampus. ZD7288 was known to disrupt theta frequency oscillations at the membrane level (Dickson et al., 2000) but was not known to have any anxiolytic action. Intra-hippocampal microinfusions of ZD7288 at 15  $\mu\text{g}$ , but not 1  $\mu\text{g}$  doses slowed brainstem-evoked hippocampal theta responses and also produced corresponding anxiolytic effects in freely behaving rats in the elevated plus maze. As a positive control condition, we microinfused diazepam, a well-established anxiolytic drug, into the hippocampus and found suppressive effects on hippocampal theta frequency, as well as anxiety-reduction in the elevated plus-maze (Yeung et al., 2013a). Taken together with our previous demonstration, these data provide converging evidence of the validity of the theta suppression model.

#### **4.) Using FG7142, Yohimbine and $\beta\text{CCE}$ to evaluate the construct validity of the theta suppression model**

In chapter 4, we examined the construct validity of the theta suppression model. Construct validity essentially asks whether the “model” measures what it is purported to measure. In this case, the question is whether theta frequency itself is a neuronal index of fear or

a reflection of some other, non-specific index of neuronal inhibition. This was done by using three behaviorally confirmed anxiogenic (i.e., anxiety enhancing) agents (FG7142 and  $\beta$ CCE - partial inverse agonists at the benzodiazepine site on the GABA<sub>A</sub> receptor (Evans and Lowry, 2007; Taguchi and Kuriyama, 1990) and yohimbine, an antagonist at pre-synaptic  $\alpha$ 2 adrenergic receptor site (Millan et al., 2000). We reasoned that if anxiolytic drugs such as diazepam *decrease* hippocampal theta, then anxiogenic agents should *increase* hippocampal theta, and produce behavioral anxiogenesis. Although all three anxiogenic drugs significantly increased behavioral measures of anxiety in the elevated plus-maze, none of the three increased the frequency of hippocampal theta oscillations in the neurophysiological model. As a positive control, we demonstrated that diazepam, a proven anxiolytic drug, decreased the frequency of hippocampal theta, as in all other studies using this model. Given this discrepancy between the significant effects of anxiogenic drugs in the behavioral model and the null effects of these drugs in the neurophysiological model, we conclude that the construct validity of the hippocampal theta model of anxiety is questionable, at best (Yeung et al., 2013b).

#### **5.) Using histamine to explore the dorsal vs. ventral hippocampus functional dichotomy in relation to the theta suppression model.**

In chapter 5 of this thesis, we explored the regional and functional differences between the dorsal and ventral hippocampus in relation to the theta suppression model. Recently, multiple lines of evidence including selective lesion and local drug infusion studies have shown that the ventral hippocampus is involved in the modulation of anxiety-related behaviors whereas the dorsal hippocampus plays a central role in mediating spatial cognition and memory (Bannerman et al., 2002; Bannerman et al., 2004; Fanselow and Dong, 2010; McEown and Treit, 2009; McEown and Treit, 2010). Nevertheless, a major limitation of current studies evaluating the theta

suppression model is the focus on recording dorsal hippocampal theta frequency while neglecting the role of the ventral hippocampus. Here, we used histamine, ( $\beta$  aminoethylimidazole), a neurotransmitter that is involved in a number of physiological and behavioral processes including the regulation of wakefulness, feeding and weight homeostasis, learning and memory, inflammatory responses and affective and emotional processes including anxiety (Brown et al., 2001; Chee and Menard, 2013; Chee et al., 2014; Haas et al., 2008). We showed that ventral hippocampal infusions of histamine produced significant increases in theta frequency and potent anxiolysis in the elevated plus-maze, while dorsal hippocampal infusions of histamine produced no changes in either the neurophysiological or behavioral assay. As a positive control, we also demonstrated that diazepam, a proven anxiolytic drug, decreased the frequency of hippocampal theta following both ventral *and* dorsal hippocampal infusions, as in all other studies using this model. Given these discordant findings between our histamine-induced increase in theta frequency and the anxiety reducing effects of histamine produced in our behavioral test, these results seriously challenge the theta frequency suppression model of anxiolytic drug action.

## **6.) Limitations of the current studies**

The technique of intracerebral microinfusions is one of the most widely used for determining the neuroanatomical and receptor specificity of anxiolytic drug effects in the brain. However, like any other technique, it is subject to a number of shortcomings. One major limitation associated with microinfusion techniques is the likelihood of drug diffusion away from the target area (Engin and Treit, 2008; Menard and Treit, 1999). Infusion sites that are in close proximity to the lateral ventricles also exacerbate this problem because they contain circulating



cerebral spinal fluid, which can carry the infused drug to almost any area in the brain. As such, the efficacy and site specificity of a particular drug may be difficult to determine and may actually reflect the effects of drug action at another site in the brain in close proximity, but functionally unrelated, to the target structure. The absence of any effect anywhere in the brain is a more intractable problem that requires other solutions.

To mitigate these problems, smaller infusion volumes (0.5-1 $\mu$ L) were chosen for microinfusions, as well as more concentrated solutions (e.g., 0.5 $\mu$ g/0.1 $\mu$ L). Given the small infusion volumes, it is estimated that the spread of infused substances will not extend beyond a 1mm radius from the site of infusion. (Myers, 1966; Myers et al., 1971; Routtenberg, 1972). Higher concentrations of the same amount of drug could help mitigate unavoidable dilution of the compound in the brain, but it would only be a first step in the solution of this problem. We also ensured location-specific effects by using experimenters blind to each rat's experimental condition to confirm cannulae placements in histological analyses. If the behavioral data from drug- infused rats with misplaced cannulae is similar to that of placebo-infused rats with well placed cannulae, we can conclude that the effects seen in drug-infused rats with accurately placed cannulae tips are due to the drug and not to some other, confounding, variable.

While the elevated plus-maze is one of the most widely used behavioral assessments of anxious behavior, having converging evidence across a number of behavioral tests would strengthen the conclusion of anxiolytic or anxiogenic effects. For example, the light-dark box and the open field test are also well established tests of unconditioned anxiety and could be included in future studies. Additionally, the elevated plus-maze also does not evaluate other aspects of fear behavior including responses to predatory stimuli, conditioned fear, and fear

extinction. As such, the incorporation of a wider behavioral battery in future studies would also allow for a more rigorous examination of the neural correlates involved in different aspects of fear and anxiety.

It is worth noting here that behavioral models of anxiety like the shock-probe burying test and elevated plus-maze have fundamental differences in the nature of their fear provoking stimuli. Burying the shock-probe, for example, involves an active coping style, while sitting still and passively avoiding contact with the probe is completely different but just as effective. These dichotomous defensive responses probably also involve different neurotransmitters and neural circuitry. For example, previous studies in our laboratory have demonstrated reliable dissociations within a particular brain area (e.g., the central amygdala versus the basolateral amygdala) with specific fear responses suppressed by lesions in one area but not the other (e.g., shock-probe avoidances versus open-arm avoidance; (Pesold and Treit, 1995).

One possible limitation in the assessment of anxiogenic drugs on theta frequency increases in chapter 4 of the thesis is that the brain-stem stimulation intensities we used to elicit hippocampal theta were too high for detecting drug-induced increases. This is a critical problem because if no pharmacological agent is actually able to enhance theta frequency, the efficacy of this model in our assessment of construct validity would be questionable. Nevertheless, this potential “ceiling effect” is unlikely for several reasons. First, hippocampal theta has a fundamental frequency ranging from 4-14Hz and the maximum frequency elicited by brainstem stimulation using similar stimulation parameters has been reported at around 15Hz (Gray and McNaughton, 2000; Green and Arduini, 1954; McNaughton et al., 2007). Although we obtained clear intensity by frequency effects, suggesting that we were stimulating relevant brainstem

regions, we did not observe stimulation-induced theta frequencies beyond 9Hz. As such, the theta frequencies elicited were well within the range of the fundamental frequencies in the theta band. More convincingly, there is substantial pharmacological evidence demonstrating that agonists of cholinergic/muscarinic neurotransmission increase theta frequencies in the urethane anesthetized preparation, either during spontaneous (Olpe et al., 1987; Rowntree and Bland, 1986) or brainstem-evoked conditions (Kinney et al., 1999). Chapter 5 of this thesis also provides powerful evidence that theta frequency can be enhanced following local infusions of histamine. Here, we demonstrate robust increases in theta frequency in both the dorsal and ventral hippocampus. Taken together, this suggests that theta frequencies can indeed be both suppressed and enhanced and the lack of theta frequency increases that we found with well validated anxiogenic drugs are indeed indicative of a lack of construct validity of the theta frequency model for behavioral anxiety *per se*.

## **7.) Theoretical implications, future directions and conclusions**

In view of findings presented in this thesis, this section consolidates these results according to current neurobiological theories of anxiety and highlights several key points which should be considered for future directions.

In chapter 1 of this thesis, we discussed the seminal work outlining the involvement of the limbic system structures (i.e. septum, amygdala and hippocampus) in the modulation of fear and anxiety. Kluver and Bucy initially highlighted the involvement of the temporal lobe structures following their studies on “psychic blindness.” Subsequently, Papez (1937) developed a neural circuit for emotional expression with the hippocampus playing a central role in the generation of an emotional experience by consolidating input from cingulate cortex, sensory

cortex and hypothalamus. Later, MacLean emphasized the role of the hippocampus as part of the triune emotional brain and modern day so called “limbic system structures.” These circuits were also supported by Flynn (1967) who was one of the first researchers to specify the function hippocampus in behavioral inhibition and Gray (1982) who constructed a theory on the neuropsychology of anxiety which amalgamated the ideas of Flynn and Papez and argued that the septo-hippocampal system was central to mediating anxiety. With regards to this, the hippocampal theta rhythm became an important integrator in Gray’s circuit, and became the foundation for the association between anxiolytic drug action and the hippocampus. Here, anxiolytic drugs alter the theta rhythm input generated by the septohippocampal system. This in turn modifies the theta rhythm output, thereby disrupting behavioral inhibition of the hypothalamus and downstream structures in the Papez circuit (i.e. cingulate cortex and anterior thalamus). Later, this idea was extended by McNaughton in the current theta suppression model of anxiolytic drug action. Recently, a functional dichotomy theory outlining the role of hippocampus in cognition and anxiety has also been presented. Specifically, it has been demonstrated that the dorsal hippocampus is involved in mediating cognition and memory while the ventral hippocampus is importantly involved in the modulation of anxiety-related behaviors (Bannerman et al., 2004; Fanselow and Dong, 2010; McEown and Treit, 2009; McEown and Treit, 2010). Nevertheless, the central role of the hippocampus in anxiety was challenged by LeDoux’s work on contextual fear conditioning. In this theory, LeDoux suggests that the hippocampus is simply a processor of complex contextual stimuli and the amygdala is central to integrating this information and producing emotional outputs.

In view of these competing theories, the role of the septum, amygdala, and hippocampus and particularly hippocampal theta, have been explored in this thesis. We concluded from

chapter 2 of this thesis that the anxiolytic effects of somatostatin are specific to the sst2 receptor and can be reversed by a selective sst2 receptor antagonist PRL2903 in both the amygdala and the septum. However, these effects have never been confirmed behaviorally in the dorsal and ventral hippocampus or in our neurophysiological assay. Demonstrating a functional dissociation between the anxiolytic action of an sst2 agonist and the absent of effects produced by the other sst agonists in the dorsal and ventral hippocampus and then subsequently reversing these effects with a selective antagonist would strengthen the association for the involvement of sst2 in mediating anxiety processes. To our knowledge, no studies have attempted to reverse the effects of theta suppression by an anxiolytic drug using a classical agonist-antagonist study. As such, the first set of experiments in this line of research should use benzodiazepine antagonist RO15-1788 to reverse theta suppression elicited by a well established anxiolytic agent such as diazepam. Establishing the exact moment at which theta suppression reversal occurs could provide important information about the relationship between theta properties and behavioral anxiolysis. Subsequently, future studies could further characterize the anxiolytic potential of somatostatin in different parts of the brain using a similar agonist-antagonist paradigm.

In chapters 3, 4, and 5 of this thesis, we critically evaluated the predictive and construct validity of the theta suppression model. The pharmacological evidence supporting the idea that theta frequency suppression is a neural marker of anxiolytic drug action has been substantial (for review, see McNaughton et al., 2007). We also demonstrated the predictive validity of this model using two drugs with no known anxiolytic effects: 1.) phenytoin, and 2.) ZD7288. While these studies provide powerful converging support for the theta suppression model, we also show that histamine, a drug with anxiolytic properties can increase the frequency of hippocampal theta. This discordant directionality between our behavioral and neurophysiological models makes it

difficult to reconcile the findings. Considering the biochemical basis of anxiety and the neurotransmitters involved in theta generation as presented in chapter 1 of this thesis, these results highlight the likelihood that multiple neurotransmitter systems (likely a complex interaction between cholinergic and GABAergic systems) may be involved in mediating these effects. With regards to this, the differences in both the behavioural and neurophysiological effects of dorsal versus ventral hippocampal infusions of histamine may be mediated by the differences in the density and distribution of H1, H2 and H3 receptors across the dorsal and ventral regions of the hippocampus and their potential interactions with cholinergic afferents (Alvarez and Banzan, 2001; Haas et al., 2008). Given that the pharmacological evidence for the theta suppression model has primarily been established using only classic anxiolytic drugs targeting GABAergic and serotonergic neurotransmitter systems, the involvement of histaminergic systems in behavioral anxiolysis and the modulation of theta may require further attention and incorporation in a revised version of this model.

Based on our critical evaluation of the theta suppression model, the utility of the model as a predictive assay for anxiolytic drug action or as an assay of anxiety *per se* now remains questionable. Nevertheless, there are a number of future directions that should be considered and empirically tested in accordance with current conceptual theories of anxiety. With regards to this, one important idea is the concept of passive vs. active avoidance behavior as presented by Miller (1944) and Flynn (1967). In a later modification, Gray and McNaughton (1983) concluded that anxiolytic drugs and hippocampal lesions both produced changes to passive avoidance behaviors (i.e. freezing), but not active avoidance behaviors (i.e. fleeing). Recently, another revision was made by McNaughton and Corr (2004) to construct a hierarchically organized model of defensive behaviors in relation to fear and anxiety disorders. This 2D (defense) system arranges

fear and anxiety reactions and their neural bases according to the complexity of the threat (defensive distance) and the type of behavioral reaction or defensive direction (defensive approach vs. defensive avoidance behaviors). Accordingly, shorter defensive distances or immediate threats require the recruitment of lower level structures such as the PAG, while longer defensive distances or distant threats require processing by higher level structures such as the pre-frontal cortex (McNaughton and Corr, 2004).

Reconciling our findings with the ideas presented in this model, the incorporation of the shock-probe burying test as a behavioral assay in future studies would be useful. The shock probe test has intrinsic components built in to measure defensive approach (burying) and defensive avoidance (avoidance of probe contacts). In chapter 3 of this thesis, we demonstrated that ZD7288 had anxiolytic properties and suppressed theta frequency. In chapter 4 of this thesis, we conclude using three well established anxiogenic agents FG7142, yohimbine and  $\beta$ CCE, that the theta suppression model lacks construct validity. Finally in chapter 5, we showed that histamine produced anxiolytic effects and theta frequency increases. In view of McNaughton and Corr's model, it would be interesting to evaluate the effect of these drugs (ZD7288, FG7142, yohimbine,  $\beta$ CCE and histamine) on defensive approach and defensive avoidance behaviors.

Presumably, ZD7288 and histamine should have similar anxiolytic effects on defensive avoidance behaviors (e.g. probe contacts) but have little or no effect on defensive approach behaviors (burying). Convergent findings with a well established benzodiazepine (i.e. diazepam or midazolam), SSRI (i.e. Fluoxetine) or 5HT1a agonist (i.e. Buspirone) would help to establish the role and similarity between histamine and other neurotransmitter systems involved. Conversely, FG7142, yohimbine, and  $\beta$ CCE should modulate burying time but not probe contacts. While these effects would likely be site specific (i.e. midazolam has no effect on

defensive burying in the amygdala, but impairs shock probe contacts in the central but not basolateral amygdala, Pesold and Treit, 1995), a systematic evaluation of these drug by site interactions in paradigms measuring different anxiety behaviors would have important theoretical implications and further our understanding of the neural control of anxiety.

Within this theoretical framework, the relationship between hippocampal theta and the expression of behavioral anxiety is another area that requires further exploration. Thus far, there is inconclusive evidence about the directionality of theta modulation by anxiety related drugs in freely moving behavioral preparations. For example, it has been demonstrated that i.p infusions of nicotine increased theta frequency while systemic infusions of pilocarpine, a muscarinic receptor agonist, and tetrahydroaminoacridine (a cholinesterase inhibitor) significantly suppressed theta frequency (Yamamoto, 1998). Additionally, the benzodiazepine anxiolytics diazepam and nozepam have been shown to decrease theta frequency following systemic infusions in dogs (Chilingarian and Bogdanov, 1998). Nevertheless, the moment to moment expression of anxiety and the changes to hippocampal theta following the administration of an anxiolytic or anxiogenic drug still requires systematic investigation. Given that anxiety is a conscious behavioral state, studies using freely moving preparations would further our understanding about the neural mechanisms underlying theta and anxiolysis

Additionally, the synchronization and cooperation of limbic system structures like the medial prefrontal cortex (mPFC), the dorsal and ventral hippocampus, and the amygdala during the expression of behavioral anxiety, learning, memory and cognition, and the relationship with theta is still unclear. Recently, the mPFC has emerged as a focal point in the neural circuitry of anxiety and cognition. The mPFC has diverse bidirectional projections throughout the brain



including direct projections from the ventral hippocampus (Verwer et al., 1997), indirect afferents from the dorsal hippocampus (Burwell et al., 1995) and reciprocal connections with the amygdala (Vertes et al., 2004). Given the widespread neuroanatomical connections of the mPFC, its role in affective and cognitive functions has been well documented. For example, our laboratory has shown that mPFC infusions of GABAA agonists midazolam and muscimol produced anxiolytic effects in the elevated plus-maze and shock probe burying tests (Shah et al., 2004a; Shah et al., 2004b; Shah and Treit, 2004). At the neurophysiological level, theta-frequency synchrony has been demonstrated between the mPFC and dorsal hippocampus during tasks of working memory (Jones and Wilson, 2005). Finally, it has been shown that theta power - as well as coherence between the mPFC and the ventral hippocampus - was significantly increased in freely moving rats in the open field and elevated plus-maze (Adhikari et al., 2010; Gordon et al., 2005), suggesting that specific properties of theta can be associated with situations designed to promote anxiety.

Given the complexity and heterogeneity of the expression of behavior and anxiety and its association with theta properties, delineating the synchrony and coherence of these limbic system structures and the changes to theta properties within each structure in an anxiety provoking environment and during a cognitive task would further our understanding of the neurobiological mechanisms of anxiety and cognition. In view of the parallels in the neural circuitry involved in mediating both cognition and anxiety, assessing the changes to theta properties and the brain structures involved in the modulation of cognition and anxiety will reveal similarities and differences in the neural and pharmacological mechanisms involved. Finally, these experiments would also be useful for the development of therapeutics for anxiety disorders and disorders characterized by deficits in cognition (i.e. Alzheimer's Disease).

In conclusion, our neurophysiological and behavioral findings provide a systematic evaluation of the theta suppression model, and advance our knowledge about the underpinnings of the neurobiology of anxiety. Nevertheless, the increases to theta frequency in conjunction with anxiolytic effects in the elevated plus-maze following the application of histamine (Yeung et al., 2014, submitted) into the hippocampus are difficult to reconcile, especially when compared to our earlier findings of predictive validity following the application of phenytoin (Yeung et al., 2012) and ZD7288 (Yeung et al., 2013). While these discrepancies suggest that the theta suppression model may not be considered a true assay of anxiolytic drug action or an assay for behavioral anxiety given its lacks of construct validity, it also highlights the complexities of the hierarchical and/or parallel neural circuitry and neurotransmitters involved in the modulation of mechanisms underlying theta and behavioral anxiety.

Within our critical evaluation of the theta suppression model, two primary but opposing sets conclusions can be generated. Given that histamine has been reliably shown to increase theta frequency and produces anxiolysis in two separate instances (Yeung et al., see chapter 5, Chee et al., 2014), we can conclude that these findings are not spurious or simply a result of a false negative. This is also borne out by the dissociation of histamine's effect at different levels of the hippocampus (dorsal vs. ventral). As such, it would be easy to conclude that the theta suppression model should be abolished since we are the second group of researchers to show that it is not a reliable or predictive assay of anxiolytic drug action. Nevertheless, I believe that several questions need to be carefully examined and considered in future research before this happens. Firstly, the receptor specificity of histamine's effects on theta frequency increases and its effects on downstream neurotransmitter systems need to be systematically evaluated. Given

the heterogeneity and complexity of the histaminergic system in anxiety, these paradoxical effects may be a result of different interactions between histamine receptors and other neurotransmitter systems. Secondly, histamine and other anxiolytic drugs targeting different neurotransmitter systems should be infused into different brain areas and tested in behavioral paradigms measuring different parameters related to anxiety (i.e. passive vs. active avoidance). Anxiety is mediated by an extensive global and targeted neural network and these drug, site, sub-nuclei and behavioral interactions need to be carefully considered before further conclusions can be made. Finally, future studies should also assess the moment to moment changes to theta and anxiety in freely moving preparations and delineate the similarities and differences in the neural correlates and neurotransmitters involved in theta generation and anxiety. These findings amalgamated as a whole, would provide concrete evidence for the application of the theta suppression model as an assay of anxiolytic drug action.

Taken together, the findings from these future studies and the ones in the present thesis may illuminate a more complete understanding of the causal relationship between theta and anxiety. This in turn may lead to the discovery of novel biomarkers in the neurobiology of anxiety, which may or may not be common to those involved in theta modulation.

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