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Novel Models and Mechanisms in the Neurobiology of Anxiety

by

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Abstract

Chapter 1 will provide a brief introduction to the neurobiology of anxiety, with an emphasis on three issues that have guided the current research. First, behavioral models of anxiety that have been useful in understanding its biological bases will be described. Second, the roles of the septum, hippocampus and amygdala in anxiety will be briefly summarized. Third, the antianxiety (anxiolytic) potential of a number of novel compounds will be described, as well as their effects in animal models of anxiety and in human anxiety.

Chapter 2 describes four studies of the anxiolytic effects of the cyclic polypeptide somatostatin in two, extensively validated rat models of anxiolytic drug action: the elevated plus-maze and the shock-probe burying test. In particular, two isoforms of somatostatin (SST 14 and SST28) were microinfused into the central amygdala, the lateral septum, and the striatum (Yeung et al., 2011), with anxiolytic-like effects found in the first two structures but not the last, thus demonstrating the site-specificity of the results.

In chapter 3, I conduct a critical test of Neil McNaughton's hippocampal theta model of anxiolytic drug action. McNaughton and colleagues have repeatedly demonstrated that clinically proven anxiolytic drugs, regardless of their individual mechanisms of action, reliably suppress brain-stem evoked hippocampal theta. Such demonstrations, however, offer only weak evidence of the validity of any particular model of anxiolytic drug action, be it behavioral or neurophysiological. Phenytoin, a well-known antiepileptic drug with no known anxiolytic potential, was used to test the predictive validity of the theta suppression model.

Chapter 4 summarizes the major results of these studies and their implications for current neuropharmacology theories of anxiety.

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Figure 3.1: **A)** Raw LFP traces of evoked theta following 90 mA stimulation pre and post administration of phenytoin are shown in both upper and lower panels (the top panel shows the response to 10 and the bottom 50mg/kg doses). **B)** Spectra components of the respective pre-post drug treatment traces shown in panel **A** are overlaid. Suppression of the evoked frequency can be seen for the 50, but not the 10mg/kg dose in both the raw traces and the spectra. **C)** Average data across all experiments showing the frequency of evoked theta at each level of stimulus intensity as measured in threshold units (1x, 2x, 3x threshold value) as a function of drug treatment. The top panel shows a lack of effect of 10mg/kg of phenytoin while the bottom shows a prominent and significant effect of the 50 mg/kg dose. Black squares represent means (±SEM) of evoked theta frequency pre administration of phenytoin. White squares represent means (±SEM) of evoked theta frequency post administration of phenytoin *Significantly different ($p<0.01$).

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List of Abbreviations

The following table describes the various abbreviations and acronyms used throughout this thesis

Abbreviation	Meaning
5-HT	5-hydroxytryptamine
AC	Alternating Current
ANOVA	Analysis of Variance
AP	Anterior-Posterior
CAPS	Clinically Administered PTSD Scale
CCK	Cholescystokinin
CNS	Central Nervous System
CRF	Corticotrophin-Releasing Factor
CSF	Cerebrospinal Fluid
DIAZ	Diazepam
DMSO	Dimethyl Sulfoxide
DSM	Diagnostic and Statistical Manual of Mental Disorders
DV	Dorsal-Ventral
EC50	Median Effect Concentration required to induce a 50% effect
EPM	Elevated Plus Maze

FFT	Fast Fourier Transform
GABA	Gamma-Aminobutyric Acid
GIRK	G-protein-receptor linked, inward rectifying K+
HPA	Hypothalamic-pituitary-adrenal axis
ICV	Intracerebroventricular
INAP	Persistent Sodium Current
ML	Medial-Lateral
NMDA	N-Methyl-D-Aspartate
NPY	Neuropeptide Y
PHT	Phenytoin
PNS	Peripheral Nervous System
PTSD	Post-traumatic Stress Disorder
SEM	Standard Error of the Mean
SPB	Shock Probe Burying Test
SSR	Somatostatin Receptor
SSRI	Selective serotonin reuptake inhibitors
SST	Somatostatin

Chapter 1: Introduction

Fear and anxiety are closely related emotions that elicit a similar set of behavioral and physiological responses elicited by stimuli perceived as dangerous or harmful. The resulting responses in mammals include sympathetic nervous system activation (e.g increased heart rate, pupil dilation) neurohormonal release of 'stress' (e.g., coritosteroids), as well as species specific defense responses such as "freezing" "thigmotaxis," and defensive "burying." These physiological and behavioral responses are product of evolution and presumably increase the organism's probability of survival. Perhaps because of this similarity, fear and anxiety are often used interchangeably to describe each other. For example, in the DSM IV, anxiety disorders are divided into categories such as "generalized" anxiety, "panic" and "phobias" but are clinically described with the terms such as "fear," "dread" and "terror." All anxiety disorder are described in the DSM IV as somehow triggered or related to fear (Gelder, Mayou and Geddes, 2005). In addition, all anxiety disorders can be effectively treated in the short-term with indirect agonists of the GABAa receptor subtype (e.g., valium®).

Nevertheless, fear and anxiety are also viewed as two, distinct psychological states. Fear can be seen as an adaptive, "normal," and transient response to a perceived threat, whereas anxiety can be viewed as a maladaptive, "abnormal," and persistent response that occurs in the absence of a "realistic," immediate threat. For example, the sight of a snake elicits *fear* and triggers escape behavior in "normal" people, but the memory or misapprehension of a snake elicits *anxiety* and maladaptive avoidance behavior in persons with a "snake phobia." This avoidance behavior may escalate to the extent that it disrupts "normal" every-day life (going to the zoo, etc). Nevertheless, the distinction between fear and anxiety can often break down or blur in specific contexts, and it is by no means a fully accepted distinction in the literature.

Therefore, for expedience, I will be using the terms fear and anxiety interchangeably throughout the rest of this thesis.

Animal Models of Anxiety

The development of various animal models of anxiety has been driven in part by the general utility of animal models in medical research (e.g., Markou et al., 2009). One important aim when developing animal models of anxiety is establishing a correspondence between the etiological, pathological and behavioral representations of "anxiety" in humans and similar constructs in the animal model (Markou et al., 2009; Treit et al., 2010 For example, a behavioral correspondence between human and animal anxiety is simple avoidance of the threatening stimulus. Other "defensive" behaviors in rodents can be used for the same purpose, as analogies of particular aspects of human anxiety, as can their physiological responses to the similar threats. Depending on their adaptive value, we might expect evolutionary conservation of the neural mechanisms of defense and anxiety across animal species, even between rats and humans (LeDoux, 2000). Two current examples of animal models of anxiety are described below.

The elevated plus-maze (EPM) test is a single trial paradigm that assesses untrained fear reactions in rodents (File, 1985). The EPM is made of four elevated arms in the shape of a plus sign (+), two adjacent arms of which are open and two enclosed with walls. The model relies on a rodent's intrinsic aversion of open spaces (thigmotaxis), leading to a preference for the enclosed arms (Treit et al., 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 (Pellow et al., 1985). These fear reactions can be reversed by drugs that suppress anxiety in humans (e.g., diazepam). An increase the proportion of open arm entries and time

spent in the open arms are the primary measures of fear-reduction in the plus-maze. The number of closed arm entries serves as a measure of general activity (see also Treit and Menard, 2000).

The *shock-probe burying test (SPB).* In this model, rats are shocked by contact with a stationary, electrified probe inside a Plexiglas chamber. The chamber floor is covered with bedding material, which the rat can use to spray and cover aversive stimuli such as the shock-probe (Treit et al., 1981; Treit and Fundytus, 1988). Rats accomplish this with rapid, alternating, forward thrusts of their forepaws, toward and over the shock-probe. Rats shocked from the probe also show increased plasma corticosteroids and adrenaline, and carefully avoid the probe while they bury it. All of these fear responses can be suppressed by standard anxiolytic drugs (e.g., diazepam) and enhanced with "anxiogenic" drugs (e.g., Yohimbine). Resting quietly on the chamber floor is taken as an inverse measure of general activity (Treit, 1990; Treit and Menard, 2000).

The next section is a brief overview of neuroanatomical and neurochemical substrates of anxiety, using the same sorts of animal models as those described above (i.e., the untrained fear reactions of rats), as well as early, unstructured behavioral observations that have stimulated later, experimental research.

The neural mechanisms of anxiety: Amygdala

One of the first indications that sub-cortical temporal lobe structures such as the amygdala or hippocampus might be involved in fear or anxiety came from the early work of Kluver and Bucy (1937) with rhesus monkeys. After the monkeys had recovered from bilateral lesions of the temporal lobes, Kluver and Bucy noted that they displayed a peculiar pattern of behaviors. They 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 animals of opposite sex, or with other species entirely (Kluver and Bucy, 1937). 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.

Modern research has supported and refined these early conceptualizations of the "emotional brain." For example, many studies have shown that amygdalar 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 1988, 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 (e.g., Riolobos et al., 1987). Similarly, when the amygdala is lesioned with a neurotoxin such as 5,7 dihydroxytrpytamine, anxiolytic effects fail to occur in the elevated plus maze, whereas 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 $GABA_A$ 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, 2008). Specific targeting of receptor sub-types 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-amygdalar microinfusion of benzodiazepines produce anxiolytic effects in a number of rat anxiety models, presumably through positive allosteric modulation of the $GABA_A$ receptor, which in turn increases neural inhibition through increasing the frequency of chloride channel openings (Engin and Treit, 2008). These general findings have been corroborated using different benzodiazepine receptor agonists, such midazolam, (e.g., Green and Vale, 1992; Zangrossi and Graeff, 1994) and direct $GABA_A$ 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, 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.

Neuropeptides are made up of small strings of 2-50 amino acids that can function either like classical, small molecule neurotransmitters (e.g., GABA) or as neuromodulators, acting indirectly through the classical neurotransmitter systems (Hokfelt et al 2000). The effects of neuropeptides on brain function in general and on anxiety in particular is still a relatively new area of investigation. Not unexpectedly, the anxiolytic-like effects of neuropeptides, when they occur, are often complex and varied. Neuropeptides, for the most part, have had unreliable effects in models such as the plus-maze after microinfusion into the amygdala. For example, neuropeptide Y (NPY) or its synthetic agonists have produced both anxiolytic and anxiogenic effects in the elevated plus-maze, while NPY receptor antagonists have produced anxiolytic effects, null effects, and anxiogenic effects Although some of these inconsistencies might be due to dose or site-specific effects, the evidence in favor of these methodological explanations weakened by the fact that opposite effects on anxiety have been found for both agonists and antagonists of NPY (Engin and Treit, 2008).

The neural mechanisms of anxiety: The Septum and Hippocampus

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 their abnormal stimulation 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 circuit (1937). Consistent with the psychodynamic theories of the time,

MacLean proposed that emotional disorders occurred as a result of impaired integration of the "viscera l brain" (e.g., hippocampus) with the "word brain" (neocortex).

Gray (1982) provided early evidence that the septum and hippocampus played an important role 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 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 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 (e.g., 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 (e.g., 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, 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., Pesold and Treit, 1990). More importantly, intra-septal microinfusions of $GABA_A$ 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; 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 recalled, 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 μl) 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 effects anxiolytic drugs accurately microinfused into either the medial *or* the lateral septum could ultimately be due to actions at the non-targeted sub-nucleus.

The effect of neuropeptides on anxiety is generally variable, but some of the results found in the septum are relatively straight forward. For example, intra-septal NPY (the endogenous agonist) 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 (Kask et al., 2001; Echevarria et al., 2005). Agonists of CRF, which itself begins the HPA hormonal "stress response," produced predictable 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).

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 (O'Keefe and Nadel, 1978; Morris, 1982). And by the end of the 20th century, the role the hippocampus in cognitive 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; Treit et al., 1993). Pharmacological inhibition of hippocampal function through microinfusion of $GABA_A$ agonists, GABA reuptake inhibitors, and Na+ 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; Engin and Treit, 2007; Liberato et al., 2006; McEown and Treit, 2009; Menard and Treit, 2001; Pesold and Treit, 1996).

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, 2005. Smialowska et al., 2007 found that NPY infusions into the hippocampus

produced anxiolysis in the elevated plus maze, while both NPY1 and NPY2 receptor antagonists reversed these effects. More recently, Engin and Treit have shown 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). Finally, Engin et al., found that intra-ventricular microinfusion of somoatostatin 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.

Hippocampal Theta Rhythm and Anxiety

Hippocampal theta rhythm plays myriad of roles in a variety of behavioural, cognitive and physiological functions, including sensorimotor integration (Bland and Oddie, 2001), sensory inhibition (Sainsbury, 1998), novelty detection (Gray, 1982; Naber et al., 2000; Lever et al., 2010) behavioural inhibition (Gray, 1982), arousal (Green et al., 1954), orienting (Grastyan et al., 1966), classical conditioning (Griffen et al., 2004; Seiger et al., 2002), instrumental learning (Adey et al., 1966), attention (Bennet et al., 1971; 1975; Kemp et al., 1975), spatial navigation (O'Keefe and Burgess, 1999), recognition memory (Givens 1996) working memory (Meltzer et al., 2008), and episodic memory (Hasselmo, 2005; 2009).

The work on the hippocampal theta model of anxiety was first initiated in experiments by McNaughton and Sedgwick (1978). In this model, McNaughton et al. proposes that the suppression of evoked hippocampal theta rhythm through reticularly activated stimulation is a predictive neurophysiological index of anxiolytic drug action (McNaughton and Sedgwick,

1978; McNaughton, Gray and McNaughton, 2000; McNaughton et al., 2007). There is also a plethora of pharmacological evidence suggesting that these reductions in the frequency of the theta rhythm only occurs after the administration of clinically effective anxiolytic drugs (e.g. benzodiazepines, 5HT1A agonists, SSRIs) (McNaughton et al., 2007). Furthermore, drugs that are not used primarily as therapeutic in anxiety (e.g antipsychotics, anticonvulsants, or precognitive drugs), do not have this effect on hippocampal theta rhythm.

Recently, there has been some evidence showing that drugs with no known effects on anxiety depressing the frequency of hippocampal theta. For example, a few studies have found that microinfusions of the cyclic neuropeptide somatostatin, and a ligand for the SSR2 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). In addition Yeung et al., 2011, found that a clinically effective dose of phenytoin, an anti-convulsant drug commonly used as a therapeutic for epileptic seizures, suppressed evoked theta to levels similar to that of diazepam and had robust anxiolytic effects in the elevated plus maze. In conclusion, these results provide additional support for the validity McNaughton's hippocampal theta model of anxiolytic drug action as an index of experimental anxiety.

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Chapter 2: Anxiolytic-like effects of somatostatin isoforms SST14 and SST28 in two animal models *(Rattus Norvegicus)* after intra-amygdalar and intra-septal microinfusions

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ABSTRACT

Rationale and objectives: Somatostatin (SST) isoforms SST 14 and SST 28 inhibit regulatory hormones in the periphery (e.g., growth hormone) and are widely distributed in the brain. In recent experiments, intracerebroventricular (ICV) somatostatin produced anxiolytic-like effects in both behavioral and electrophysiological models. The sites of action of these anxiolytic effects in the brain, however, and the relative contributions of SST 14 and SST 28 to these effects are unknown.

Materials and methods: Anxiolytic effects were assessed in the plus-maze and shock-probe tests after 1) intra-amygdalar microinfusion of SST 14 (0.5 or 3µg per hemisphere) or SST 28 (3µg per hemisphere), 2) intra-septal microinfusion of SST 14 (0.5 or 1.5 µg per hemisphere) or SST 28 (1.5 µg per hemisphere) or 3) intra-striatal microinfusion of SST 14 (3µg per hemisphere).

Results: Intra-amygdalar and intra-septal microinfusions of SST 14 and SST 28 produced robust anxiolytic-like effects in the behavioural tests, unlike intra-striatal microinfusions. The magnitude of the anxiolytic effects in the amygdala and septum were comparable to those found previously with ICV SST 14, ICV L-779976, a somatostatin (sst2) receptor agonist, and ICV diazepam, a classical benzodiazepine anxiolytic.

Conclusions: Somatostatin receptors in the septum and amygdala are responsive to both SST 14 and SST 28, but not those in the striatum. Although no obvious differences in the anxiolytic-like effects of the isoforms were detected, quantitative or even qualitative differences in their specific

anxiolytic effects may occur in different sub-regions of the septum and amygdala, as has been found for benzodiazepine anxiolytics.

Introduction

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 prosomatostatin, whereas SST 14 is synthesized from either prosomatostatin or by proteolytic conversion of SST 28. Peripherally, 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 cholecystokinin from the gastrointestinal tract (Moller et al. 2003). While somatostatin controls hormone function in 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).

Although it seems possible that SST 28 is merely a non-functional precursor of SST 14, SST 28 modulates similar hormonal functions, albeit with different potencies. For example, Mandarino et al. (1981) found that SST 28 suppressed insulin release with twice the potency of SST 14, whereas SST 14 suppressed glucagon release with six times the potency of SST 28. SST 14 and SST 28 bind at equally high levels in the cortex and in 'limbic' structures such as the septum, hippocampus, and amygdala (Leroux et al. 1985). SST is often co-localized with γ-amino butyric acid (GABA; Sur et al. 1994; Saha et al. 2002), an inhibitory neurotransmitter that has long been implicated in anxiety (Tallman and Gallager 1985). 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).

SST 14 and SST 28 can act as neurotransmitters or neuromodulators in the brain, modifying neuronal excitability. For example, Meis et al. (2005) found that SST 14 induced a G-protein-receptor linked, inward rectifying K+ (GIRK) current within the lateral amygdala, dampening cell excitability. Wang et al. (1989) found that SST 14 increased voltage-dependent K+ currents in cortical neurons, while SST 28 *decreased* K+ currents in the same neurons. These effects of SST 14 and SST 28 on K+ currents showed no cross-desensitization, suggesting actions at different somatostatin receptor subtypes (Wang et al. 1990). Karschin (1995) showed that stimulation of the sst1 receptor in oligodendrocytes also decreased GIRKs. Finally, Kreienkamp et al. (1997) found that the EC50 values for the activation of inhibitory GIRK currents by SST 14 and SST 28 could differ by as much as an order of magnitude, depending on their action at somatostatin receptor subtypes (sst1-5). Thus, in addition to differences in the magnitude of the hormonal effects of SST 14 and SST 28, their effects on neuronal excitability can also differ dramatically (Kreienkamp et al. 1997; Meis et al. 2005).

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 et al. 1990), spatial memory (Dutar et al. 2002), and emotion (Engin et al. 2008). 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-HT1A agonists, and SSRIs; McNaughton et al. 2007).

Whether or not brain SST 28 is also involved in anxiety is unknown, as are the sites of action in the brain where somatostatin produces its anxiolytic-like effects. In addition, it would be useful to demonstrate anxiolytic effects in more than one model. Accordingly, the purpose of experiment 1 was to compare the effects of intra-amygdalar microinfusions of the SST 14 and SST 28 in two animal models, the elevated plus-maze and shock probe burying test. The amygdala was chosen as the target site for three reasons. First, the involvement of the amygdala in the regulation of fear and anxiety is well documented (e.g.,LeDoux 2000; Pesold and Treit 1995). Second, somatostatin in some amygdalar neurons is both co-localized and co-released with GABA (Batten et al. 2002). GABA, through the $GABA_A$ receptor and an allosteric binding site for benzodiazepines, modulates both experimental anxiety in animals and clinical anxiety in humans (Treit et al. 2010). Third, SST receptor gene expression in the amygdala is increased by predatory stress (Nanda et al. 2008).

The septum also has high levels of both somatostatin and GABA, and has been independently implicated in anxiety (e.g., Degroot and Treit 2004; Engin and Treit 2008b; Gray and McNaughton 2000; Pesold and Treit 1994; 1996; Shin et al. 2009; Treit and Menard 1997; 2000). Thus, it seemed likely that somatostatin microinfusions into the septum would also produce anxiolysis. Thus, the purpose of experiment 2 was to corroborate the findings in experiment 1 in another limbic structure implicated in anxiety, extending our neuroanatomical understanding of somatostatin's anxiolytic effects. The purpose of experiment 3 was to assess the effects of a low dose of SST 14 in the amygdala and the septum, and experiment 4 addressed the site- specificity of the anxiolytic effects of SST 14 in the amygdala and septum by infusing it

into nearby areas in the striatum. The striatum also synthesizes somatostatin, and expresses various somatostatin receptor sub-types, including sst2 (Santis et al. 2009), which was previously implicated by Engin et al. in anxiety (Engin et al., 2008; Engin and Treit, 2009).

Materials and Methods

2.1. Subjects

Subjects were 98 male, Sprague-Dawley rats, weighing 200-300g at the time of surgery. Rats were individually housed in 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. 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 assigned to surgery conditions (amygdala, septum, striatum).

2.2. Surgery

Rats were anesthetized with isofluorane (5% induction, 1.5% maintenance in 67% N2O and 33% O2; Halocarbon Product Corp. River Edge NJ, USA), injected with atropine sulfate (0.1 mg/0.2 mL i.p.; Bimeda-MTC, Cambridge, Ontario, Canada) and Marcaine (1.5 mg/0.3mL s.c. just under the cranial skin; Hospira, Quebec, Canada), and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). Following hydration with 0.9% saline (3cc, i.p.), an incision was made to expose 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), the septum (AP: 0.7 mm, ML: -2.6, DV: -4.2, angled 22° towards the midline) or the striatum (AP +0.5, ML +/- 3.0 DV -5.6). 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 surgery area was treated with 2.5 mg carprofen (Rimadyl©, Pfizer; 2.5 mg/0.5 mL s.c. on the head). Following the surgery, the subjects were placed in a warm environment until they regained consciousness. Rats were then allowed to recover for at least 5 days in their home cages before the start of behavioral testing.

2.3. Infusion procedure

Seventy-five rats from the amygdalar and septal surgical groups were randomly assigned to receive SST 14, SST 28, or vehicle prior to behavioral testing. Both SST 14 (Sigma, St. Louis, MO, USA) and SST 28 (AnaSpec, Fremont, CA, USA) were dissolved in a 5% DMSO vehicle at concentrations of 3 μg/μL (1.5 μg per hemisphere) and 6 μg/μL (3.0 μg per hemisphere). Smaller groups of amygdalar (n = 7) and septal implanted (n=8) rats received 1 μg/μL of SST 14 (0.5 μg per hemisphere) or vehicle before testing, while a striate-implanted group (n = 8) received 6 μg/μL of SST 14 (3.0 μg per hemisphere) or vehicle before testing. The drugs were infused bilaterally (0.5, 1.5 or 3.0 μg/ hemisphere) via an infusion pump (Harvard Apparatus 22, MA, USA) at a rate of 1μL/min for 30 sec per hemisphere. SST 14, SST 28, and vehicle solutions were infused 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 40 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 (for details see Treit et al. 1993; Treit and Pinel 2005). The experimenter handled each of the rats for 5min, checking the cannulae tracts for blockage and habituating the rats to the infusion procedures, on each of the 4 consecutive days prior testing. All behavioral testing occurred in a quiet testing room between 0900 h and 1700 h and was recorded on videotape. Testing started 10 min after the end of infusion procedure, as described in previous studies (e.g., Engin et al. 2008). The subjects were assigned to the same drug treatment groups for both behavioral tests. The plus-maze test occurred first, followed seven days later by the shock probe test.

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 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. A higher 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 et al. 1986).

2.4.2. Shock-probe burying

Three days after the elevated plus-maze test, the rats began habituations for the shock-probe burying test. The 40×30×40 cm Plexiglas shock-probe chamber contained wood-chip bedding material distributed evenly on the floor of the chamber. Rats were habituated individually in the shock-probe chamber, without the probe in place, for 30 min on each of the 4 consecutive days before the test. On the test day, rats were placed individually on the floor of the chamber, which now had an electrified probe (6.5 cm long and 0.5 cm in diameter) protruding from one of the walls, 2cm above the bedding material. Each time the rats came into contact with the probe they received a shock (2 mA). Current was generated with an AC shocker (H13-15 precision regulated shocker, Colbourn Instruments, Allentown, PA, USA). The 15 min test period began when the first shock was received, and the probe remained electrified throughout the testing period. During this period, the following measures were taken: (1) total amount of time spent spraying bedding material towards or on top of the shock probe, with rapid, alternating pushing movements of the forepaws (i.e., burying behavior); (2) number of shocks received due to contact with the probe; (3) amount of time spent immobile (e.g., rest, sleep); and (4) reactivity to shock, which was measured on a four-point scale: (1) flinch involving only head or forepaw, (2) whole body flinch, with or without slow ambulation away from the probe, (3) whole body flinch, and/or jumping, followed by immediate ambulation away from the probe, and

(4) whole body flinch and jump (all four paws in the air), followed by immediate and rapid ambulation (i.e. running) to the opposite end of the chamber. An average reactivity score was computed for each animal by summing up reactivity scores for all the shocks taken and dividing this by total number of shocks taken. Total amount of time spent burying the probe was taken as a measure of anxiety, with reduced burying indicating anxiolysis. The number of contact-induced probe-shocks was also used as a measure of anxiety, with increased contacts indicating reduced anxiety. Time spent immobile (e.g., resting on the floor of the chamber) was an inverse index of general activity. Finally, mean shock reactivity was used as a measure of pain sensitivity (for details see Treit et al. 2010). All testing took place between 0900 and 1700 h. The bedding material was cleaned between animals and smoothed to an even thickness before the next animal was tested.

2.5. Histology

Following behavioral testing, rats were euthanized with an overdose of sodium pentobarbital (Nembutal) and perfused intracardially with 0.9% (wt/vol) saline followed by 4% (vol/vol) formaldehyde. Post-fixation, the brains were removed from the skull and placed in a 4% formaldehyde solution for at least 48 h. The brains were then frozen with dry ice and cut into 60-µm sections with a sliding microtome (Model 860, American Optical Company, Buffalo, New York). Every second section was collected and mounted onto a microscope slide and later stained with thionin. 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 from both tests were analyzed with planned comparisons (Keppel and Zedeck 1989; ANOVA; $\alpha = 0.05$)

Results

3.1 *Histology.* Figure 1 shows the approximate infusion sites of rats with amygdalar cannulae included in the behavioural analysis of experiment 1. Most of the cannulae tips were bilaterally clustered in the medial and lateral divisions of the central amygdala, as well as the basolateral amygdala. Four out of thirty six rats, however, were excluded from the main behavioral analyses as a result of misplaced cannulae (indicated by the black squares in Figure 1). Their data were later compared to that of the included control rats in a small, post hoc analysis of site-specificity. Figure 2 shows the approximate infusion sites of rats with septal cannulae included in the behavioural analysis of Experiment 2. All 39 rats in this surgical group had cannulae tips bilaterally centered in the intermediate and dorsolateral regions of the septum. Three rats in experiment 2 fell off the plus-maze so their data was excluded from that analysis. The same 3 rats were tested in the shock-probe without incident and their data were included in that analysis. Figure 3 shows the approximate infusion sites of rats with striatal cannulae included in an a priori analysis of site-specificity in experiment 4. All placements were bilaterally centered in striatum.

3.2 Experiment 1: Intra-amygdalar infusions

Elevated Plus-maze. As can be seen in Figure 4, both SST 14 and SST 28 produced robust anxiolytic-like effects in the elevated plus-maze. Planned comparisons showed that both the SST 14 group ($F_{(1,30)} = 4.21$; p=0.044), and the SST 28 group ($F_{(1,30)} = 7.52$; p=0.001) had significantly higher percentages of open-arm entries, compared to the vehicle control group. Likewise, both the SST 14 ($F_{(1,30)} = 5.37$; p=0.012), and SST 28 ($F_{(1,30)} = 8.14$; p=0.001) groups spent a significantly higher percentage of time in the open-arms than the vehicle control group. Neither the percentage of open-arm entries nor the percentage of time spent in the open-arms differed significantly between the SST 14 and SST 28 groups (% open-arm entries ($F_{(1,30)}$ = 3.31, p>0.11; % open-arm time ($F_{1,30}$) = 2.77, p>0.17). There were no differences between the groups on either measure of general activity (closed arm entries: $F_{(1,30)} = 2.10$, p>0.30; total arm entries $F_{(1,30)} = 1.92$, p>0.46; see Table 1). When the 4 rats with misplaced cannulae (data not shown) were directly compared to the original vehicle control group (see Figure 4), there were no significant differences in the percentage of open-arm entries ($F_{(1, 13)}$=0.59, p>.5) or percentage of open-arm time ($F_{(1,13)}$=0.25, p>.5), suggesting some degree of site-specificity in the anxiolytic effects of SST in the amygdala.

Shock Probe Test. Figure 5 shows that SST 14 and SST 28 produced clear anxiolytic-like effects in the shock probe burying test. Planned comparisons revealed a significant suppression of burying behavior in both the SST 14 group ($F_{(1,30)} = 5.09$; p=0.016) and SST 28 group ($F_{(1,30)}$ = 4.65; p=0.027), compared to the vehicle control group. A direct comparison of the effect of SST 14 and SST 28 in the shock-probe test was not significant ($F_{(1,30)} = 0.44$, p>0.87). Measures of general activity ($F_{(1,30)} = 0.38$, p>0.85), number of probe contacts ($F_{(1,30)} = 0.62$, p>0.76) and reactivity to shock ($F_{(1,30)} = 1.35$, p>.51), did not differ between groups (Table 2). There was no

significant difference in the duration of burying behavior between the 4 rats with misplaced cannulae (data not shown) and the vehicle infused controls ($F_{(1,13)}$=4.16, p>0.06)

3.3 Experiment 2: Intra-septal infusions

Elevated Plus-maze. Similar to the findings of Experiment 1, figure 6 shows that intra-septal infusion of either SST 14 or SST 28 produced unambiguous anxiolytic-like effects in the elevated plus-maze. Planned comparisons showed that the percentages of open-arm entries for the SST 14 group ($F_{(1,33)}$ = 6.44; p=0.01), and the SST 28 group ($F_{(1,33)}$ = 9.61; p=0.004) were significantly higher than the vehicle control group, as were the percentages of open-arm time [SST 14: ($F_{(1,33)}$ = 7.29; p=0.011; SST 28: ($F_{(1,33)}$ = 14.13; p=0.001]. Neither measure of anxiolysis differed significantly between the SST 14 and SST 28 groups (% open-arm entries: F $_{(1,33)}$ = 0.38, p>.50; % open-arm time: ($F_{(1,33)}$ = 2.38, p>.24). There were no significant between-group differences in closed entries ($F_{(1,33)}$ = 0.51, p>.80), or total arm entries($F_{(1,33)}$ = 0.49, p>0.81; Table 3).

Shock Probe Test. The anxiolytic effects of intra-amygdalar SST 14 or SST 28 in the shock probe burying test in Experiment 1 were replicated by intra-septal infusions of the same compounds in Experiment 2 (see Figure 7). Here, a significant suppression of burying behavior occurred in both groups compared to control [SST 14: ($F_{(1,36)}$ = 7.95; p=0.008; SST 28: ($F_{(1,36)}$ = 6.47; p=0.01]. Defensive burying did not differ significantly in rats given SST 14 compared to rats given SST 28: $F_{(1,36)}$ = 0.28, p>.89). Measures of resting behavior ($F_{(1,36)}$ = 1.96, p>0.33), number of probe contacts ($F_{(1,36)}$ = 1.99, p>.33) and reactivity to shock ($F_{(1,36)}$ = 2.08, p>0.31), did not differ significantly from control (Table 4).

3.4 Experiment 3: Intra-amygdalar infusions II: low dose.

Table 5 shows the mean $(\pm SEM)$ differences in open arm activity and burying behaviour in rats given intra-amygdalar infusions of vehicle or a low dose of SST 14 (0.5µg bilateral). Neither the percentage of open-arm entries ($F(_{1,5})$=3.37, p>.5) nor the percentage of open-arm time ($F(_{1,5})$ = 2.59, p>.5) were significantly different between the groups. Importantly, the direction of the mean differences in the plus-maze is opposite to that of an anxiolytic effect. The mean difference in the duration of burying behavior seen in Table 5 was also not significant ($F(_{1,5})$=1.18, p>.5). Taken together with the positive results of experiment 1, experiment 3 provides preliminary evidence that intra-amygdalar doses of SST 14 below 1.5 µg per hemisphere may not be sufficient to reliably produce anxiolytic effects in the plus-maze and shock-probe tests.

3.5 Experiment 3: Intra-septal infusions II: low dose.

Table 6 shows mean $(\pm SEM)$ differences between the low dose (0.5 µg per hemisphere) SST 14 groups and the vehicle control groups in the plus maze and the shock-probe burying tests. None of these differences were statistically significant [% entries:($F(_{1,6})$=0.26, p>.5) % time:($F(_{1,6})$=0.1, p>.5; duration of burying: ($F(_{1,6})$=0.93, p>.5)]. In the shock-probe burying test, he mean differences are opposite to an anxiolytic effect (Table 6).

Combined with the null effects of intra-amygdalar microinfusions of a 0.5µg bilateral dose of SST 14, described above, the null effects of the same microinfusion regimen in the septum suggest that doses higher than 0.5 µg per hemisphere may be necessary to produce reliable anxiolytic effects in the plus-maze and shock-probe tests.

3.6 Experiment 4: Intra-striatal infusions: high dose

The striatum synthesizes somatostatin and expresses somatostatin receptor sub-types (e.g., sst2; Santis et al. 2009). Be this as it may, neither the percent open-arm entries ($F_{(1,6)}$=1.10, p>.3), percent open-arm time ($F_{(1,6)}$=1.31, p>.3), nor the duration of burying behavior ($F_{(1,6)}$=2.57, p>.5) differed significantly between the SST 14 group (3.0 μg per hemisphere) and the vehicle control group (Table 7). These findings add evidence of site-specificity, since the same dose of SST 14 microinfused into the amygdala or septum in Experiments 1 and 2 produced reliable anxiolytic effects in both behavioural tests.

Discussion

SST 14 and SST 28 both had potent anxiolytic-like effects in the elevated-plus-maze and shock-probe burying tests, after microinfusions into two brain areas consistently implicated in anxiety: the amygdala and the septum. The increase in open- arm activity produced by intra-amygdalar or intra-septal microinfusion of SST 14 or SST 28 was comparable to that previously observed following ICV infusions of somatostatin 14, L-779976, (a synthetic sst2 agonist), and diazepam, a classical anxiolytic drug (see Engin et al. 2008; Engin and Treit 2009). The anxiolytic effects somatostatin appeared to be site-specific, since they did not occur in the striatum, an area that also expresses the sst2 subtype. Finally, the results show that the shock-probe burying test is also sensitive to the anxiolytic effects of SST agonists, providing converging evidence for their anxiolytic effects.

The anxiolytic-like effects of SST 14 and SST 28 are not readily explained by non-specific effects on general activity. Neither intra-amygdalar nor intra-septal SST 14 or SST 28

significantly changed measures of general activity in either test (see Tables 1-4). It is also unlikely that their anxiolytic-like effects in the shock-probe burying test were confounded by prior drug experience in the first test (elevated plus-maze). This would have required the development of drug tolerance (or sensitization) after a single, microgram dose of somatostatin, and the maintenance of this drug-induced change across the 7 day interval between the first test and the second. Nor does it seem plausible that DSMO, the vehicle used to dissolve somatostatin, could have produced neurotoxic effects that somehow led to the anxiolytic-like effects found in the plus-maze and shock-probe tests, for three reasons. First, all drug conditions were compared to surgically equivalent DSMO vehicle control groups. Second, doses of DSMO used for medical treatments (e.g., 2-6 g/kg/20 days; Brien et al. 2008), which have been associated with some adverse side-effects, are thousands of times higher than those used in the present experiments. Third, much higher doses of DMSO than used here, administered to adult rats for long durations, produced little or no evidence of neurotoxic effects (Authier et al. 2002).

The site-specificity of the anxiolytic effects of somatostatin found in the amygdala and septum was tested by microinfusing an effective dose (3.0 μg/ hemisphere) of SST 14 into the striatum, in an area approximately 2.5 mm posterior to the emergence of central and basolateral sub-regions of the amygdala, and 2.0 mm lateral to the septum (Paxinos and Watson 1986). The absence of an anxiolytic effect of this dose of SST 14 in the striatum is consistent with the view that the anxiolytic effects previously observed amygdala and septum were site-specific.

More questionable is the lack of difference in the anxiolytic effects of SST 14 and SST 28. Based on relative their potency differences in the periphery, and their varied effects on potassium channel currents in the brain (e.g., Karschin 1995 Kreienkamp et al. 1997; Wang et al. 1989; 1990), we expected some differences in their anxiolytic effects. Higher doses of SST 14

and SST 28 might have revealed these differences, but pilot studies showed doses higher than 3µg/per hemisphere (e.g., 8-12 µg) produced adverse side effects (e.g., sedation, ataxia). On the other hand, we found that a lower dose (0.5 µg bilateral) of somatostatin was not anxiolytic in the plus-maze or the shock-probe tests (Experiment 3).

Another possibility is that metabolic factors obscured differences in the anxiolytic effects of the two isoforms. SST 14 is produced through two biosynthetic pathways: direct enzymatic processing of prosomatostatin and/or the proteolytic conversion of SST 28 to SST 14 (Patel, 1999; Zingg and Patel 1982). Both conversion pathways are rapid in cell preparations (direct: 4 minutes; indirect: 8 minutes). Therefore, given the 10 min interval between microinfusion and behavioral testing in these experiments, enough time had passed for SST 28 to have been completely converted into SST 14 before the tests began, explaining the similarity of anxiolytic effects. The relative importance of these biosynthetic pathways, however, is highly dependent on the brain area under examination (Patel 2004; Robbins et al. 1983; Zingg and Patel 1982). Directly quantifying the conversion kinetics of the two pathways in the amygdala and septum would be less feasible than two other approaches. The simplest is to reduce the time between microinjection and behavioural testing to 3 min, thus reducing the possibility of conversion of SST 28 to SST 14. The second is to combine microinfusion of SST 28 with hexa-d-arginine, a known inhibitor of PACE-4, the enzyme that converts SST 28 into SST 14 (Hall et al. 2007; Schindler et al., 1996). Anxiolytic effects that survive a reduction of the injection-test interval and/or enzyme inhibition could then be attributed to SST 28.

SST 14 and SST 28 have similar affinities for each of the five somatostatin receptor subtypes, with the exception of sst5, where SST 28 has a 10-50 fold greater affinity than SST 14 (Olias et al. 2004; Patel 1999). However, only an sst2 receptor agonist reduced anxiety in

previous experiments; agonists of sst1,sst3, sst4 and sst5 were not effective (Engin and Treit 2009). Given the similar affinities of SST 14 and SST 28 for the sst2 sub-type, and the similar densities of sst2 receptors in the amygdala and septum (Bassant et al. 2005; Breder et al. 1992), commonalities in their anxiolytic effects might be expected. Nevertheless, previous experiments have found differences in the anxiolytic effects of $GABA_A$ receptor agonists (e.g., midazolam) microinfused into the septum and the amygdala, as a function of the specific sub-regions into which there were infused (e.g., Pesold and Treit 1995; 1996). Thus it seems possible, given overlap in the functions and distributions of somatostatin and $GABA_A$ receptors in these regions, that similar sub-regional differences in the anxiolytic somatostatin might also be discovered.

Synthetic analogues of somatostatin have been developed that already play a useful role in the treatment of a number of medical disorders, including tumour cell proliferation (e.g., de Jong et al. 1999; Hofland et al. 1992). As well, there is emerging evidence that somatostatin could play a role in CNS and PNS disorders, such as epilepsy, pain, and perhaps dementia (Chrubasik and Ziegler 1996; Pinter et al. 2006). In view of the present results, psychiatric disorders such as anxiety might be another class of therapeutic targets for somatostatin and its analogues (Weckbecker et al. 2003)

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Figure Captions

Fig 1. Schematic diagram of coronal sections of the rat brain illustrating the approximate locations (black circles) of amygdalar infusion sites in Experiment 1. The black 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. Schematic diagram of coronal sections of the rat brain illustrating the approximate locations (black circles) of septal infusion sites in Experiment 2. 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.

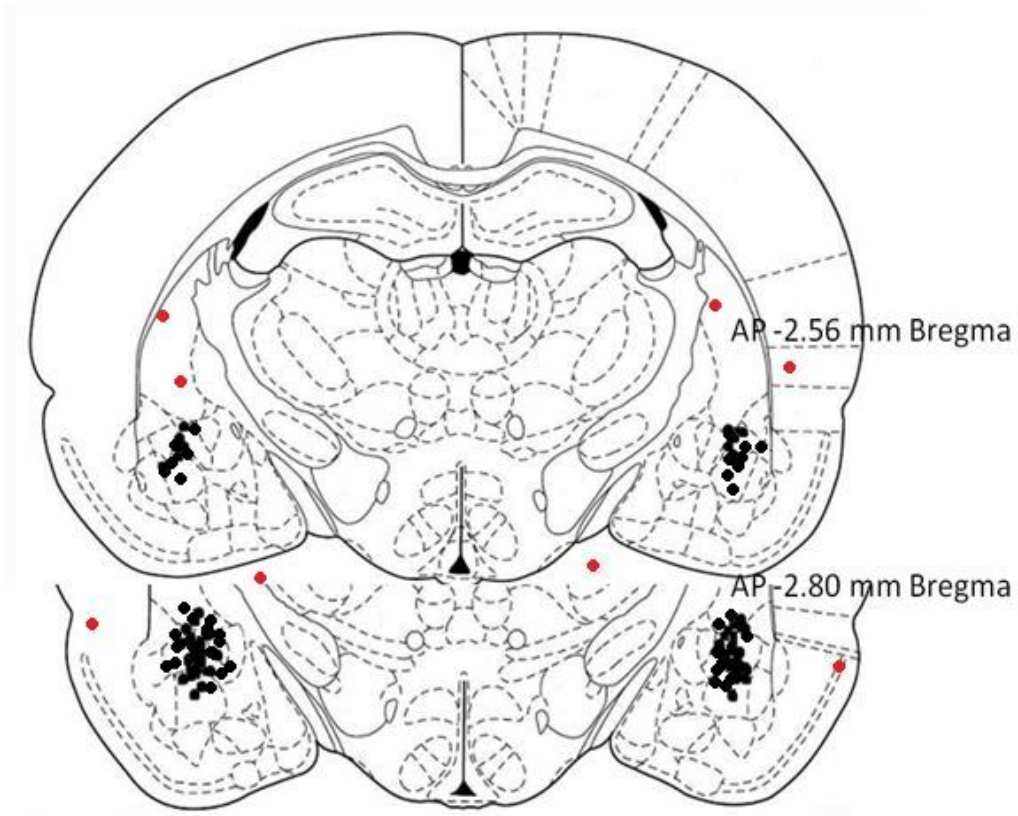
Fig 3. Schematic diagram of coronal sections of the rat brain illustrating the approximate locations (black circles) of striatal infusion sites in Experiment 4. There were no misplaced cannulae. The atlas plates are adapted from Paxinos and Watson (1986). Below is a representative photomicrograph of striatal cannulae sites

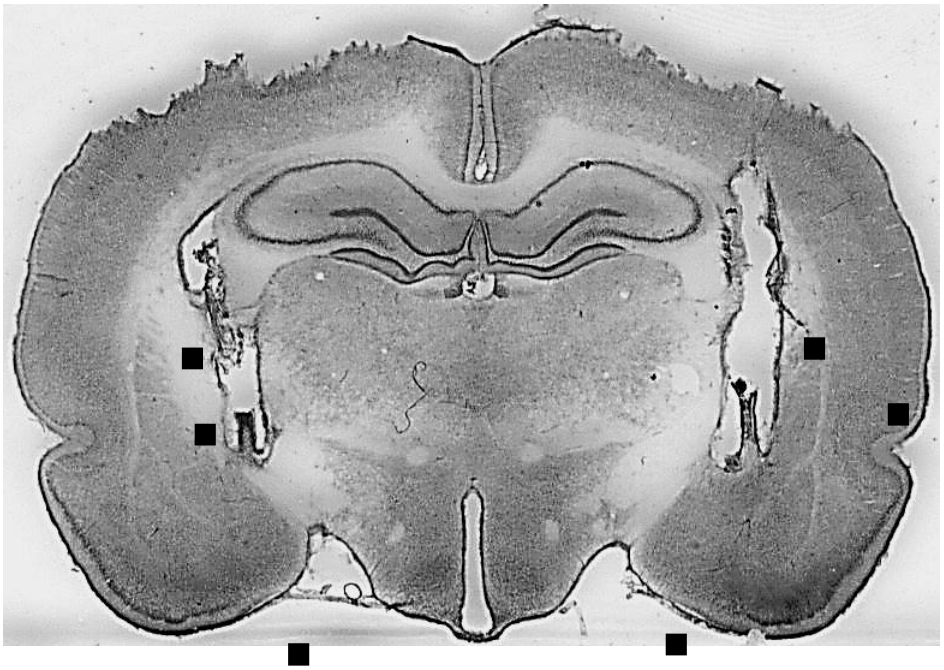
Fig 4. Open-arm activity following intra-amygdalar microinfusions of vehicle, 3μg bilateral of SST 14, or 3μg bilateral of SST 28 in Experiment 1. Black bars represent mean (±SEM) percentage of open-arm entries, white bars represent mean (±SEM) percentage of open-arm time. *Significantly different from the vehicle control group ($p<0.05$). †Significantly different from the vehicle control group ($p<0.01$).

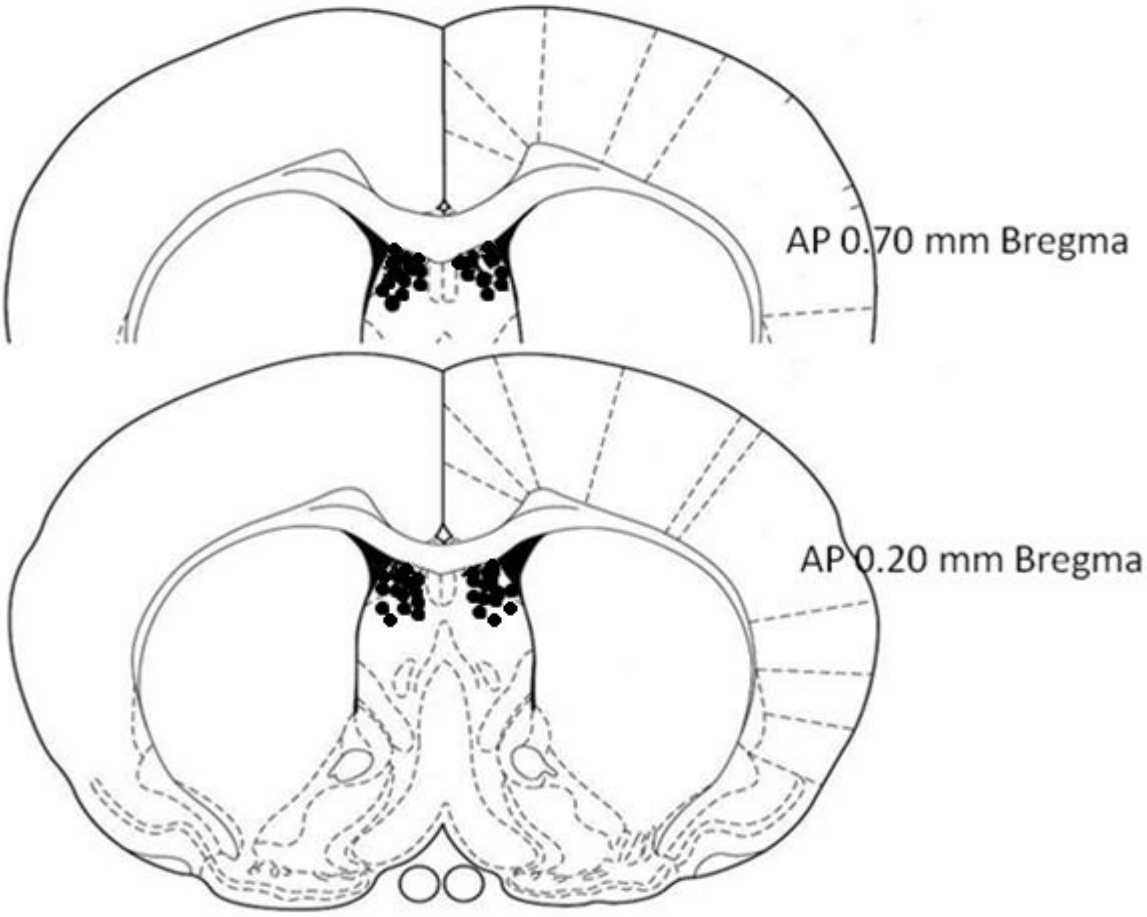
Fig 5. Mean duration (±SEM) of burying behavior following intra-amygdalar microinfusions of vehicle, 3 μg bilateral of SST 14, or 3 μg bilateral of SST 28 in Experiment 1. *Significantly different from the vehicle control group ($p<0.05$). †Significantly different from the vehicle control group ($p<0.01$)

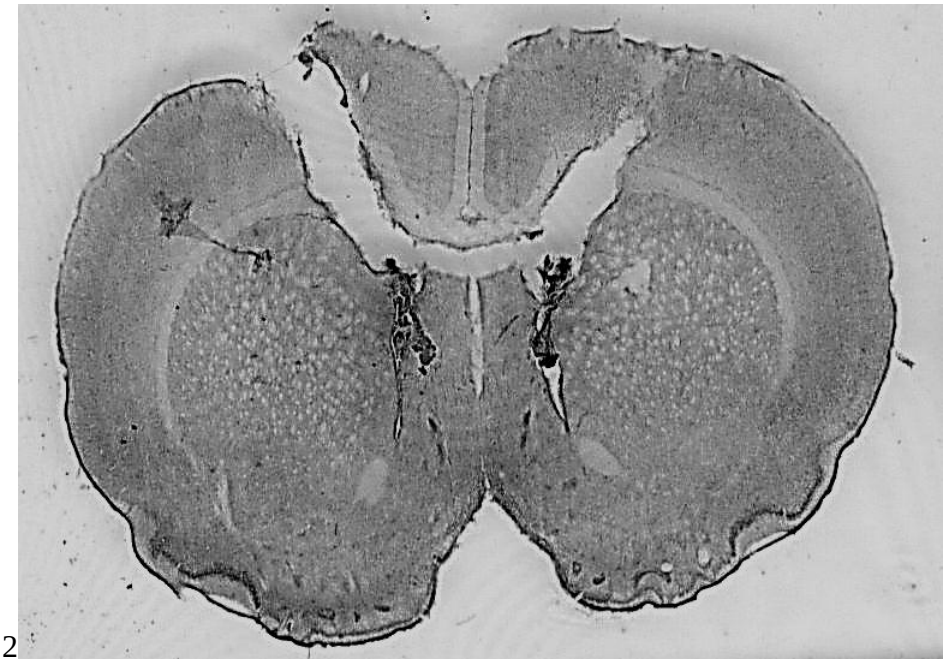
Fig 6. Open-arm activity following intra-septal microinfusions of vehicle 1.5μg bilateral of SST 14, or 1.5μg bilateral of SST 28 in Experiment 2. Black bars represent mean (±SEM) percentage of open-arm entries, white bars represent mean (±SEM) percentage of open-arm time. *Significantly different from the vehicle control group ($p<0.05$). †Significantly different from the vehicle control group ($p<0.01$)..

Fig 7 . Duration (±SEM) of burying behavior following intra-septal microinfusions of vehicle or 1.5 µg bilateral of SST 14 or 1.5 µg bilateral of SST 28 in Experiment 2. *Significantly different from the vehicle control group ($p<0.05$). †Significantly different from the vehicle control group ($p<0.01$)









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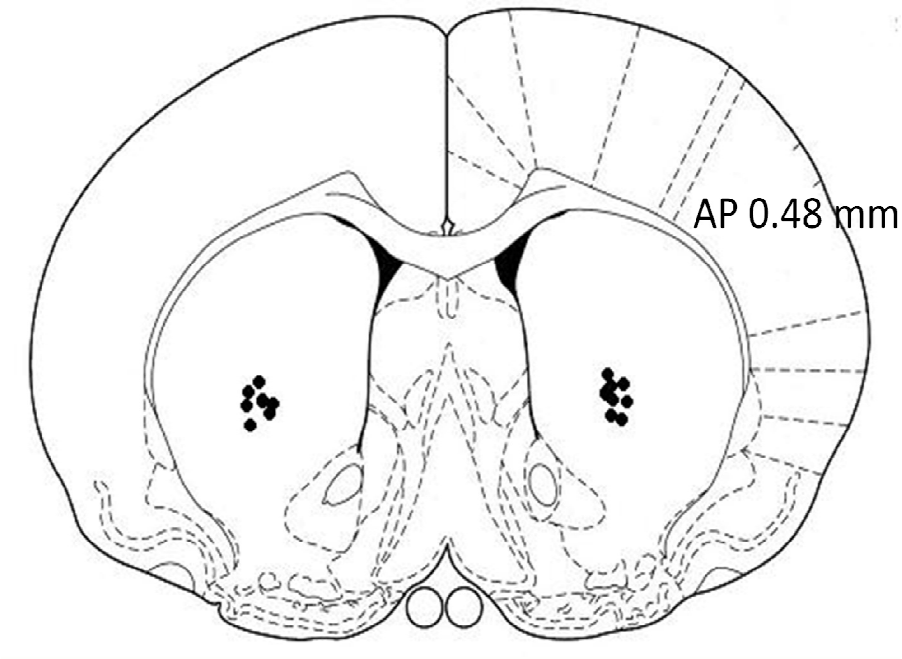

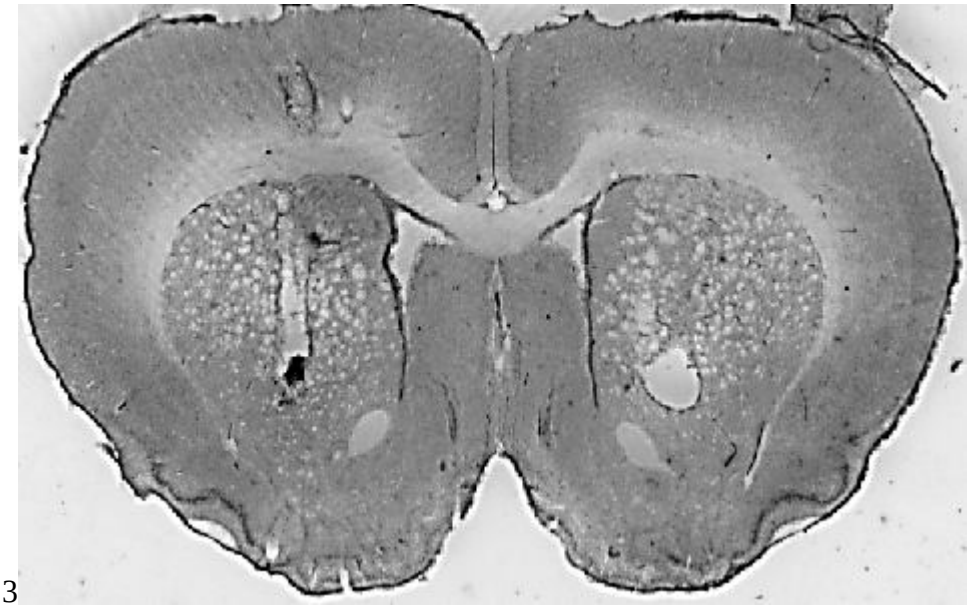

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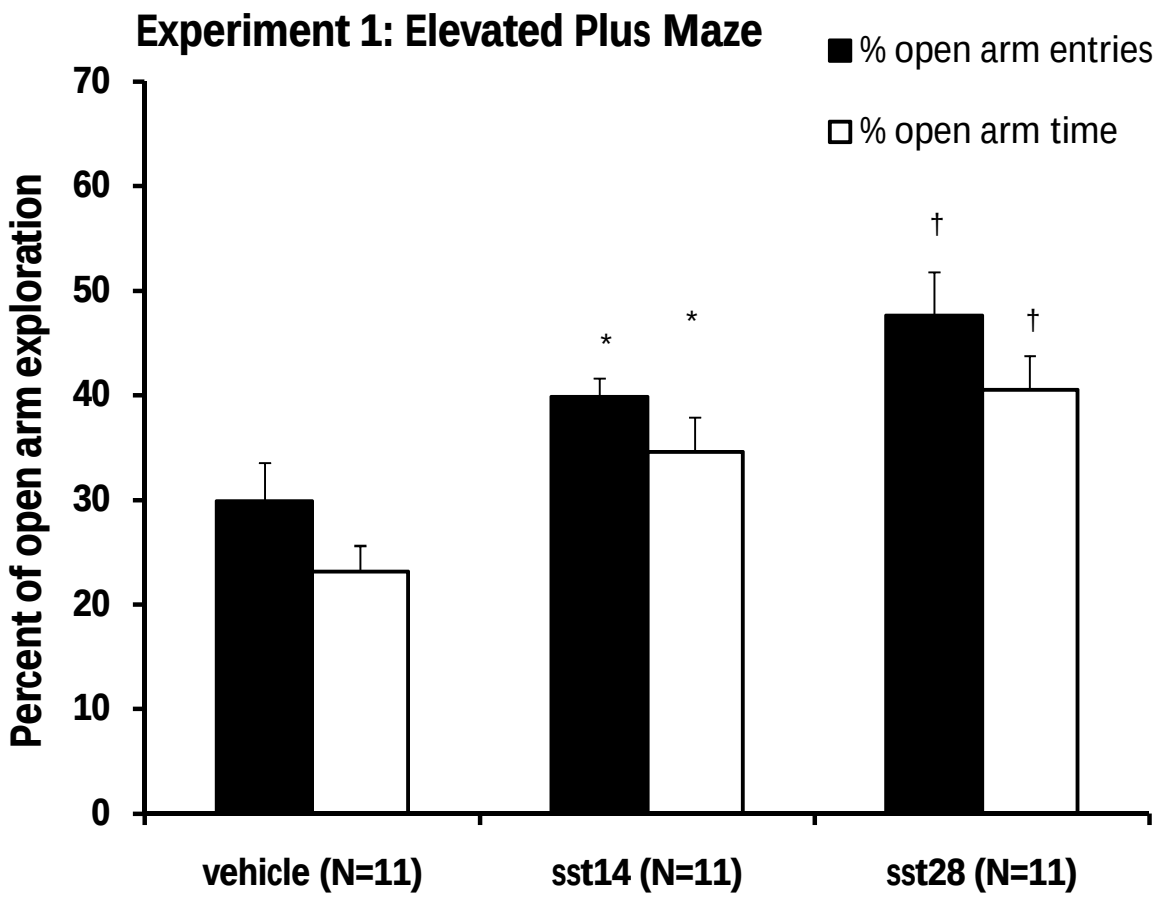
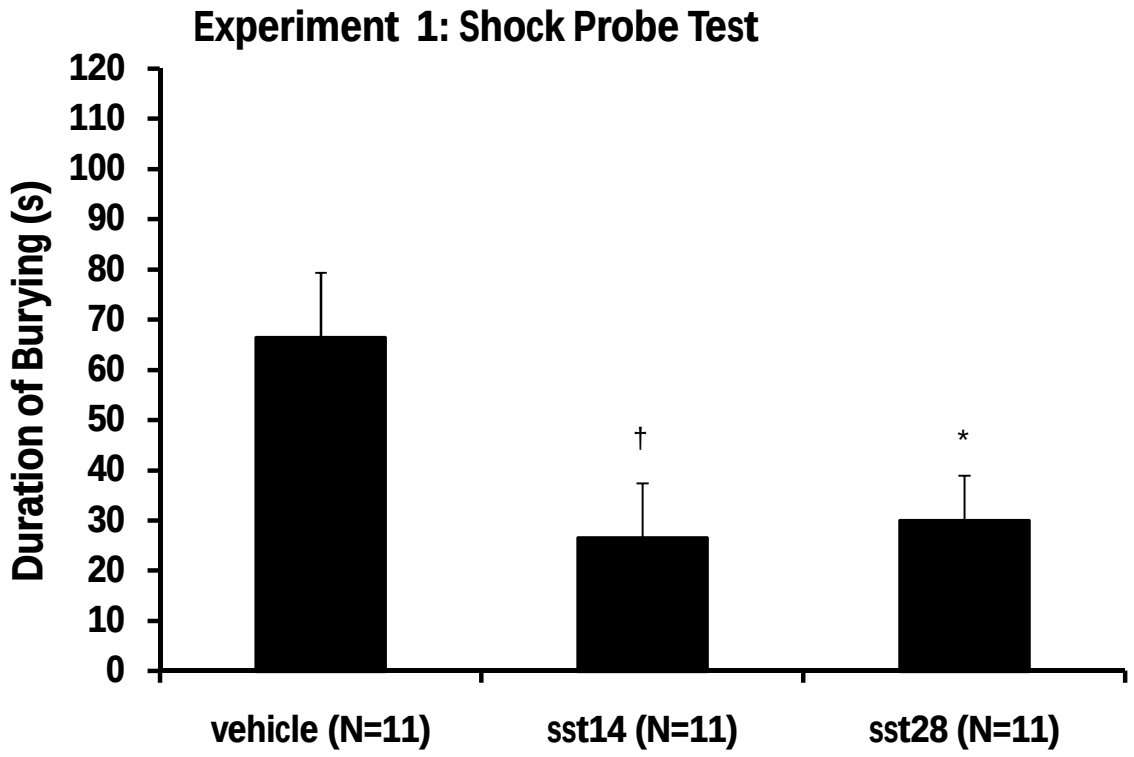
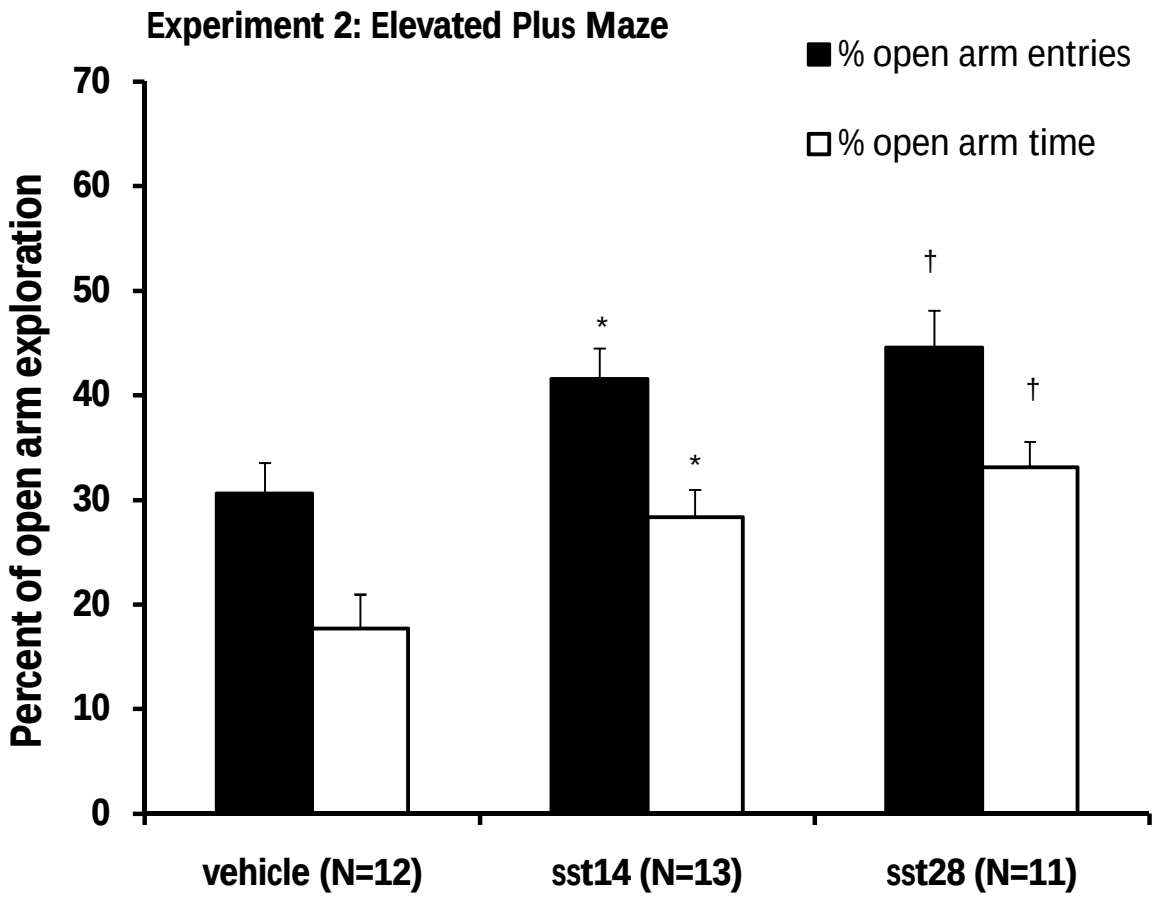

Experiment 1: Elevated Plus Maze
% open arm entries
% open arm time
Percent of open arm exploration
70
60
50
40
30
20
10
0
*
*
†
†
vehicle (N=11)
sst14 (N=11)
sst28 (N=11)

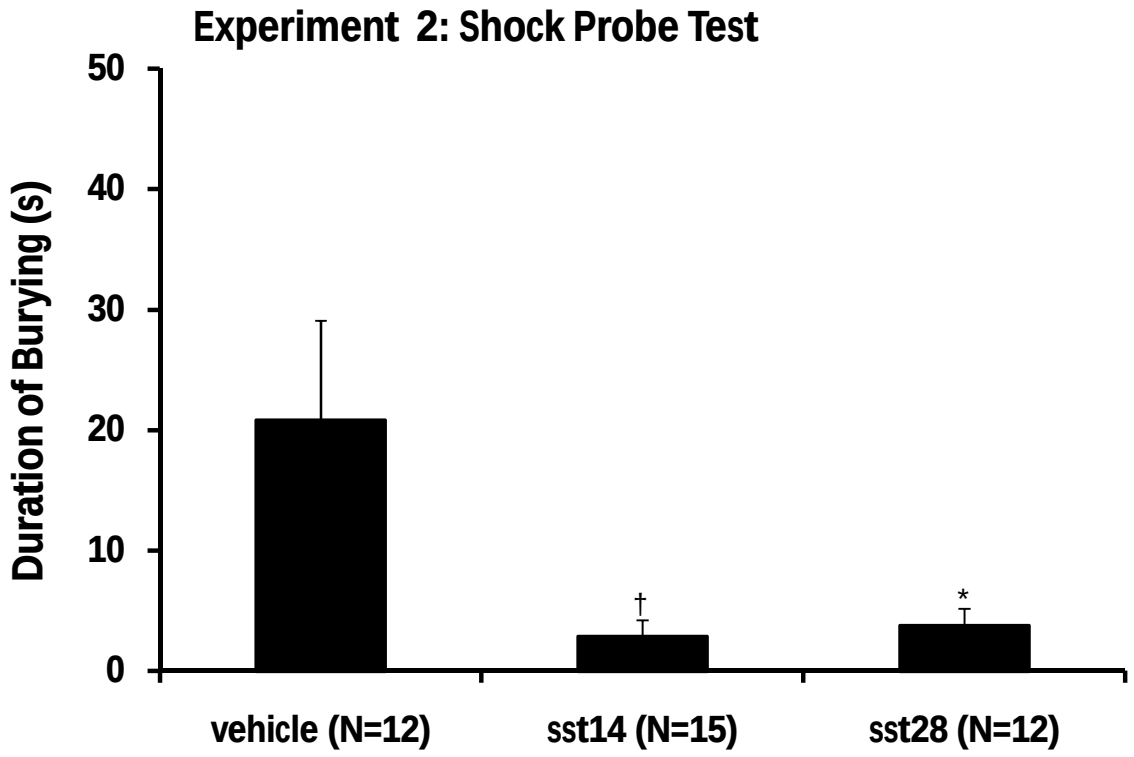
4


Experiment 1: Shock Probe Test
Duration of Burying (s)
120
110
100
90
80
70
60
50
40
30
20
10
0
†
*
vehicle (N=11)
sst14 (N=11)
sst28 (N=11)

5


Experiment 2: Elevated Plus Maze
% open arm entries
% open arm time
Percent of open arm exploration
70
60
50
40
30
20
10
0
*
*
†
†
vehicle (N=12)
sst14 (N=13)
sst28 (N=11)

6


Experiment 2: Shock Probe Test
Duration of Burying (s)
50
40
30
20
10
0
†
*
vehicle (N=12)
sst14 (N=15)
sst28 (N=12)

7

Table 1. General activity measures (Means ±SEM) in the elevated plus-maze test following intra-amygdalar microinfusions (3 μg/hemisphere) in Experiment 1.

Drug	Number of Closed Arm Entries	Total Number of Entries
Vehicle Control	9.09± (1.17)	12.64±(1.40)
SST14 (6 μg)	7.91±(0.73)	13.36±(1.38)
SST28 (6 μg)	6.36±(0.97)	11.36±(1.64)

Table 2. General activity and shock reactivity measures (Means ±SEM) in the shock probe burying test following intra-amygdalar microinfusions (3 μg/hemisphere) in Experiment 1

Drug	Still Time	Number of Probe Contacts	Shock Reactivity
Vehicle Control	48.91± (37.76)	1.81± (0.30)	0.73±(0.10)
SST14 (6 μg)	76.45±(35.64)	1.27±(0.14)	1.01±(0.12)
SST28 (6 μg)	85.36±(23.10)	1.36±(0.15)	1.09±(0.20)

Table 3. General activity measures (Means ±SEM) in the elevated plus-maze test following intra-septal microinfusions (1.5 μg/hemisphere) in Experiment 2

Drug	Number of Closed Arm Entries	Number of Total Entries
Vehicle Control	6.33± (0.85)	9.00±(1.07)
SST14 (3 μg)	6.92±(0.99)	11.92±(0.83)
SST28 (3 μg)	6.63±(0.56)	11.55±(1.35)

Table 4. General activity and shock reactivity measures (Means ±SEM) in shock probe test following intra-septal microinfusions (1.5 μg/hemisphere) in Experiment 2

Drug	Still Time	Number of Probe Contacts	Shock Reactivity
Vehicle Control	99.83± (46.41)	1.42± (0.51)	1.33±(0.44)
SST14 (3 μg)	136.67±(40.20)	1.60±(0.91)	1.10±(0.28)
SST28 (3 μg)	78.67±(40.04)	1.33±(0.49)	1.25±(0.40)

Table 5. Mean (±SEM) % open arm exploration in the plus-maze and mean (±SEM) duration of burying behavior following intra-amygdalar microinfusions of SST 14 (0.5 μg/hemisphere) in Experiment 3

Drug	% open arm entries	% open arm time	Duration of burying behavior (sec)
Vehicle	57.0 (± 14.5)	39.0 (± 3.1)	78.7 (± 39.5)
SST 14 (1 μg)	25.0 (± 5.2)	14.5 (± 5.6)	34.8 (± 19.6)

Table 6. Mean (±SEM) % open arm exploration in the plus-maze and mean (±SEM) duration of burying behavior following intra-septal microinfusions of SST 14 (0.5 µg/hemisphere) in Experiment 3

Drug	% open arm entries	% open arm time	Duration of burying behavior (sec)
Vehicle	49.3 (± 11.2)	39.0 (± 3.1)	8.0 (± 8.3)
SST 14 (1 µg)	41.3 (± 14.5)	35.8 (± 9.7)	49.3 (± 41.9)

Table 7. Mean (±SEM) % open arm exploration in the plus-maze and mean (±SEM) duration of burying behavior following intra-striatal microinfusions of the high dose of SST 14 in Experiment 4

Drug	% open arm entries	% open arm time	Duration of burying behavior (sec)
Vehicle	33.2 (± 5.9)	24.7 (± 7.0)	41.0 (± 40.0)
SST 14 (6 µg)	25.0 (± 5.2)	14.5 (± 5.6)	19.0 (± 10.9)

Chapter 3: A Critical Test of the Hippocampal Theta Model of Anxiolytic Drug Action

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ABSTRACT

Hippocampal theta rhythms have been associated with a number of behavioural processes, including learning, memory and arousal. Recently it has been argued that the suppression of hippocampal theta is a valid indicator of anxiolytic drug action. Like all such models, however, it has relied almost exclusively on the experimental effects of well known, clinically proven anxiolytic compounds for validation. The actual predictive validity of putative models of anxiolytic drug action, however, cannot be rigorously tested with this approach alone. The present study provides a stringent test of the predictive validity of the theta suppression model, using the drug phenytoin. Phenytoin has two important properties that are advantageous for assessing the validity of the theta suppression model: 1) it is a standard antiepileptic drug with no known anxiolytic effects, and 2) its primary mechanism of action is through suppression of the persistent sodium current, an effect that should also suppress hippocampal theta. Because of the latter property, we also directly compared the effects of phenytoin in the theta suppression model with it effects in the most widely tested behavioural model of anxiolytic drug action, the elevated plus-maze. While an anxiolytic-like effect of phenytoin in the theta suppression model might be expected simply due to its suppressive effects on sodium channel currents, anxiolytic effects in both tests would provide strong support for the predictive validity of the theta suppression model. Surprisingly, phenytoin produced clear anxiolytic-like effects in both neurophysiological

and behavioural models, thus providing strong evidence of the predictive validity of the theta suppression model.

Introduction

Hippocampal theta rhythm has been suggested to play a myriad of roles in brain and behavioural functions, including learning and memory (Griffin et al. 2004; Adey et al. 1966; Givens 1996; Meltzer et al. 2008; Hasselmo, 2005; 2009), spatial navigation (O'Keefe and Burgess, 1999) and more generally, sensorimotor integration (Bland and Oddie, 2001). An earlier line of research, however, viewed theta as a cortical representation of arousal (Green et al. 1954). This general idea of "arousal" was later delimited to "anxiety" by Jeffery Gray (1982), who further specified that the behavioural expression of anxiety corresponded to theta band activity in the septo-hippocampal system. Within this framework, anxiety *reduction* should be produced by manipulations that impair hippocampal theta, such as lesions to the septo-hippocampal region (Degroot and Treit 2004; Gray and McNaughton 2000; Treit and Menard, 2000), or anxiolytic drug administration (Munn and McNaughton, 2008; McNaughton and Coop, 1991; Pesold and Treit, 1996).

In a recent review, McNaughton et al. (2007) summarized an impressive array of data showing that all clinically viable anxiolytic drugs inhibit rat hippocampal theta, whether they are $GABA_A$ receptor agonists (e.g., diazepam), 5-HT_{1A} receptor agonists (e.g., Buspirone), or SSRIs (e.g., fluoxetine). Since this review, it has also been shown that the anticonvulsant and neuropathic analgesic drug pregabalin (Lyrica®) which is structurally similar to GABA and has a clinically proven anxiolytic efficacy (e.g., Rickels et al. 2005) also inhibits hippocampal theta

(Siok et al. 2009). Based on these and other findings, McNaughton has concluded that suppression of hippocampal theta is a novel and reliable test of anxiolytic drug action. Like most animal models of anxiolytic drug action, however, the validity of this electrophysiological model has been almost exclusively based on the effectiveness of clinically successful anxiolytic drugs (true positives), rather than successful detection of a truly novel compound with previously unknown anxiolytic properties (*c.f.* Treit et al. 2010). The latter approach represents *prima facie* evidence of a valid model of anxiolytic drug action (whether behavioural or electrophysiological), but this exacting criterion is extremely difficult to satisfy (Treit et al. 2010).

Here we attempt to satisfy the *prima facie* criterion by testing a well-known antiepileptic, phenytoin, in the theta suppression model. Phenytoin is used for the treatment of epilepsy as well as cardiac arrhythmias (McNamara 2006). Although the mechanisms of its anticonvulsant and antiarrhythmic actions are not entirely understood, several possibilities exist (Tunnicliff, 1996). The primary mechanism for its anticonvulsant effect is considered to be due to its inhibitory action on the persistent sodium current (I_{NaP}: e.g., Segal and Davis, 1997; Mantegazza et al. 2010). Since I_{NaP} is vital for the theta rhythmical responses of single neurons and for neuronal membranes (e.g. subthreshold oscillations, bursting and resonance; Alonso and Llinás, 1989; D'Angelo et al. 2001; Hutcheon et al, 1996), it is likely that systemic phenytoin would depress population theta rhythms.

At the same time--and importantly for the present purpose--phenytoin has never been recognized as a clinically effective anxiolytic drug (e.g., McNamara 2006). Thus, phenytoin could provide a strong test of the theta suppression model. In the present study we test the influence of phenytoin at clinically relevant doses on evoked hippocampal theta and corroborate

these with behavioural measures of anxiolysis using the elevated plus-maze, a widely recognized animal model of anxiolytic drug action (Treit et al. 2003; 2010). A diazepam condition was included as a positive control in the case of null effects in both behavioural and electrophysiological models. An anxiolytic-like effect in the theta suppression model in the absence of an anxiolytic effect in the elevated plus-maze would be evidence of a false positive. Conversely, an anxiolytic effect in the behavioural model but not in the electrophysiological model would represent a false negative in the theta model. Anxiolytic effects in *both* models, however, would satisfy the *prima facie* test of the validity of the theta suppression model. Thus, our experimental design provides a critical test of the theta suppression model of anxiolysis.

Materials and Methods

1.1 Subjects

Subjects were 71 male Sprague-Dawley rats, weighing 200-300g, 15 of which were randomly assigned to the theta suppression test, and 56 of which were assigned to the elevated plus-maze test. Rats were group-housed in 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 experiments to minimize the number of animals used and all possible measures to minimize suffering and stress were taken during the experiments.

1.2 Drug Administration

Just prior to surgery, rats were randomly assigned to one of three drug conditions: 1) low dose phenytoin (10mg/kg), 2) clinically effective dose of phenytoin (50mg/kg), 3) diazepam (2mg/kg). In addition to our three drug conditions, two vehicle conditions were added in our behavioural experiments. Phenytoin was obtained from Sigma-Aldrdich (ON, Canada), and was suspended in 20% DMSO in H_2O solution. 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_2O. In all conditions, i.p injections were made 30 min prior to evaluation.

Neurophysiology Experiments

2.1 Anesthesia, surgery and recording.

Animals were initially anesthetized with isoflurane in 100% O_2 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: 1.3± 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 withdrawal was observed. Body temperature was maintained at 37 °C using a heating pad and rectal probe (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 ±2.2 mm, DV -2.7 mm) and a

bipolar stimulating electrode was lowered into the brain stem (AP -8.0 mm, ML ±1.6 mm, DV -6.5-7.3 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 500 Hz using a differential AC amplifier (Model, 1700, A-M Systems Inc., Carlsborg, WA, USA). Signals were digitized on-line (sampling frequency: 1 kHz) with a Digidata 1322 A A-D board connected to a Pentium PC running the AxoScope acquisition program (Axon Instruments, Union City, CA, USA).

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 (40.7 ± 9.6 mV). 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, and 3 times 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. 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 4% v/v formaldehyde.

2.2 Data analysis.

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 was extracted from the resulting spectrum and plotted as a function of stimulation intensity. The average of the peak frequency at the three different time points pre-infusion and post-infusion was calculated and used as raw data. A priori, pairwise, within-subjects comparisons of peak frequencies pre- and post-drug infusion were conducted. These assessed whether the average peak frequency at each intensity was significantly suppressed after drug administration. A priori, pairwise, between-subjects comparisons of the peak frequencies under each of the three drug conditions (i.e., diazepam 2mkg, phenytoin 10mg/kg; phenytoin 50mg/kg) were also conducted. These evaluated the significance of differences between the average peak frequency at each threshold stimulus intensity as a function of the three different drug conditions.

3.1 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 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. A higher percentage of open-arm time or open-arm entries are taken

as measures of anxiety-reduction (anxiolysis). In addition, the total number of closed arms entered was taken as an index of general activity (Pellow and File, 1986; Hogg, 1996).

3.2 Data Analysis

The results from the elevated plus maze test and were expressed as means and standard errors of the mean (S.E.M). Behavioural measures were analyzed with planned comparisons (Keppel and Zedeck, 1989; ANOVA; $\alpha = 0.05$). Four rats were excluded from the analysis of the plus-maze data because the number of times they fell off the maze was more than three standard deviations from the mean.

Results

Neurophysiology.

As previously shown (McNaughton et al. 2007) the frequency of brainstem-evoked hippocampal theta showed a positive linear relationship with increasing intensities of stimulation during baseline conditions in all animals tested (see Figure 1). Perhaps not surprisingly, neither this relationship, nor the absolute frequency for each level of stimulus intensity was altered by i.p. injections of the low (10mg/kg) dose of phenytoin. Specifically, differences in the measure of evoked theta before and after the 10 mg/kg phenytoin injection were not significant at any level of intensity (all probabilities>0.05). In contrast, a significant reduction in evoked theta frequency was observed at all stimulation intensities following the administration of the clinically relevant dose (50 mg/kg) of phenytoin (1x threshold stimulation intensity: $F_{(1,5)} = 68.88$; $p<0.001$; 2x threshold stimulation intensity: $F_{(1,5)} = 46.78$; $p=0.001$; and 3x threshold stimulation intensity: $F_{(1,5)} = 48.83$; $p=0.001$).

Consistent with previous findings (McNaughton et al. 2007), we confirmed that diazepam (2 mg/kg) also significantly reduced the frequency of evoked theta at all stimulation intensities (1x threshold stimulation intensity: $F_{(1,4)} = 29.59$; $p=0.006$; 2x threshold stimulation intensity: $F_{(1,4)} = 68.90$; $p=0.001$; 3x threshold stimulation intensity: $F_{(1,4)} = 18.26$; $p=0.013$). We also compared the degree of suppression in evoked theta frequencies across all three drug conditions (Figure 2). Planned comparisons revealed that diazepam produced a greater suppression of theta frequency in comparison to both 50mg/kg of phenytoin: $F_{(1,12)} = 7.78$; $p=0.016$, and 10mg/kg of phenytoin: $F_{(1,12)} = 19.31$; $p<0.001$ at 1x threshold stimulation intensity. However, at the higher threshold stimulation intensities (2x and 3x), both 50mg/kg of phenytoin (threshold stimulation intensity 2x = $F_{(1,12)} = 19.89$; $p<0.001$; threshold stimulation intensity 3x = $F_{(1,12)} = 7.38$; $p=0.019$), and 2mg/kg diazepam (threshold stimulation intensity 2x = $F_{(1,12)} = 30.51$; $p<0.001$; threshold stimulation intensity 3x = $F_{(1,12)} = 9.28$; $p=0.010$) suppressed theta frequency significantly more the 10mg/kg phenytoin condition.

Elevated Plus-Maze.

As can be seen in Figure 3, both 50mg/kg of phenytoin and 2mg/kg of diazepam produced robust anxiolytic effects in the plus-maze. Planned comparisons revealed that both the phenytoin 50mg/kg group ($F_{(1,51)} = 22.71$; $p<0.001$), and the diazepam 2mg/kg group ($F_{(1,51)} = 40.99$; $p<0.001$) significantly increased the percentage of open arm entries compared to the vehicle control group. In addition, both the phenytoin 50mg/kg ($F_{(1,51)} = 5.87$; $p=0.019$), and diazepam 2mg/kg ($F_{(1,51)} = 23.07$; $p<0.001$) groups showed a significantly greater increase in the percentage of time rats spent in the open arms of the plus-maze, compared to the vehicle control group. A limited indication of the dose-effect relationship for phenytoin in the plus maze was also suggested by the significantly greater percentage of open arm entries in the 50mg/kg group

compared to the 10mg/kg group ($F_{(1,51)} = 15.11$; $p<0.001$). Neither the percentage of open arm entries nor percentage of open-arm time differed significantly between the phenytoin10mg/kg group and the vehicle control group ($p>0.05$). Although both the diazepam and phenytoin 50mg/kg groups showed increased levels of general activity (greater number of closed arm entries), a Pearson Product Moment Correlation between closed arm entries and percent of open arm entries was actually negative ($r = -0.628$, $n=56$, $p<0.001$).

Discussion

Phenytoin at 50mg/kg produced robust anxiolytic effects in both the neurophysiological and behavioural models of anxiety, while phenytoin at 10 mg/kg did not. The decrease in theta frequency and the increase in open- arm activity produced by the 50 mg/kg dose were comparable to that of 2 mg/kg of diazepam, a clinically proven anxiolytic drug. Although both phenytoin and diazepam increased measures of general activity in the plus-maze, this did not explain the increases in the percent open-arm activity they produced in the plus-maze. More importantly, apart from the fact that phenytoin is known to suppress the persistent sodium current--which is a critical mechanistic element of theta rhythmicity--there was little reason to expect that phenytoin would produce both suppression of theta *and* increased open-arm exploration. It is the concordance of these two effects that provide *prima facie* evidence of the validity of the theta suppression model of anxiolytic drug action.

It is important to again stress that previous research has not revealed the anxiolytic-like effects of phenytoin, either in animal models (Munro et al. 2007) or in human patients (Mula et al. 2007). Indirect evidence of such an effect exists, however. For example, it has been shown that phenytoin can reverse the *anxiogenic* effects of RO 5-4864 in the rat social interaction test of anxiety (File and Lister, 1983) and reduce isolation-induced aggression in rats (Keele, 2001).

Stress-induced deficits in rat spatial learning have also been ameliorated by phenytoin (Luine et al, 1994). Interestingly, phenytoin reduced stress-induced hippocampal atrophy in rats, a condition that has been associated with PTSD in humans (Luine et al. 1994; Bremmer et al. 2004). To the best of our knowledge, however, there have been no clinically controlled trials of the effects of phenytoin on human anxiety. One non-controlled, open label pilot study with nine subjects has been published, however. Unfortunately, all of the subjects had comorbid psychiatric disorders, in addition to PTSD, and were all being treated with other medications at the time of the phenytoin trial. Nevertheless, Bremmer et al. (2004) found that variable doses of phenytoin reduced post-traumatic stress disorder (PTSD), as determined by the DSM IV and the Clinically Administered PTSD Scale (CAPS). While these findings are potentially interesting, they have not encouraged more controlled, clinical studies of the putative anxiolytic effects of phenytoin.

It is still somewhat surprising that phenytoin has not received more attention as a potential treatment for anxiety and other mood disorders, since a number of other anticonvulsant drugs have proven useful in this regard. For example, carbamazepine, valproate, lamotrigine, and oxcarbazepine have proven to be effective in the treatment of mania and bipolar disease, while gabapentin, pregabalin and tiagabine are reported to have anxiolytic effects, in reasonably well-controlled clinical trials (Ettinger and Argoff, 2007). Although pharmacological factors may ultimately account for this differential attention, there may be other reasons for pursuing the "off-label" benefits of newer as opposed older anticonvulsant drugs. Without patent protection, for example, there is little reason to study novel therapeutic actions of phenytoin in the clinic.

In any case, phenytoin in the present study has provided invaluable, *prima facie* evidence of the predictive validity of the theta suppression model. Other drugs with no known anxiolytic

properties that target different intrinsic membrane currents known to influence theta rhythmicity could also be useful in this context. One example is I_h, the hyperpolarization activated inward current, which is crucial for both theta resonance and rhythmicity at the single cell (Hutcheon, 1996; Dickson et al, 2000) and network (Kocsis et al, 2004) levels. This current is inhibited in a relatively specific fashion by ZD7288 (BoSmith et al, 1993) so this drug would provide another *prima facie* test of the theta suppression model of anxiolysis. Certainly, the combination of neurophysiological and behavioural techniques used here will directly advance our understanding of the neurochemical and neurophysiological bases of anxiety.

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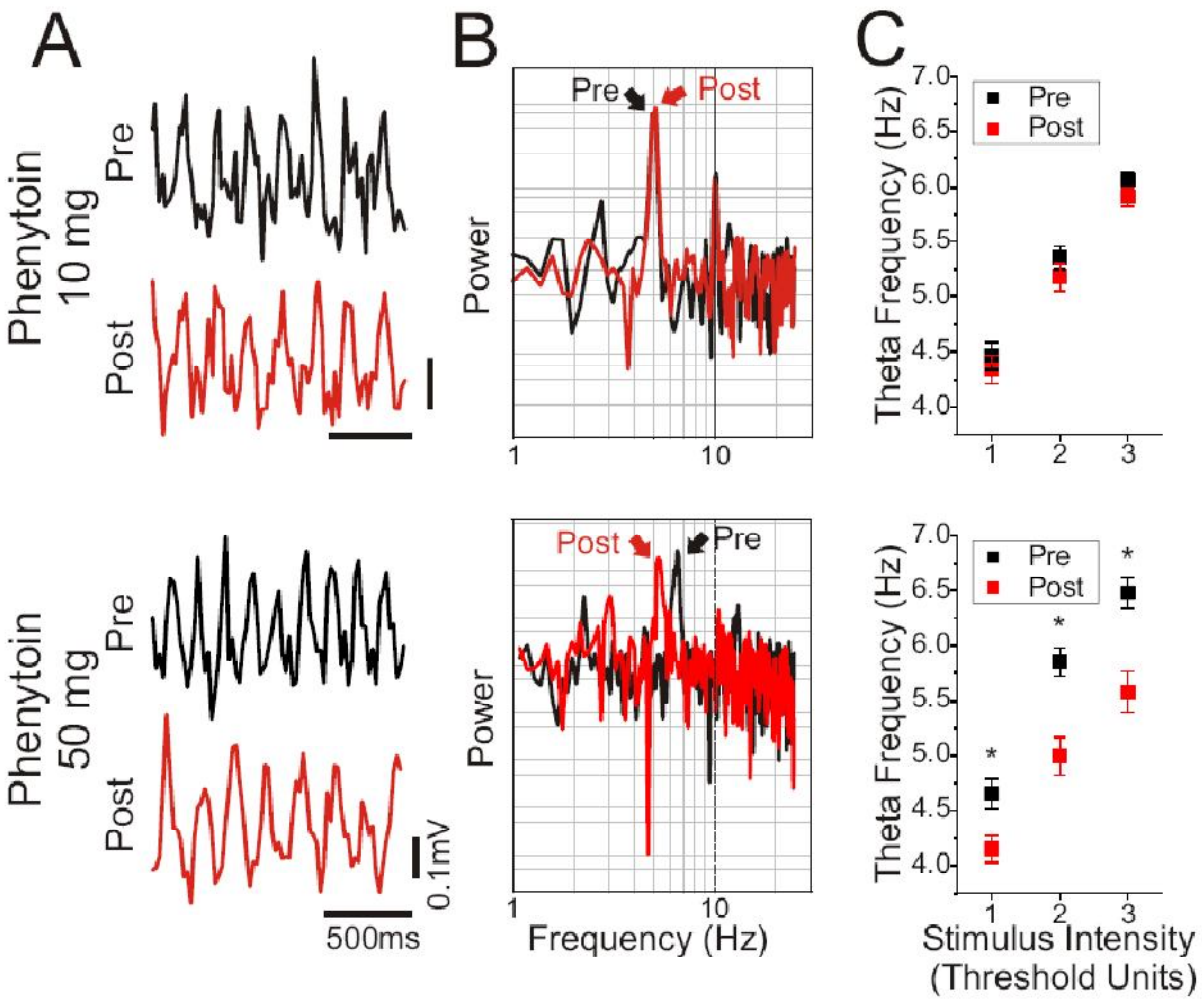
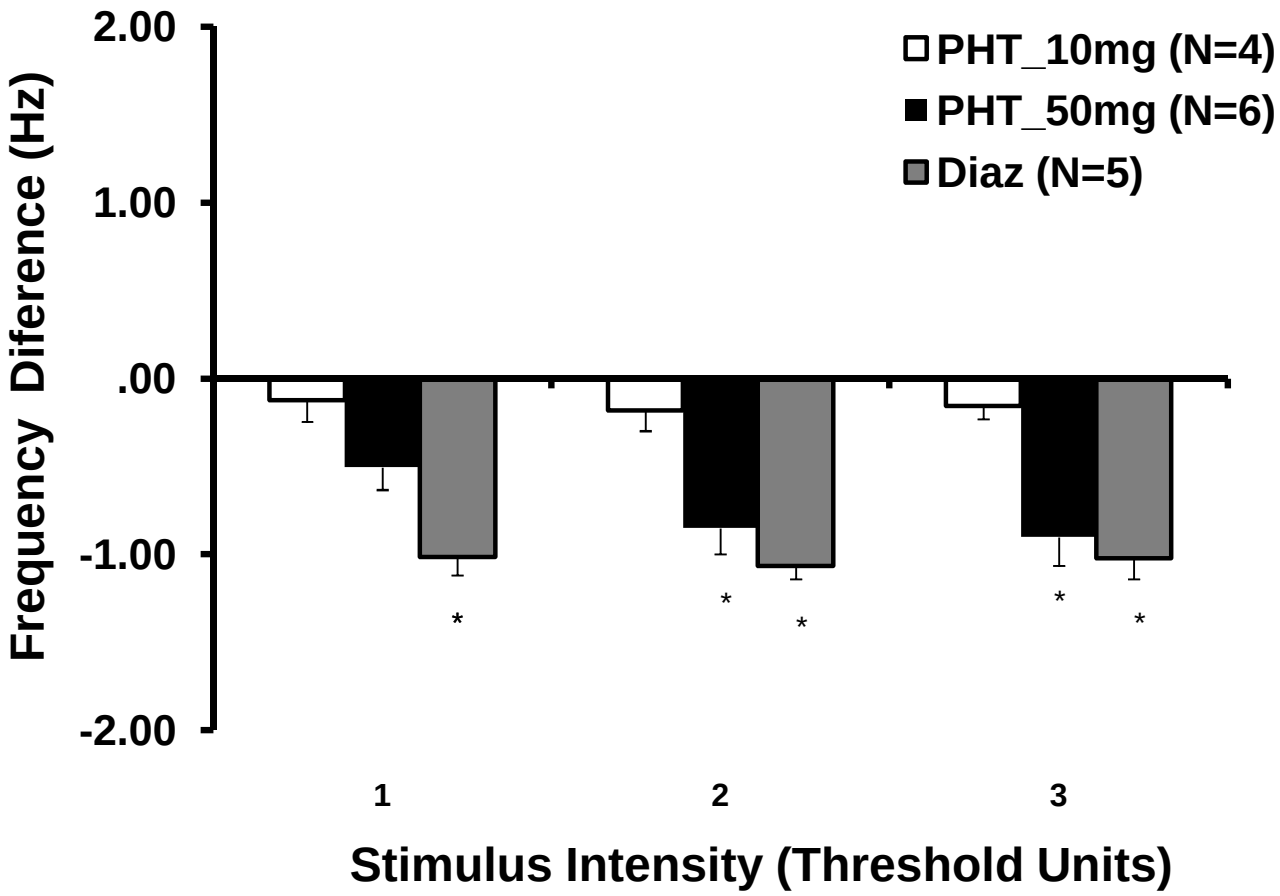
Figure Captions

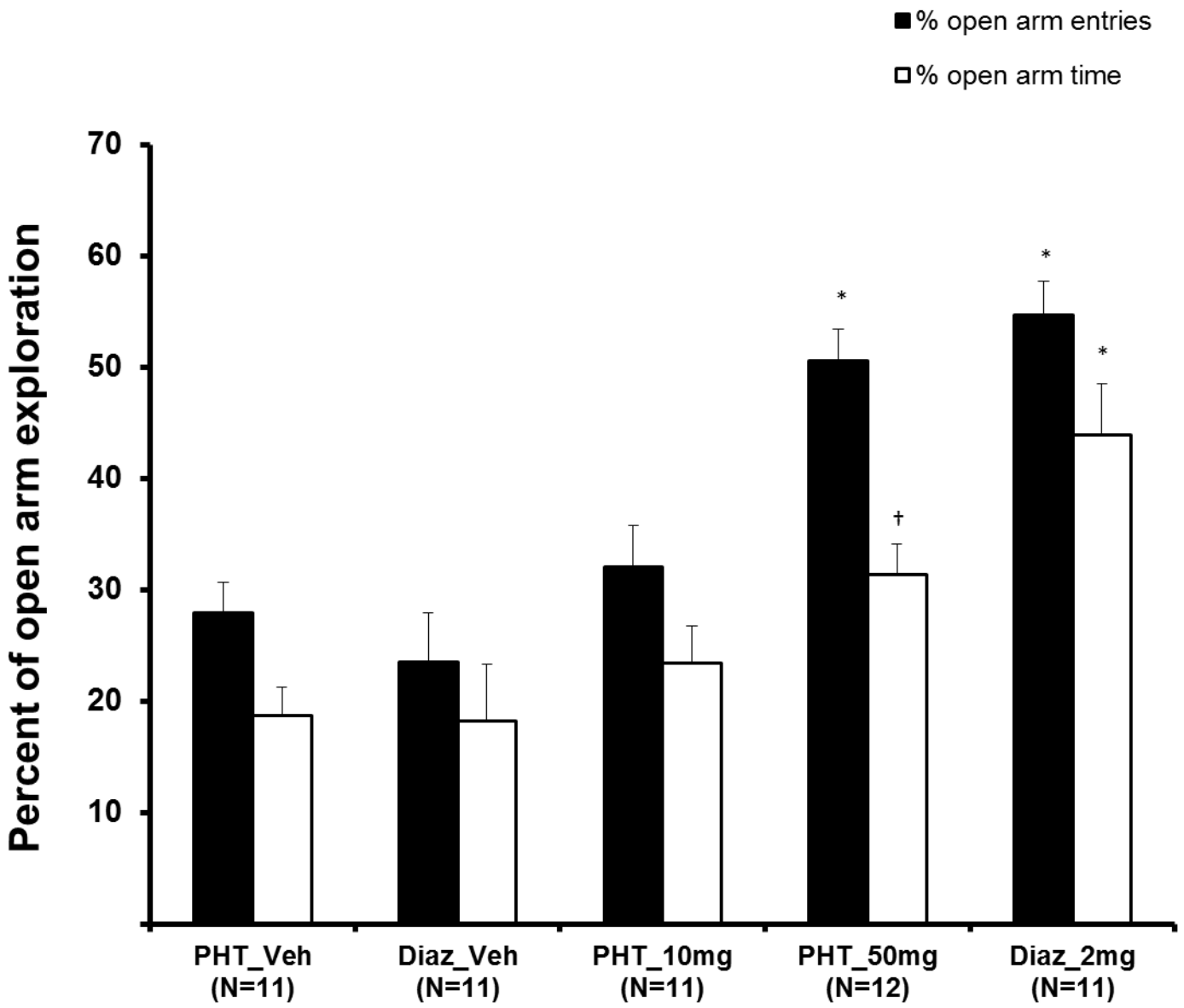
Fig. 1. **A)** Raw LFP traces of evoked theta following 90 mA stimulation pre and post administration of phenytoin are shown in both upper and lower panels (the top panel shows the response to 10 and the bottom 50mg/kg doses). **B)** Spectra components of the respective pre-post drug treatment traces shown in panel **A** are overlaid. Suppression of the evoked frequency can be seen for the 50, but not the 10mg/kg dose in both the raw traces and the spectra. **C)** Average data across all experiments showing the frequency of evoked theta at each level of stimulus intensity as measured in threshold units (1x, 2x, 3x threshold value) as a function of drug treatment. The top panel shows a lack of effect of 10mg/kg of phenytoin while the bottom shows a prominent and significant effect of the 50 mg/kg dose. Black squares represent means (±SEM) of evoked theta frequency pre administration of phenytoin. White squares represent means (±SEM) of evoked theta frequency post administration of phenytoin *Significantly different ($p<0.01$).

Fig. 2 Difference in evoked theta frequency (Hz) at 1x, 2x, 3x threshold units of stimulus intensity pre and post i.p administration of 10 mg/kg phenytoin, 50 mg/kg phenytoin, or 2 mg/kg diazepam. White bars represent mean differences (±SEM) of evoked theta frequency pre and post administration of pht10. Black bars represent mean differences (±SEM) of evoked theta frequency pre and post administration of pht50. Grey bars represent mean differences (±SEM) of evoked theta frequency pre and post administration of diazepam. *Significantly different ($p<0.01$).

Fig. 3 Open-arm activity following i.p injections of vehicle (phenytoin), vehicle (phenytoin), 10 mg/kg phenytoin, 50 mg/kg phenytoin, or 2 mg/kg diazepam in experiment 2. Black bars represent means (±SEM) percentage of open arm entries; white bars represent means (±SEM) percentage of open arm time. †Significantly different from the vehicle control group ($p<0.05$). *Significantly different from the vehicle control group ($p<0.01$).


A
Phenytoin
10 mg
Pre
Post
B
Pre
Post
Power
1
10
C
Theta Frequency (Hz)
7.0
6.5
6.0
5.5
5.0
4.5
4.0
Pre
Post
1
2
3
Phenytoin
50 mg
Pre
Post
0.1mV
500ms
Post
Pre
Power
1
10
Frequency (Hz)
*
Stimulus Intensity
(Threshold Units)


2.00
1.00
.00
-1.00
-2.00
Frequency Diference (Hz)
PHT_10mg (N=4)
PHT_50mg (N=6)
Diaz (N=5)
*
*
*
*
1
2
3
Stimulus Intensity (Threshold Units)


% open arm entries
% open arm time
Percent of open arm exploration
70
60
50
40
30
20
10
*
*
*
†
PHT_Veh
(N=11)
Diaz_Veh
(N=11)
PHT_10mg
(N=11)
PHT_50mg
(N=12)
Diaz_2mg
(N=11)

Chapter 4: General Discussion

In Chapter 2, we found robust anxiolytic effects following microinfusions of either SST14 or SST28. These effects appeared to be both dose and site specific, as only intra-amygdalar or intra-septal infusions of a high dose of SST14 or SST28 increased open arm activity in the elevated plus maze, and decreased burying behavior in the shock probe test. These effects were not confounded by changes in general activity and were absent following intra-striatal microinfusions (Yeung et al., 2011). The present findings are also consistent with the anxiolytic results found in previous studies using I.C.V infusions of somatostatin and a selective SST2 receptor agonist, L-779976 (See Engin et al., 2008; Engin and Treit, 2009).

While the present study provides clear evidence for the anxiolytic potential of both isoforms of somatostatin, there are a few issues that need to be addressed in future studies to better understand the anxiolytic profile of somatostatin. First, SST14 and SST28 modulate a number of hormonal, neurophysiological, and behavioral functions independently and with different potencies (Leroux et al., 1985; Mandarino et al., 1981; Meis et al., 2005; Semenova et al., 2010; Vecsei et al., 1990). Therefore, it is also possible that SST14 and SST28 may mediate anxiety differentially.

While it is possible that the behavioral techniques used in these experiments may have been too insensitive to detect differences between SST14 and SST28, future studies may reveal these differences in the same modes but at other doses of the two ligands. It is also possible that differences could be detected in the theta suppression model. Specifically, the electrophysiological signatures of the two isoforms in this model could differ quite dramatically, as previously shown in other electrophysiological measures (Kreienkamp et al. 1997; Wang et al., 1989). If this outcome occurs, further studies using the theta suppression model could

compare the effects of SST14 and SST28, microinfused directly into the amygdala, septum and striatum, to examine the neuroanatomical specificity of their different effects.

In addition, the receptor-specificity of the effects of SST 14 and SST 28, after both central and peripheral administration, needs to be assessed using sub-type select SST receptor antagonists. Given the evidence indicating that SST2 receptors might be mediating the anxiolytic effects of somatostatin (Engin et al., 2009), a starting point would be to show that a somatostatin antagonist can reverse the anxiolytic effects of a selective SST2 receptor agonist. While both SST14 and SST28 bind to the SST2 receptor within limbic structures with a similar affinity, SST28 has a higher affinity for the SST5 receptor (O'Carroll et al., 1992; Kreinkamp et al., 1997). Thus it is possible that the anxiolytic effects of SST depend to some extent on the SST5 receptor. Co-infusions of SST with a selective SST5 receptor antagonist could yield important results in this regard. Alternatively, a reversal or reduction in the anxiolytic effects of SST by a SST5 receptor *antagonist* would also indicate that SST5 plays some role in the anxiolytic effects of SST. Conversely, the absence of a reversal with co- infusions of the SST5 antagonist would indicate that other receptor sub-types are more in play (e.g., SST2). All of these experiments should be replicated in the theta suppression model of anxiolytic drug action, with the same hypotheses in mind,

Finally, it is important to extend our finding to clinical settings. To date, there have been numerous correlational reports that CSF somatostatin levels are decreased among populations with depression and increase following treatment (Bissette et al., 1986; Gerner and Yamada, 1982; Rubinow et al., 1986). However, CSF somatostatin levels pre and post treatment among patients with anxiety disorders is often obscured or absent entirely. Therefore, to initiate a causal analysis of the relationship between CSF somatostatin levels and anxiety, I could measure rat

CSF somatostatin levels while animals are exposed to a number of different stressors, and compare difference in CSF somatostatin to pre and post levelschanges post treatment. Taken together, these results from these experimental studies and studies with anxious patients can be used to support or refute the putative relationship between CSF somostatin levels and anxiety.

Chapter 3 demonstrated that phenytoin, an anticonvulsant drug with no known anxiolytic properties, suppressed evoked hippocampal theta and increased open arm activity in the elevated plus maze. These findings appear dose dependent and only a clinically effective dose of phenytoin (50mg/kg, and not phenytoin 10 mg/kg) had robust anxiolytic effects comparable to that of diazepam (Yeung et al., 2011). Additionally, these findings are also comparable to studies using lamotrigine and riluzole, anticonvulsant drugs with mechanisms of action akin to phenytoin, which have consistently shown anxiolytic effects in a number of studies (Blackburn-Munro et al., 2002; Mirza et al., 2005). Nevertheless, results were surprising because the primary mechanism by which phenytoin elicits anticonvulsant effects is through the inhibition of a sodium channel current, INAP (Segal and Davis, 1997; Mantegazza et al., 2010). Given that INAP promotes theta rhythmicity, it may not be too surprising that phenytoin suppressed theta (D'angelo et al., 2001). However, there was no evidence to suggest that INAP currents are associated with anxiety, or anxiety reduction. Thus, the anxiolytic effects of phenytoin in a well validated behavioral model of anxiety would not be unexpected. Nevertheless, these behavioral and electrophysiological effects of phenytoin provide prima facie evidence of the validity of McNaughton's hippocampal theta model of anxiety

Despite the strong, converging evidence suggesting that phenytoin has anxiolytic properties, comparable to that of classical benzodiazepine agonists (e.g. diazepam), there are a number of interesting questions left to answer. First, the effects of central and peripheral

infusions of phenytoin should be assayed in a larger battery of behavioural models (e.g. elevated plus maze, shock probe test, social interaction test, light-dark box, etc.). This is important in order to assess the generality of phenytoin's anxiolytic effects across different behavioral indices of anxiety. In addition, because some anxiolytic drugs (e.g., SSRIs) have paradoxical anxiogenic effects in some animal models, this possibility also need to be assessed for phenytoin in a battery of tests.

Second, the pharmacological specificity of phenytoin in behavioral and electrophysiological models of anxiety needs to be assessed. While the depression of INAP may be the primary mechanism of phenytoin's anticonvulsant drug action, it is not necessarily the primary mechanism of anxiolytic-like effects. One possibility is that the anxiolytic-like effects of phenytoin are related to its ability to increase or decrease the reuptake of GABA and glutamate (e.g., Tatsuoka et al., 1984; Weinberger et al. 1976), since both of these neurotransmitters are involved in anxiety modulation (Degroot et al., 2001; Matheus and Guimaraes, 1997; Molchanov and Guimaraes, 2002). In either case, if there is a significant difference in the rate of the two reuptake functions that favors inhibition (i.e. faster reuptake of Glutamate than GABA), this could represent the mechanism by which phenytoin reduces anxiety. To begin to explore this possibility, I will co-infuse phenytoin with a selective GABA receptor antagonist, or a glutamate receptor agonist, to see whether the anxiolytic effects of phenytoin can be reversed. Should a reversal occur, this might suggest that phenytoin mediates convulsions and anxiety through distinct neurochemical mechanisms.

Third, the brain sites that mediate the anxiolytic effects of phenytoin on elicited hippocampal theta need to be explored. For example, simultaneous microinfusions of phenytoin into the left or right hippocampus while recording hippocampal theta on the other side could be

an interesting starting point for a number of similar studies. Finally, ZD7288, a drug that inhibits theta resonance and rhythmicity through hyperpolarization activated current (Ih), but with no known anxiolytic effects, could also be used to test McNaughton's theta suppression model (BoSmith et al., 1993). Furthermore, the phenytoin mechanism (INAP) of theta inhibition could be directly with the putative ZD7288 mechanism (Ih) of theta suppression, with the possibility that ZD7288 is also anxiolytic in behavioral models such as the elevated plus maze. There are also other drugs with no known anxiolytic properties that suppress hippocampal theta. For example, reserpine, once used to treat psychosis, decreases theta frequency. (Nakagawa, 2000). In contrast, psychoactive dopaminergic receptor agonists (e.g. quinpirole, SKF38393 methamphetamine) reverse reserpine-induced theta suppression (Nakagawa et al., 2000). Taken together, these studies would further our understanding of the neurophysiological and neurochemical bases of anxiety, provide additional, critical tests of the theta suppression model.

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