“I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale.”

Marie Curie

“The most exciting phrase to hear in science, the one that heralds new discoveries, is not Eureka! (I found it!) but rather, "hmm.... that's funny...."”

Isaac Asimov

“Science is a wonderful thing if one does not have to earn one's living at it.”

Albert Einstein

University of Alberta

The effects of neurosteroids and neuropeptides on anxiety-related behavior

by

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For Nur and Marc

You are the light in my mind,

the warmth in my heart.

Abstract

Anxiety disorders are the most prevalent of all psychiatric conditions. However, current pharmacological treatments for anxiety disorders are characterized by one or more of the following deficiencies: 1) unwanted side effects, 2) partial efficacy, 3) addictive potential, and 4) delayed onset of therapeutic effects. These therapeutic liabilities motivate the search for better pharmacological treatments. This research effort has been concentrated in three broad, neuropharmacological domains: 1) Sub-unit specific $GABA_A$ receptor agonists, 2) Neurosteroids, and 3) Neuropeptides. The general purpose of this thesis was to advance our understanding of the putative anxiolytic potential of neurosteroids and neuropeptides, and their neural mechanisms of action, as revealed by intracerebral infusion studies in animal models of anxiety.

Chapter 1 of this thesis will provide a systematic review of what is now known about the behavioral effects of intra-cerebrally infused agonists and antagonists of anxiolytic compounds in animal models of anxiety. A theoretical context in which to view the empirical work is also outlined. Chapter 2 will provide a brief introduction to neurosteroids and neuropeptides, and their potential as anxiolytic drugs as suggested by the current literature. In Chapter 3, the anxiolytic-like effects of the neurosteroid allopregnanolone were examined in the amygdala, the hippocampus or the medial prefrontal cortex. Allopregnanolone had site- and test-specific

anxiolytic effects, causing anxiolysis following infusion into the amygdala and the medial prefrontal cortex. In Chapter 4, the anxiety-related effects of two receptor antagonists of the neuropeptide arginine vasopressin were investigated in the hippocampus. Anxiolytic effects were specific to both receptor sub-type and by infusion site. In chapter 5, the putative anxiolytic and antidepressant effects of the neuropeptide somatostatin were investigated. Intracerebroventricular microinfusion of somatostatin produced anxiolytic-like and antidepressant-like signatures in distinct domains. In chapter 6, selective agonists for each of the 5 G-protein coupled somatostatin receptors were administered to rats. Intracerebroventricular administration of an sst2 agonist produced anxiolytic-like effects, whereas an antidepressant-like effect was observed following the administration of both sst2 and sst3 agonists.

In summary, the present thesis provides important clues to the neurochemical correlates of anxiety, and its potential treatment with alternative compounds such as neuropeptides.

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morning and whether I am getting enough vitamin C. Thank you for keeping the child in me always alive, and thank you for worrying about everything, so that there is not much left for me to worry about. I know the compromises you have made for us and I will be forever grateful.

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5-HT	5-hydroxytryptamine
AC	alternating current
ACTH	adrenocorticotropic hormone
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ANOVA	analysis of variance
AP	anterior-posterior
AVP	arginine vasopressin
Avpr	arginine vasopressin receptor
BDZ	benzodiazepine
BLA	basolateral amygdala
BNST	bed nucleus of stria terminalis
cc	cubic centimeter
CCK	cholecystokinin
CeA	central nucleus of the amygdala
cm	Centimeter
CNS	central nervous system
CRF	Corticotrophin releasing factor
dH	dorsal hippocampus
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone – sulphate
DRN	dorsal raphe nucleus
DV	dorsal-ventral
EEG	Electroencephalography
EPM	elevated plus maze
g	Gram
GABA	γ-aminobutyric acid
h	Hour

HPA	hypothalamic-pitiutary-adrenal (axis)
Hz	Hertz
i.c.	intra-cerebral
ICV	intracerebroventricular
i.p.	intraperitoneal
kg	Kilogram
kHZ	kilohertz
LD	light-dark (test)
LS	lateral septum
LSD	least significant difference (post-hoc test)
mA	Milliampere
MA	medial amygdala
MAOI	monoamine oxidase inhibitor
mg	Milligram
mGlu R	metabotropic glutamate receptor
Min	Minute
ML	medial-lateral
mm	Millimeter
mPFC	medial prefrontal cortex
MRN	medial raphe nucleus
mRNA	messenger ribonucleic acid
ms	Millisecond
MS	medial septum
NA	Noradrenaline
ng	Nanogram
NK	Neurokinin
NMDA	N-methyl-D-aspartate
NPY	neuropeptide Y
OF	open field (test)

OVX	Ovariectomy
PAG	periaqueductal gray
PREGS	pregnanolone sulfate
PVN	periventricular nucleus
REM	rapid eye movement
s.c.	Subcutaneous
SCN	Subchiasmatic nucleus
sec	Second
S.E.M.	standard error of the measure
SI	social interaction (test)
SON	suboptic nucleus
SP	substance P
SPA	shock-probe avoidance
SPB	shock-probe burying
SSRI	selective serotonin reuptake inhibitor
SST	Somatostatin
TH DOC	tetrahydrodeoxycorticosterone
TH PROG	tetrahydroprogesterone
TTX	Tetrodotoxin
USV	ultrasonic vocalization
VC	Vogel's conflict (test)
vH	ventral hippocampus
vol	Volume
VTA	ventral tegmental area
wt	Weight
µg	Microgram
µl	Microliter
µm	Micrometer

CHAPTER 1

GENERAL INTRODUCTION:

THE EFFECTS OF INTRA-CEREBRAL DRUG INFUSIONS ON ANIMALS' UNCONDITIONED FEAR REACTIONS

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1. General Introduction

Anxiety refers to the collection of behavioral and physiological responses of an organism to perceived danger. This set of responses may involve adaptive alterations such as increases in heart rate, blood pressure, glucocorticoid release; piloerection; fight, flight or freezing behaviors. Several researchers have made distinctions between anxiety and fear based on the existence of a physical threat (i.e., actual versus implied/possible presence), the distance of the threat (i.e., distal versus proximal) or the reaction of the organism (i.e., approach versus avoidance); however a generally agreed-upon distinction between the two terms is not available (Barlow, 1988; Blanchard and Blanchard, 1990; McNaughton and Corr, 2004). Thus, for the purposes of this thesis, the two terms will be used interchangeably, referring to the adaptive responses of an organism to perceived threat.

Over the past four decades, animal indices of anxiety have been used as simple "screening tests" of potential anxiolytic compounds, and as "models" to study the neural mechanisms of anxiety and anxiolytic drug action, (Bourin and Hascoet, 2003; Griebel, 1995; Treit, 1985). Three criteria distinguish simple screening tests from models specifically used to study the neural bases of anxiety: 1) correspondence in form between the expression of fear in the animal model and its expression in humans 2) continuity of function between fear in animals and anxiety in humans, and 3) conservation of the underlying brain mechanisms of fear and anxiety across mammalian species. While these criteria are theoretical imperatives for animal models of anxiety, in practice they are difficult to satisfy unambiguously (Treit, 1985). The very best animal models of anxiety are incomplete approximations of the human condition. The hope is that some aspect of the model will ultimately relate to the behavioral and neural correlates of 'anxiety' in humans, normal or pathological.

There is a vast literature in which animal fear reactions have been used to study the effects of peripherally administered anxiolytic drugs (for reviews, see File and Seth, 2003; Graeff, 2002; Griebel, 1995; Igor et al., 2001;

Rodgers, 1997; Treit, 1985). While these studies have provided a wealth of information about the behavioral effects of anxiolytic drugs (e.g., Valium®, BuSpar®), the widespread distribution of these drugs after peripheral administration obscures their site-specific effects in the brain. Another, more direct approach for studying the neural mechanisms of anxiety is to lesion selected brain structures and/or neurotransmitter systems and to examine the effects on animal fear reactions (e.g., File et al., 1979; Shah and Treit, 2003). The specificity of brain lesioning techniques, however, is often inadequate for the unambiguous assessment of brain function. Even relatively specific lesions of cell bodies, axons, or neurotransmitter systems provide limited information about the function of specific receptor populations within the denervated brain area. This is particularly important given that the function of well-defined receptor systems (e.g., $GABA_A$) can vary across subregions of a single, neuroanatomically defined structure (Kaufmann et al., 2003). Intra-cerebroventricular (i.c.v.) administration of specific receptor agonists or antagonists provides more detailed neurochemical information, but the anatomical specificity of this technique is weak. Most compounds are distributed more or less evenly throughout the brain following i.c.v. administration.

A third technique—site-specific intra-cerebral (i.c.) microinfusion of selective receptor agonists or antagonists—seems to combine the utility of other techniques used to stimulate or inhibit brain function, with a physiologically more selective and subtle effect. Although not without its own complexities and pitfalls (see Greenshaw, 1998; Menard and Treit, 1999), in principle i.c. infusion techniques can provide detailed information about both the anatomical and neurochemical substrates of anxiety, as expressed in animal models. Thus, findings from i.c. infusion studies provide a general picture of the brain structures and transmitter molecules that contribute to the behavioral expressions of anxiety in animal models.

Most animal models have been designed to represent either conditioned (e.g., Geller conflict paradigms) or unconditioned anxiety (e.g., elevated plus-maze; for reviews see Treit, 1985; Treit et al., 2003). Because animal models of unconditioned fear or anxiety do not explicitly require learning or memory, the effects of neuropharmacological interventions on anxiety-related behaviors in these models can be more easily separated from effects on more complex, cognitive processes. Thus, a combination of i.c. infusion techniques and "ethologically-inspired" behavioral techniques may provide relatively specific and unique neuroanatomical and neurochemical insights into the neural mechanisms of anxiety.

Accordingly, next I would like to present a review of the behavioral effects of intra-cerebral microinfusions of selective agonists and/or antagonists, in five widely used models that are explicitly based on animals' untrained defense reactions: the elevated plus-maze test [EPM], shock-probe burying test [SPB], light/dark exploration test [LD], social interaction test [SI], and the separation-or shock-induced ultrasonic vocalization test [USV].

2. Intra-Cerebral Infusion Studies and Brain Anxiety Circuits

The empirical findings reviewed below are organized anatomically, along a caudal–rostral axis, from the brainstem (locus coeruleus, raphe nuclei, periaqueductal gray) to the forebrain (hypothalamus, amygdala, septum, hippocampus, medial prefrontal cortex), mainly for convenience. Nonetheless, all of the structures in this neural hierarchy are interconnected, and have been implicated in fear and anxiety using complementary methodologies such as Fos immunohistochemistry, receptor knockout, electrophysiology, and functional brain imaging (for reviews see Singewald et al., 2003; Singewald and Sharp, 2000; Finn et al., 2003; Linden, 2006; Ressler and Mayberg, 2007; Davidson, 2002). A brief summary of the neuroanatomy, neurochemistry and

receptor distributions of each of these target structures will be presented at the start of each section, with citations to reviews. In each of the target structures, the behavioral effects of i.c. infusions of receptor agonists and/or antagonists of amino acid, acetylcholine, monoamine, and neuropeptide neurotransmitter systems will be provided. Because the implications of the behavioral findings often depend on knowledge of specific fear reactions in each model, I will begin with a description of the animal models, and the behaviors that serve as indices of fear or anxiety in each.

2.1. Animal Models of Unconditioned Anxiety Reactions

The elevated plus-maze (EPM) is a plus-shaped apparatus with two open and two enclosed arms, each with an open roof, elevated 40–70 cm from the floor. The model is based on rodents' aversion of open spaces and the resulting thigmotaxic behavior that restricts their movement to the enclosed arms (Pellow et al., 1985; Treit et al., 1993; Rodgers, 1997; Carobrez and Bertoglio, 2005). Anxiety reduction in the plus-maze is indicated by an increase in the proportion of time spent in the open arms (time in open arms/total time in open or closed arms), and an increase in the proportion of entries into the open arms (entries into open arms/total entries into open or closed arms). Total number of arm entries and number of closed-arm entries serve as indices of locomotor activity (for a review of methods and procedures see Hogg, 1996).

In *the social interaction test (SI)*, naïve rats are placed in pairs in an open arena, and the time they spend in active social interaction (e.g., sniffing, grooming) is measured. Social interaction is suppressed when animals are tested under bright lights or in an unfamiliar test environment, relative to low light/familiar conditions. This suppression is the index of anxiety (File and Hyde, 1979). Line crossings are counted as a measure of non-specific changes

in locomotor activity. Disinhibition of social interaction is the measure of anxiety reduction in this test (for a review see File, 1985).

In *the light–dark exploration test (LD)*, rodents avoid the brightly lit side of a two-compartment chamber, spending most of their time exploring the dimly lit side. Anxiety reduction is indicated by increased transitions between the two compartments and/or increased exploration (i.e., time spent and number of line crossings) in the bright compartment, whereas non-specific effects are indicated by changes in general locomotor activity (Blumstein and Crawley, 1983; Crawley and Goodwin, 1980; Crawley, 1981; for a recent review, see Bourin and Hascoet, 2003).

In *the shock-probe burying test (SPB)* rats are shocked from a stationary, electrified probe inside of a Plexiglas chamber with bedding material on the floor. After a brief withdrawal to the back of the chamber, a typical rat approaches the shock-probe, backs away suddenly, approaches again, and within 5 min begins to spray bedding material toward or over the probe, with rapid, alternating movements of the forepaws (i.e., "burying behavior"; Pinel and Treit, 1978). All the while rats avoid further contact with the shock-probe. A reduction in the duration of probe-burying, in the absence of a decrease in general activity, is the primary index of anxiety reduction (Treit et al., 1981). An increase in the number of contact-induced probe shocks is a second index of anxiety reduction, and indicates a deficit in shock-probe avoidance (SPA). For reviews see Treit and Pinel (2005), De Boer and Koolhaas (2003), Treit and Fundytus (1988), Treit (1985).

The ultrasonic vocalization test (USV) measures high-frequency (30–50 kHz) 'distress' calls from rat pups separated from their mother and littermates (Noirot, 1972). Anxiolysis is indicated by a reduction in high-frequency vocalization, in the absence of behavioral sedation (Gardner, 1985a,b; Insel et al., 1986). Another USV test has been developed in which adult rats are given inescapable foot-shocks (DeVry et al.,1993). During a separate test period, foot-shocks are again administered and the duration of ultrasonic vocalizations

is measured during inter-shock intervals. The duration of these localizations is used as the index of "anxiety." Anxiolytic-like effects are indicated by a reduction in ultrasonic vocalizations (for a recent review see Igor et al., 2001).

It bears repeating that all of these "ethologically-inspired" models of anxiety include control measures for non-specific drug effects on general activity, and the SPB test also includes a measure for nonspecific drug effects on shock-sensitivity. Most importantly, none of the models is motivated by food reward, or dependent on the acquisition and/or retention of a learned response. Accordingly, drug effects on food- motivation, or learning and memory cannot be confounded with selective effects on fear or anxiety (Treit and Menard, 2000). Finally, each of these models has undergone extensive pharmacological validation: i.e., all of the index-measures of fear or anxiety in each model are reliably suppressed by peripheral administration of standard anxiolytic drugs (e.g., i.p. diazepam). These features, along with their convenience, are the main strengths of the five models described above (Treit et al., 2003).

2.2. Effects of Intra-Cerebally Infused Compounds in Animal Models of Anxiety

2.2.1. Periaqueductal gray (PAG)

The PAG receives information from, and sends information to most areas of the brain (Beitz, 1982, 1995). In particular, it is interconnected through efferent and afferent pathways with other structures also implicated in anxiety or fear, including the locus coeruleus, raphe nuclei, hypothalamus, amygdala, septum, and prefrontal cortex. The PAG receives noradrenergic and serotonergic innervations from the locus coeruleus and raphe nuclei, respectively, as well as histaminergic fibers from the hypothalamus. Intrinsic processes include cholinergic, glutamatergic, and GABAergic cell bodies and

receptors, both ionotropic and metabotropic (Beitz, 1995; Barbaresi, 2007; Lima et al., 2008). There is also evidence suggesting the presence of both mu and delta opioid receptors on PAG neurons projecting to the rostral ventromedial medulla (Wang and Wessendorf, 2002). Other neuropeptides such as neurotensin, substance P, CCK and CRF have also been detected in the PAG (Beitz, 1995; Behbehani, 1995). The area has been extensively implicated in fear and defense, particularly in response to painful stimuli (e.g., Behbehani, 1995; Vianna and Brandao, 2003).

Microinfusion of direct and indirect $GABA_A$ receptor agonists into either the dorsal or the ventrolateral PAG decreased anxiety-related behavior in the elevated plus-maze and the ultrasonic vocalization tests, as did glutamate antagonists of either NMDA or AMPA receptors (e.g., Matheus and Guimaraes,1997; Molchanov and Guimaraes, 2002; Russo et al.,1993; Walker and Carrive, 2003). These findings are uniform and comparable to the effects of permanent lesions of the PAG, which also block unconditioned fear responses (for a review see Behbehani, 1995).

The effects of cholinergic and monoaminergic compounds microinfused into the PAG have not been studied in any of the five models of unconditioned anxiety. There is, however, indirect evidence for the involvement of serotonergic systems. Lesions of the dorsal raphe, which is a major source of serotonergic projections to the rest of the brain, inhibited serotonergic activity in the dorsal PAG and produced a corresponding reduction in “anticipatory anxiety” while at the same time increasing “panic” (Sena et al., 2003). Studies involving direct microinfusion of serotonergic compounds into the dorsal PAG, however, did not entirely support this view of the role of serotonin (Graeff, 2004). Nevertheless, the effects of intra-PAG microinfusions of selective serotonergic compounds should be pursued in models of unconditioned anxiety. It is quite possible, for example, that the anxiety-related effects of serotonergic activation in individual subregions of the PAG (e.g., medial, dorsal, dorsolateral and ventrolateral; Beitz, 1982,

1985) may vary, with some regions mediating the expression of some fear reactions, and other regions mediating different fear reactions. Cholinergic and histaminergic neurotransmitter systems within the PAG also need to be investigated for their potential roles in unconditioned anxiety.

There is evidence that a variety of neuropeptides (NPY, CRF, substance P) modulate anxiety-related behavior in the social interaction and plus-maze tests when infused into the PAG. Neuropeptide Y (NPY) is found throughout the CNS and plays important roles in a number of behavioral and physiological functions such as feeding, energy balance, and stress (Eva et al., 2006). NPY1 receptor blockade in the PAG exacerbated anxiety-related behavior in the plus-maze and social interaction tests (Kask et al., 1998a,b), suggesting that NPY receptor agonists might produce anxiolytic-like effects. CRF, a key substrate of the Hypothalamus–Pituitary–Adrenal (HPA) "stress" pathway, decreased open-arm activity in the plus-maze when infused into the PAG (Martins et al., 1997). This anxiogenic effect of CRF has been replicated in the amygdala (Sajdyk et al., 1999), BNST (Sahuque et al., 2006) and septum (Kask et al., 2001). The receptor specificity of the anxiogenic effect of CRF was investigated in a recent study showing that cortagine, a specific CRF 1 receptor agonist, increased burying behavior (Litvin et al., 2007). Nevertheless, demonstrating that intracerebral microinfusion of CRF receptor antagonists has anxiolytic effects in models of unconditioned anxiety has proven difficult (Martins et al., 2000). Although Martins et al. (2000) have shown that intra-PAG CRF receptor antagonists can block "stress-potentiated" fear behavior in the elevated plus-maze, they failed to do so in the standard version of this test (i.e., without a pre-test stressor such as immobilization). This finding might seem curious in light of the fact that i.c.v. or peripheral (i.p.) administration of CRF receptor antagonists can produce reliable anti-stress and/or anti-anxiety effects in all of the animal models reviewed here (e.g., Heinrichs et al., 2002). But even in these cases it has been argued that the most robust anxiolytic-like effects are seen when a stressor (e.g., physical restraint) is administered prior

to the standard test (see Heinrichs et al., 2002; Overstreet et al., 2005). Nevertheless, the precise role of "pre-test stress" in the anxiolytic effects of CRF antagonists remains to be determined, especially given the fact that convincing evidence of the sensitivity (or lack thereof) of pre-test stress to the anxiolytic effects of standard anxiolytic compounds such as diazepam has not yet been demonstrated. Substance P, the "preferred" neurokinin (NK) 1 receptor agonist, had a clear anxiogenic effect in the plus- maze and USV tests when microinfused into the PAG (Aguiar and Brandao, 1996; Bassi et al., 2007). Furthermore, this anxiogenic effect seemed to depend on the C-terminal fragment of substance P. When the N-terminal fragment was infused, it produced an anxiolytic effect (De Araujo et al., 1999, 2001). These and other observations have led to an intense effort to demonstrate a possible role for NK receptor antagonists in the pharmacological treatment of anxiety and depression (McLean, 2005). The role of opioid peptides has also been investigated in the PAG. The stimulation of mu-opioid receptors in the dorsal PAG with morphine or the mu-opioid receptor agonist DAMGO reduced anxiety-like behavior in the elevated plus-maze (Motta and Brandao, 1993; Motta et al., 1995). On the other hand, kappa-opioid receptor agonism in the dorsal PAG produced an anxiogenic effect, indicating the receptor specificity of opioid-related effects in this area (Motta et al., 1995).

In summary, the evidence suggests that $GABA_A$ and NMDA receptors within the PAG play an important role in the expression of anxiety-related behavior. The PAG opioid system also seems to be involved, with kappa receptor agonists enhancing and mu receptor agonists inhibiting the expression of anxiety reactions. While other neuropeptide receptor systems such as CRF may play a role in anxiety in the PAG, the evidence is not entirely systematic or clear. The role in anxiety of monoamines in the PAG, especially catecholamines, has yet to be systematically investigated in models of unconditioned anxiety.

2.2.2. Raphe nuclei and locus coeruleus

Other brainstem structures, such as the raphe nuclei and the locus coeruleus are sources of modulatory monoamine pathways, which project to each other and to various forebrain structures that have been implicated in anxiety (e.g., hypothalamus, amygdala, BNST, septum, hippocampus and medial prefrontal cortex; Aston-Jones et al., 1995; Vertes et al., 1999; Vertes, 1991). In general, output from the raphe nuclei is serotonergic (5-HT), and output from the locus coeruleus is noradrenergic (NA). Nevertheless, there is evidence that each of these brainstem nuclei house a variety of neurotransmitters and receptor systems, including GABA, glutamate, NA , 5-HT, opiate, acetylcholine, substance P, CRF, galanin, as well as others (for overviews see Aston-Jones et al., 1995; Harsing, 2006; Van Bockstaele, 1998).

The raphe nuclei and the locus coeruleus in particular have long been implicated in anxiety (e.g., Tanaka et al., 2000; Lechin et al., 2006). A brief synopsis of the microinfusion data related to these areas follows.

Full agonists of the benzodiazepine receptor (e.g., diazepam) produced reliable anxiolytic effects in several of the tests when microinfused into the dorsal or median raphe (Costall et al., 1989; Gonzalez and File, 1997; Gonzalez et al., 1998). Flumazenil, a benzodiazepine receptor antagonist, produced no effects by itself but reversed many of the anxiolytic effects found with full agonists (Gonzalez et al., 1998; Gonzalez and File, 1997). As might be expected, the benzodiazepine inverse agonist β-CCM produced an anxiogenic effect when infused into the dorsal raphe (Hindley et al., 1985). Endogenous GABAergic input is known to inhibit the activity of serotonergic neurons in raphe nuclei (Adell et al., 2002). Thus, an enhancement of GABAergic activity in the raphe through the infusion of $GABA_A$ receptor agonists would result in an inhibition of serotonergic projections from the raphe nuclei to areas such as PAG, the amygdala and the hippocampus. Serotonergic efferent inhibition to these “upstream” structures could explain the anxiolytic effects of $GABA_A$ receptor agonists in the raphe. Conversely,

stimulation of these serotonergic projections should increase anxiety (Wise et al., 1972).

Evidence that is consistent with this general hypothesis also comes from the effects of partial or full agonists of the 5-HT_{1A} receptor. 5-HT_{1A} receptor agonists stimulate presynaptic autoreceptors, which in turn reduce serotonin output (e.g., Sharp et al., 2007). As expected from the model outlined above, these receptor agonists produced anxiolytic-like effects when infused into the raphe nuclei (Andrews et al., 1994; Carli and Samarin, 1988; DeAlmeida et al., 1998; File and Gonzalez, 1996; File et al., 1996; Higgins et al., 1988; Merali et al., 2006; Picazo et al., 1995; Schreiber and DeVry, 1993;). One exception to this uniform pattern of anxiolytic effects, however, is the elevated plus-maze, where intra-raphe 5-HT_{1A} receptor agonists have inconsistent effects, similar to those seen in the plus-maze after peripherally-injected 5-HT_{1A} receptor agonists (e.g., Treit et al., 2003). Furthermore, WAY100635, a selective 5-HT_{1A} antagonist, which increases serotonin output presynaptically, actually produced anxiolytic effects in the plus-maze when microinfused into the median raphe (Canto-De-Souza et al., 2002). Intra-raphe 5-HT_3 receptor antagonists (which also dampen serotonergic activity) were uniformly ineffective in the social interaction test (e.g., Higgins et al.,1991), despite some hint that they might be anxiolytic in the light–dark test (Costall et al., 1989a,b). Clearly, while some of these results are consistent with the hypothesis that increases and decreases in serotonergic output from the raphe translate into anxiogenic and anxiolytic effects, respectively, the overall results are not entirely consistent with this view. Variations of the serotonin hypothesis that appeal to differential effects of medial or dorsal raphe manipulations, or presynaptic versus postsynaptic 5-HT_{1A} receptor stimulation (e.g., File et al., 1996) have met with limited success. Part of the problem is the actual complexity of 5-HT inhibitory mechanisms, which extend beyond presynaptic autoreceptors and include, at a minimum, inhibitory postsynaptic 5-HT heteroreceptor mechanisms involving 5-$HT_{1–7}$ receptors, as well as

GABAergic inhibitory mechanisms feeding back from local neurons in the raphe nuclei, as well as distant postsynaptic sites such as the medial prefrontal cortex (for recent review, see Sharp et al., 2007). Clearly, any model of the anxiety-related effects of 5-HT in the brain must encompass this multiplicity of neuronal feedback mechanisms to remain viable.

Intra-DRN infusion of the cholinergic agonist nicotine produced dose-dependent effects in the social interaction test, with low doses producing an anxiolytic effect, medium doses producing no effect, and high doses producing anxiogenesis (Cheeta et al., 2001a,b).

The only neuropeptides that have been investigated in the raphe nucleus are bombesin and ghrelin, both of which seem to have an anxiogenic effect (Carlini et al., 2004; Merali et al., 2006).

The locus coeruleus has been almost totally neglected as a target of intra-cerebral infusion studies. Considering the important role of the norepinephrine system in anxiety-related responses to stress (e.g., Morilak et al., 2005; Debiec and LeDoux, 2004), it is surprising that there has not even been one study of the effects of monoaminergic compounds, of any kind, microinfused into the locus coeruleus. The one microinfusion study that exists showed that, whereas NPY and an NPY2 receptor agonist were anxiolytic in the plus-maze, NPY1 agonists and antagonists had no effect. These results, although limited, suggest that in the locus coeruleus the anxiolytic effect of NPY is due to its action at NPY2 receptor sites (Kask et al., 1998c). Nevertheless, the neuropharmacology of the locus coeruleus and its role in anxiety need far more attention than they are currently receiving.

2.2.3. Hypothalamus

The hypothalamus is densely interconnected with many anxiety-related areas in both the brain stem and the forebrain, including the locus coeruleus, raphe nuclei, periaqueductal gray, amygdala, septum, hippocampus and medial

prefrontal cortex, and has been repeatedly implicated in fear and defense (e.g., Risold et al., 1997). Neuronal fibers within the hypothalamus contain classical neurotransmitters such as catecholamines, acetylcholine and serotonin, and cell bodies and their axons contain glutamate, GABA, neurotensin, CRF and Neuropeptide Y, as well as their associated receptors (e.g., Abrahamson and Moore, 2001; Gerashchenko and Shiromani, 2004; Fetissov et al., 2004; Hrabovsky et al., 2004).

Temporary inhibition of the hypothalamus by direct or indirect $GABA_A$ receptor agonists increased rats' open-arm time in the plus maze, and increased contacts in the social interaction test, suggesting that the hypothalamus is necessary for the appropriate expression of fear reactions (Jardim and Guimaraes, 2001; Jardim et al., 2005; Rivera-Arce et al., 2006; Shekar, 1993; Shekar and Katner, 1995). NMDA receptor antagonism seems to have similar effects to $GABA_A$ agonism, but those findings proved difficult to replicate (Jardim and Guimaraes, 2001; Jardim et al., 2005).

An agonist at the cholinergic muscarine receptors produced an anxiogenic effect in the USV test when infused into the preoptic area (Brudzynski,1994). This study has been the whole extent of research regarding the cholinergic system in the hypothalamus.

An NPY receptor antagonist had no effect in the elevated plus-maze when infused into the paraventricular nuclei (Kask et al., 1998b). Although an oxytocin receptor antagonist microinfused into the paraventricular nucleus of the hypothalamus increased adrenocorticotropic hormone (ACTH) release in male and female rats, suggesting an anxiogenic effect, it had no behavioral effect on anxiety in the elevated plus-maze (Neumann et al., 2000; Bale et al., 2001). These are the only intra-cerebral microinfusion study of oxytocin ligands in behavioral models of unconditioned anxiety. Clearly, more extensive studies of these and other neuropeptides are warranted. Similarly, intra-hypothalamic microinfusion studies of cholinergic and aminergic receptor agonists and antagonists are needed to establish their role in unconditioned

anxiety. In contrast, the anxiolytic-like effects of $GABA_A$ receptor agonists in the hypothalamus seem fairly clear.

2.2.4. Amygdala

The amygdala and parts of the "extended amygdala" (e.g., bed nucleus of the stria terminalis; BNST) have long been viewed as neural substrates for fear and anxiety (for reviews see LeDoux, 2000; Davis and Whalen, 2001). In addition, these areas are strongly interconnected with all of the other regions that are the focus of this review, including the locus coeruleus, raphe nuclei, PAG, hypothalamus, septum, hippocampus and medial prefrontal cortex (Swanson and Petrovich, 1998; McDonald, 1998). These amygdalar structures also contain a variety of monoaminergic, amino acid and peptidergic neurotransmitters and receptors (Sah et al., 2003). A large number of studies have established the importance of the amygdala in Pavlovian conditioned fear (LeDoux, 2000; McNaughton and Corr, 2004). The role of the amygdala in the expression of animals' fear reactions in models of unconditioned anxiety, however, is complex, and seems to depend on specific amygdalar sub-nuclei, as well as particular behavioral tests or indices. For example, local administration of an indirect $GABA_A$ receptor agonist (midazolam) into the central nucleus of the amygdala (CeA) had no effect on open arm activity in the plus-maze, but significantly blocked passive avoidance of the electrified shock-probe in the burying test, an anxiolytic effect (Pesold and Treit, 1994, 1995). Conversely, the same dose of midazolam infused into the BLA did not affect shock-probe avoidance in the burying test, but selectively impaired open-arm avoidance in the plus-maze, another anxiolytic-like effect. None of these drug treatments significantly affected burying behavior, suggesting that the shock-probe avoidance deficit found in this test was not due to a failure to associate the shock with the probe (see also Lehmann et al., 2000a, 2003). Furthermore, the receptor specificity of the anxiolytic effects of infused

midazolam in the two tests was confirmed by the fact that they were blocked by co-infusion of the receptor antagonist flumazenil. In both paradigms, anxiety reduction was indicated by a passive avoidance deficit (open-arms versus shock probes), suggesting that the response requirements of the two tests did not differentiate the functions of the two amygdalar nuclei. Instead, the results suggest that specific benzodiazepine receptor populations in the amygdala may mediate defensive responses to different sorts of environmental threat (indistinct, potential threat [open arms] versus discrete, unambiguous threat [shock-probe]). A number of other studies of the anxiolytic-like effects of benzodiazepine agonists infused into the central and basolateral amygdala are in general agreement with this conclusion (e.g., Green and Vale, 1992; Gonzalez et al., 1996; Zangrossi and Graeff, 1994; see, however, Moreira et al., 2007). Unlike $GABA_A$ receptor agonists and antagonists, amygdalar infusion of $GABA_B$ receptor agonists and antagonists seem to be without effect, at least in the social interaction test (Sanders and Shekhar, 1995). To the best of our knowledge, however, this is the only microinfusion study of the effects of $GABA_B$ ligands in animal models of unconditioned anxiety. Nevertheless, the results are consistent with other pharmacological studies, which suggest that the role of $GABA_B$ receptors in anxiety is not entirely clear (Cryan and Kaupmann, 2005).

Both NMDA and AMPA receptor antagonists infused into the amygdala had anxiolytic-like effects in the social interaction test, and amygdalar infusions of a metabotropic glutamate receptor antagonist of mGlu R5 were also anxiolytic, in a variety of tests (De la Mora et al., 2006; Lehmann et al., 2000b; Sajdyk and Shekar, 1997b). Interestingly, agonists of other mGlu receptor subtypes were also anxiolytic in these models (Wieronska et al., 2005).

Amygdalar infusion of various α and β adrenergic receptor antagonists did not produce anxiolytic-like effects, except in the elevated plus-maze test, where β2 receptor antagonists infused into the CeA increased open arm

exploration (Cecchi et al., 2002a,b). As in other brain areas, the effects of adrenergic receptor stimulation or inhibition in the amygdala have been under-studied in animal models of anxiety. Evidence of a possible anxiolytic effect of D1 receptor antagonists infused into the amygdala (de la Mora et al., 2005) also requires additional study and corroboration.

While a number of studies have examined the behavioral effects of full or partial agonists of the 5-HT_{1A} receptor on rats' untrained fear reactions, their effects in the amygdala have only been clearly anxiolytic in the ultrasonic vocalization test (Schreiber and DeVry, 1993). This general result is mirrored in these tests by systemic administration of 5-HT_{1A} agonists (Treit et al., 2003). The anxiety related effects of ligands of other serotonin-receptor subtypes (5-HT_{1C}, 5-HT_{2A}, 5-HT_{2B}) have been inconsistent after intra-amygdalar infusion, with the notable exception of 5-HT_3 receptor antagonists, which seem to be uniformly anxiolytic in a number of models (e.g., Higgins et al., 1991; Costall et al., 1989b).

Intra-amygdalar microinfusion of neuropeptide ligands has variable effects in animal models of anxiety. For example, microinfusion of the opiate antagonist naloxone, into either the central or the basolateral nucleus, is without effect in the plus-maze test, suggesting that opioid receptors in the amygdala may not be involved in unconditioned anxiety. On the other hand, the robust anxiolytic effects of peripherally-injected diazepam in the plus-maze were blocked by intra-CeA (but not intra-BLA) infusions of the same opioid receptor antagonist, suggesting that GABA and opioid receptor systems in the amygdala interact in the control of anxiety (Burghardt and Wilson, 2006). NPY or NPY1 receptor agonists were anxiolytic in the plus-maze, but only when infused into the right amygdala (Kokare et al., 2005). The anxiety-related effects of NPY1 receptor antagonists were mixed, as were the effects of intra-amygdalar infusions NPY 2 receptor agonists, with reports of both anxiolytic and anxiogenic effects (Sajdyk et al., 2002a,b). An NPY 5 antagonist failed to produce any effect in the social interaction test when infused into the BLA.

While the intra-amygdalar infusion of galanin receptor ligands produced null effects in the plus-maze and social interaction tests (Khoshbourei et al., 2002), Salome et al. (2006) showed that a selective antagonist of the vasopressin receptor subtype Avpr1b was anxiolytic in the plus maze when infused into the BLA, but not when infused into CeA, results that parallel those found with the $GABA_A$ receptor agonist midazolam. The neuroanatomical specificity of the anxiolytic effect of Avpr1 antagonism in the BLA was further reinforced by the finding that Avpr1 receptor antagonism was without effect in the medial amygdala. Similarly, oxytocin was found to be ineffective when microinfused into the CeA (Bale et al., 2001); however, comparison data involving BLA and medial amygdala is unfortunately not available. Perhaps not surprisingly, CRF, which initiates a cascade of pituitary "stress" hormones such as ACTH, had anxiogenic effects in the social interaction test when infused into the BLA (Sajdyk et al., 1999). A closely related neuropeptide, urocortin 1, was also anxiogenic in the social interaction test when microinfused into the BLA (Spiga et al., 2006). A substance P/neurokinin 1 receptor antagonist produced an anxiolytic effect in the plus-maze when microinfused into the medial amygdala of pre-stressed rats, but had no effect in the plus-maze in the medial amygdala of non-stressed rats (Ebner et al., 2004). While these findings suggest that the substance P/neurokinin 1 receptor system is only active under higher stress conditions, there have been no systematic studies showing that the anxiolytic effects of standard or novel compounds in the traditional plus-maze test vary as a function of different levels of "stress," or that higher doses of the same anxiolytic compound can overcome the effects of higher levels of stress. In the absence of these data, it is difficult to evaluate the effects of drugs in this so-called, "stress-potentiated" plus-maze test. Finally, ghrelin, a peptide found in the gut and brain, produced an anxiogenic effect in the elevated plus-maze when microinfused into the BLA (Carlini et al., 2004).

Several findings indicate that the bed nucleus of stria terminalis [BNST] is involved in Pavlovian fear responses that have been specifically

conditioned to previously "neutral" stimuli such as discrete tones or lights (Waddel et al., 2006). On the other hand, there is also evidence that the BNST may be involved in unconditioned fear responses (Davis and Shi, 1999), particularly where there is a less explicit threat stimulus, such as exposure to the elevated plus-maze. Although this interesting hypothesis has not been thoroughly explored in animal models of unconditioned anxiety, it should be noted that the blockade of β2 adrenergic receptors in the BNST (or the CeA) reduced anxiety- like behavior in the elevated plus-maze (Cecchi et al., 2002a,b) In contrast, blockade of β1 or α1 adrenergic receptors in either the BNST or CeA failed to produce a significant change in anxiety responses in elevated the plus-maze or the social interaction tests (Cecchi et al., 2002a,b). Finally, intra-BNST infusion of CRF resulted in anxiogenesis (Sahuque et al., 2006), whereas a galanin antagonist had no effect in these tests (Khoshbouei et al., 2002). Whether the BNST plays a special role in unconditioned anxiety is unclear at the present time, although future microinfusion studies will likely clarify this putative relationship.

2.2.5. Septum

The septum communicates directly with the hippocampus and a number of other forebrain and brainstem structures thought to be involved in anxiety (PAG, raphe nuclei, locus coeruleus, hypothalamus, amygdala, BNST, prefrontal cortex (Jakap and Leranth, 1995). Furthermore, the septum contains a large number of neurosteroids and neuropeptides, as well as "classical" neurotransmitters such as glutamate, GABA and the monoamines (Risold and Swanson, 1997a,b). Although its role in fear and anxiety has only recently received systematic consideration, evidence continues to reinforce and refine its role in these processes (e.g., Treit and Menard, 2000). Some of the most compelling evidence comes from the intra-cerebral microinfusion studies reviewed here.

Neuronal inhibition of the lateral or medial septum by i.c. infusion of $GABA_A$ receptor agonists—direct or indirect— reduced anxiety-like behavior in the plus-maze, a finding which is also consistent with the effects of electrolytic, excitotoxic, or temporary TTX lesions of the septum (Pizzo et al., 2002; Menard and Treit, 1996; Pesold and Treit, 1994; Degroot et al., 2001; Degroot and Treit, 2004; Molina-Hernandez et al., 2006a,b; Bannerman et al., 2004; Treit and Pesold, 1990; Treit et al., 1993). Earlier evidence that the anxiolytic-like effects of intra-septal $GABA_A$ receptor agonists in the plus-maze might be specific to distinct subregions of the septum (e.g., Pesold and Treit, 1996) has not been replicated in more recent work (e.g., Degroot et al., 2001). Thus, GABAergic inhibition of either of the major sub-areas of the septum, medial or lateral, will suppress anxiety-related behavior. Intra-septal $GABA_A$ receptor agonists also completely abolish defense burying of an electrified shock-probe, but do not impair normal passive avoidance of the probe (Pesold and Treit, 1996). It is also important to recall that intra-amygdalar infusion of $GABA_A$ receptor agonists produce the opposite pattern: inhibition of shock probe avoidance, but not shock-probe burying. Taken together, these results suggest that the behavioral effects of $GABA_A$ receptor agonists in the shock-probe burying test are not due to associative learning or memory deficits. Rather, they reinforce the view that specific populations of $GABA_A$ receptors in specific regions of the brain control distinct aspects of animal defense, fear and anxiety (for earlier, more extended articulations of this view see Treit and Menard, 2000; Treit and Pesold, 1990; Treit et al., 1993a).

As in the case of GABAergic agonists, anxiolytic-like effects were observed in the plus-maze and shock-probe burying tests after intraseptal antagonism of ionotropic glutamatergic (AMPA) receptors: i.e., an increase in open-arm activity in the plus-maze test, and a decrease in burying behavior in the shock-probe test, without an effect on shock-probe avoidance (Menard and Treit, 2000). Interestingly, 5 μg of the NMDA receptor antagonist AP5

produced a clear anxiolytic-like effect in the plus-maze when infused into the medial septum (Elvander-Tottie et al., 2006), but not when infused more dorsally into the lateral septum (Menard and Treit, 2000), suggesting some site-specificity in the anxiolytic effects of intra-septal AP5 in the plus maze. Antagonism of metabotropic glutamatergic (mGlu5) receptors in the lateral septum also produced anxiolytic-like effects in the elevated plus-maze (Molina-Hernandez et al., 2006a).

Petre et al. (2005) found that a variety of adrenergic receptor antagonists (α1, β1/β2) microinfused into the lateral septum produced selective anxiolytic-like effects in the shock-probe burying test. These results contrast with those found in the amygdala, using the elevated plus-maze, where only β2 receptor antagonists produced anxiolytic-like effects (Cecchi et al., 2002a). In either case, these results suggest that further study of the effects of adrenergic compounds in different areas of the brain, and in different animal models of anxiety is warranted.

Intra-septal microinfusion of the 5-HT_{1A} receptor agonist 8-OHDPAT suppressed defensive burying in the shock-probe test, but did not affect shock-probe avoidance, plus-maze behavior, or general activity in either test (Menard and Treit, 1998). The anatomical selectivity of the anxiolytic effect of 8-OH-DPAT was demonstrated in the plus-maze after 8-OH-DPAT was microinfused into the hippocampus (Andrews et al., 1994; File et al., 1996; Menard and Treit, 1998). Here it produced a selective increase in open arm exploration, but did not affect defensive burying and did not affect shock-probe avoidance (Menard and Treit, 1998). These results provide another example of test-by-site dissociation: i.e., the same receptor system (5-HT_{1A}) in different parts of the brain controlled the expression of very specific fear reactions (Menard and Treit, 1998). Although another report (De Almeida et al., 1998) suggested that 8-OH-DPAT microinfused into the medial septum reduced open arm activity in the plus-maze—an "anxiogenic" effect—it was seen at only one dose, and was confounded by significant reductions of closed-arm and overall arm

entries. Similarly, a later report of anxiogenic effects in the plus-maze and social interaction tests after intra-septal infusions of 8-OH-DPAT is also confounded by parallel changes in measures of general activity (see Tables 1 and 3 in: Cheeta et al., 2000b). Thus, some caution is warranted in interpreting the effects of 5-HT_{1A} receptor agonists in these tests after intra- cerebral administration.

In contrast, the anxiogenic effects of nicotine infusions into the lateral septum are very consistent in the plus-maze and social interaction tests (e.g., File et al., 2000a). Intra-septal muscarinic agonists are similarly anxiogenic in the ultrasonic vocalization test (Brudzynski, 1994). Moreover, intra-septal infusion of a nicotinic antagonist is anxiolytic in the social interaction test (Ougazzal et al., 1999b).

The effects of intra-septal infusions of neuropeptide ligands are mixed, with most studies showing null, or anxiogenic effects (e.g., Everts and Koolhaas, 1999; Kask et al., 2001; Stemmelin et al., 2005). Two exceptions are: 1) the anxiolytic-like effect of intra-septal infusions of the galanin receptor antagonist M40 in the shock-probe burying test (Echevarria et al., 2005), and 2) the anxiolytic-like effect of intra-septal NPY in the social interaction test (Kask et al., 2001; Molina-Hernandez et al., 2006b). Intra-septal microinfusions of CRF or related agonists are anxiogenic, except for the CRF1 agonist cortagine, which had no significant effect in the elevated plus-maze (Tezval et al., 2004). The receptor specificity of these results awaits pharmacological verification with selective CRF receptor antagonists. Despite these somewhat scattered results, however, the septum houses a wide variety of neuropeptides, whose role in modulating anxiety invites further investigation.

2.2.6. Hippocampus

The hippocampus is massively interconnected with the septum, and has important connections with the locus coeruleus, raphe nuclei, hypothalamus,

amygdala, and medial frontal cortex, areas that are involved in anxiety (Amaral and Witter, 1995). The hippocampus utilizes a number of neurotransmitter and receptor systems, including glutamate, GABA, noradrenalin (from the locus coeruleus), serotonin (from the raphe nuclei), and acetylcholine (from the septal nuclei).

Inhibition of dorsal hippocampal neuronal function—whether by electrolytic lesions, direct or indirect $GABA_A$ receptor agonists, GABA reuptake inhibitors, or TTX blockade of Na+ channels—decreased fear reactions in the elevated plus-maze and social interaction tests (Liberato et al., 2006; Rezayat et al., 2005; Kjelstrup et al., 2002; Bannerman et al., 2002; McHugh et al., 2004; Degroot and Treit, 2004; Menard and Treit, 2001). At the very least, these studies suggest that the dorsal hippocampus plays a role in anxiety, consistent with the importance of the hippocampus in various neurobiological theories of anxiety (e.g., Gray,1982; LeDoux, 2000). On the other hand, unlike the anxiolytic effects consistently seen after infusions of ionotropic glutamatergic receptor antagonists into the PAG, the amygdala, and the septum, these glutamatergic antagonists do not seem to elicit anxiolytic-like effects when infused into the dorsal hippocampus (Hackl and Carobrez, 2007; Padovan et al., 2000). These null effects may depend on the location of infusions within the hippocampus, as Hackl and Carobrez, (2007) have found that the same dose of AP-5 is anxiolytic in the plus-maze when infused into the ventral hippocampus, but not when infused into the dorsal hippocampus. This apparent dissociation between the effects of AP-5 in the dorsal and ventral hippocampus has broader implications for the roles of these hippocampal areas in anxiety.

The effects of 5-HT_{1A} receptor agonists microinfused into the dorsal hippocampus are mixed (Alves et al., 2004; Cornelio and Nunes-de-Souza, 2007; File et al., 1996; Hogg et al., 1994; Whitton and Curzon, 1990). Of the 14 studies we reviewed, only the ultrasonic vocalization test reliably detected the anxiolytic effects of 5-HT_{1A} agonists infused into the dorsal hippocampus

(Schreiber and DeVry, 1993; Jolas et al., 1995). In the elevated plus-maze, two studies found anxiolytic effects after dorsal hippocampal infusion of 5-HT_{1A} agonists (Kotkowski et al., 1989; Menard and Treit), 1998;, while four found no effect (Belcheva et al., 1994; File and Gonzalez, 1996; Nunes-de-Souza et al., 2002; Picazo et al., 1995). In the social interaction test, 5-HT_{1A} agonists in the dorsal hippocampus had no effect in one study (Picazo et al., 1995), and anxiogenic effects in another (Andrews et al., 1994). Intra-dorsal hippocampal infusion of 5-HT_{1A} receptor antagonists, not surprisingly, were equally inconsistent, being anxiogenic in the social interaction test (Andrews et al., 1994) and without effect in the plus-maze (Nunes-de-Souza et al., 2002). Interestingly, 5-HT_{1A} receptor antagonists had anxiolytic-like effects in the elevated plus maze when microinfused into the ventral hippocampus (File and Gonzalez, 1996; Nunes-de-Souza et al., 2002). Ligands of other 5-HT receptor subtypes were either anxiogenic or, in most instances, without effect in the plus-maze (Alves et al., 2004; Cornelio and Nunes-De-Souza, 2007).

Facilitation of dorsal hippocampal acetylcholine with microinfusions of the acetylcholine esterase inhibitor physostigmine produced effects surprisingly similar to intra-septal $GABA_A$ receptor agonists: an increase in open-arm exploration in the plus-maze, and a decrease in defensive burying in the shock-probe test, without effects on shock probe avoidance or general activity (Degroot et al., 2001; Degroot and Treit, 2002, 2003; also see File et al., 2000b; Kenny et al., 2000; Tucci et al., 2003 for social interaction test findings similar to findings following nicotine infusion into the septum). Furthermore, simultaneous microinfusions of sub-effective doses of physostigmine into the hippocampus and muscimol into the medial septum summated to produce a suppression of shock-probe burying (Degroot and Treit, 2003). These results strongly suggest that the hippocampus and the septum interact in the control of anxiety (Gray, 1982). The neural mechanisms of this interaction are unknown, but one suggestion is that cholinergic agonism in the hippocampus stimulates inhibitory GABAergic projections to the medial

septum, which, when inhibited produces these anxiolytic effects. Alternatively, cholinergic agonism in the hippocampus could stimulate a glutamatergic projection to the lateral septum, which in turn sends inhibitory GABAergic fibers to the medial septum (Amaral and Witter, 1995; Degroot and Treit, 2003). In either case, we might expect intra-hippocampal physostigmine to recapitulate the well documented, anxiolytic effects of $GABA_A$ inhibition of the septum (e.g., Degroot et al., 2001; Treit et al., 2000). This scenario, although conceivable, represents only one of several possible routes by which the septum and hippocampus could interact in the control of anxiety (Tsurusakim and Gallagher, 2006).

Ventral hippocampal infusions of physostigmine also produced these anxiolytic-like effects, with the addition of an increase in shock probe contacts (Degroot and Treit, 2002). In contrast, selective stimulation of intra-dorsal hippocampal nicotinic receptors produced anxiogenesis in the social interaction test (e.g., File et al., 2000b). While it could be argued that the anxiolytic effects of intrahippocampal physostigmine in the shock-probe and plus-maze tests are the net effect of a global stimulation of all cholinergic receptor subtypes, not just one (e.g., nicotinic), this does not seem consistent with the increases in hippocampal acetylcholine seen after environmental stressors (e.g., Degroot et al., 2004). On the other hand, the stress-induced increase in hippocampal acetylcholine could represent an early adaptive response to increased stress. In any case, more work is needed using selective intra-hippocampal cholinergic receptor agonists and antagonists to assess the potential role of acetylcholine in anxiety.

Neuropeptides in the hippocampus (e.g., somatostatin) are often co-localized with some of the "classical" neurotransmitters such as GABA, presumably in a modulatory role (Vizi and Kiss, 1998). Infusion of neuropeptide Y, into either the CA1 region of the hippocampus or the dentate gyrus reduced anxiety in the elevated plus-maze (Smialowska et al., 2007). NPY1 or NPY2 receptor antagonists were without effect in either of these

areas, but they reversed the anxiolytic effect of NPY. Interestingly, in the CA1 region, the anxiolytic action of NPY was selectively blocked by the NPY1 receptor antagonist, whereas in the dentate gyrus, it was blocked by the NPY2 receptor antagonist. These apparent site-by-receptor type interactions are reminiscent of others seen throughout this review.

Finally, a cholecystokinin fragment (CCK-8; Rezayat et al., 2005), and ghrelin (Carlini et al., 2004) were anxiogenic in the plus-maze after infusions into the dorsal hippocampus. These findings are in agreement with other work showing that one or both compounds are anxiogenic after infusion into the PAG, the dorsal raphe and the amygdala.

2.2.7. Medial prefrontal cortex

The medial prefrontal cortex has efferent and afferent connections with practically all parts of the brain, including areas that are the focus of this review i.e., the hippocampus, septum, amygdala, hypothalamus, PAG, raphe nuclei, and locus coeruleus (Hoover and Vertes, 2007). The medial prefrontal cortex also contains a host of neurotransmitter and receptor systems, including dopamine, glutamate, GABA, serotonin, noradrenalin, acetylcholine, opiate and neurotensin (Steeke, 2003). Thus, it would not be too surprising to find that this cortical area is also involved in anxiety. In spite of these anatomical and neurochemical characteristics, however, as yet only a handful of investigators have microinfused receptor ligands into this area of the brain and studied the behavioral effects in animal models of unconditioned anxiety.

Lesions to the medial prefrontal cortex (mPFC), produced clear anxiolytic effects in the plus-maze (Lacroix et al., 2000; Gonzalez et al., 2000; Maaswinkel et al., 1996; Shah and Treit, 2003; but see Jinks and McGregor, 1997). Likewise, neuronal inhibition produced by $GABA_A$ receptor agonists microinfused into the mPFC increased rats' open arm activity, and decreased shock-probe burying. Shock-probe avoidance was not affected (Shah and Treit,

2004; Shah et al., 2004a). The only other study of the involvement of cortical structures in the expression of "anxiety" in these tests showed that a direct $GABA_A$ receptor agonist infused into the perirhinal region decreased open-arm avoidance in the plus-maze (Schulz-Klaus et al., 2005). Hopefully these studies will spur interest in the roles of other cortical structures in animals' untrained anxiety reactions.

A variety of anxiogenic stimuli can increase prefrontal dopamine activity, an effect that can be antagonized with $GABA_A$ receptor agonists (e.g., Tam and Roth, 1990). Although this suggests that dopamine antagonists directly infused into the mPFC might also have anxiolytic effects, only two studies have evaluated this possibility. Shah et al. (2004b) found that a D4 receptor antagonist produced anxiolytic effects in the plus-maze and shock-probe burying tests when microinfused into the mPFC. D1 and D2 receptor antagonists had no significant effect. Wall et al. (2003), on the other hand, found that a D2 receptor antagonist infused into the mPFC was effective in the plus-maze. Since the receptor specificity of dopamine antagonists is quite variable, and the D4 receptor itself belongs to a larger family of "D2-like dopamine receptors," (Sokoloff and Schwartz, 1995) further studies are needed to accurately characterize the anxiolytic effects of intra-mPFC dopamine antagonism in the plus-maze. Furthermore, these studies need to be reconciled with others that show an anxiogenic effect in the plus-maze after selective lesions of DA neurons in the mPFC (Espejo, 1997).

Finally, Wall et al. (2001) found that intra-mPFC infusions of an M1 cholinergic agonist produced anxiogenesis in the plus-maze, whereas similar infusions of an M1 antagonist produced anxiolysis. Wall and Messier (2000, 2002) have reported that antagonism of kappa-opioid receptors in the infralimbic area of the mPFC produced
anxiogenesis in mice, while kappa-opioid agonism resulted in anxiolysis. These findings directly contradict others that show kappa receptor antagonists produce anxiolysis when administered peripherally (e.g., Knoll et al., 2007).

There is also evidence that kappa receptor agonists infused into the dorsal PAG produce anxiogenisis (Motta et al., 1995). While it is possible that kappa-opioid receptors in the mPFC and PAG work in opposition to control anxiety-related responses, such hypotheses have rarely been tested using intra-cerebral infusion of selective receptor ligands.

2.3. Conclusions

The above review suggests that anxiety-related responses are controlled by a complex web of structures and neurotransmitter systems in the brain. $GABA_A$ receptor agonists produced anxiolysis in every major structure into which they were infused, and in all the models of unconditioned anxiety, suggesting this neurotransmitter system plays a global role in the neural control of anxiety. At the same time, there were a number of sub-nuclei-by-target response interactions, some of which may have theoretical implications. For the most part, antagonists of ionotropic and metabotropic glutamate receptors produced the same pattern of anxiolytic effects. These data provide a pharmacological complement to the anxiolytic effects of GABAergic agonists, and reinforce the importance of these structures in the neural control of anxiety-related behavior.

The data involving monoaminergic systems, although promising in some cases, were complicated by inconsistent results and, in the case of catecholamines, by a very limited data set (e.g., in the amygdala, the septum and the medial prefrontal cortex). A possible mitigating factor here is that brain monoamines, and for that matter, acetylcholine, typically have both excitatory and inhibitory effects on neural activity, depending upon, among other factors, receptor subtype: e.g., 5-HT_2 versus 5-HT_{1A} (for recent reviews see Parra et al., 1998; Kawaguchi and Shindou, 1998; Foehring et al., 2002). In contrast, GABA and glutamate neurotransmitter systems tend to produce only inhibition or excitation, respectively, at the majority of synapses in the adult mammalian

brain. Thus, centrally infused amine receptor ligands might be expected to produce more complex behavioral effects in animal models of anxiety, depending upon the exact target site and receptor subtype.

Cholinergic receptor systems in the septum and hippocampus appear to modulate unconditioned anxiety in opposite ways, although the cholinergic receptor-subtypes mediating these effects in the hippocampus remain to be determined.

Selective agonists and antagonists for CRF, NPY, and AVP receptors had reliable anxiety-related effects in different parts of the brain (e.g., PAG, amygdala), and in different animal models, (e.g., EPM, SI, LD). Generalizations about the anxiety-related effects of these neuropeptide receptor ligands, however, are complicated by a number of ligand-by-site interactions. For example, even in the same animal model (e.g., EPM), and after the same dose of ligand (e.g., vasopressin V1B receptor antagonist SSR149415), the anxiolytic effects of neuropeptides often depended on the specific sub-nuclei into which they are infused (e.g., basolateral versus central or medial amygdala; Solome et al., 2006). Such site-by-ligand interactions, although surprising, are not uncommon and can be accommodated in some neurobiological theories of anxiety. There are, however, instances where the same ligand (NPY2 agonist NPY3-36) in the same subnuclei (basolateral amygdala) in the same test (social interaction) has anxiolytic effects at one dose (80 pM) but anxiogenic effects at another (5 pM; Sajdyk et al., 2002a). Certainly, there may be specific ligand- receptor models that can accommodate these opposite effects. (see Sajdyk et al., 2002a). Nevertheless, the neuropharmacological specificity of such effects complicates the intense, ongoing research effort to discover neuropeptides, or their synthetic derivatives, that can serve as novel agents for the treatment of human anxiety disorders (e.g., Griebel et al., 2002; Holmes et al., 2003). Furthermore, there are number of other examples of these intricate interaction effects (e.g., Pesold and Treit, 1995), that are apparent even after microinfusion of standard

anxiolytic compounds (e.g., midazolam) into well-studied structures (e.g., amygdala). Although they impede easy generalizations, these interaction effects must be accounted for in neuropsychopharmacological theories of anxiety.

While models accommodating both the neurochemical and neuroanatomical data are yet to be developed, several earlier theories are being updated to accommodate at least the neuroanatomical aspects of these recent findings. In recent versions of Gray's (1982) original neuropsychological theory of anxiety, Gray and McNaughton (2000), and McNaughton and Corr (2004) suggest that the biological mechanisms of fear and anxiety can be understood in terms of the neural circuitry underlying animal defense reactions (e.g., avoidance, burying, freezing). This general view has currency and advocates among a number of other prominent researchers in the field (e.g., LeDoux, 2000; Blanchard and Blanchard, 1990), although the specific roles or functions they assign to each structure may differ somewhat. According to McNaughton and Corr (2004), the neural circuitry of anxiety is represented by evolutionarily conserved brain structures that are critically involved in animal defense. These structures are hierarchically organized along a caudal–rostral axis, from the periaqueductal gray, hypothalamus, and amygdala, to septum–hippocampus and the prefrontal cortices. In addition, monoaminergic projections from the locus coeruleus and raphe nuclei are important modulators of the more rostral structures in this neuroanatomical hierarchy (see Gray and McNaughton, 2000 and McNaughton and Corr, 2004 for more detailed descriptions). Although this neuroanatomical "hierarchy" was used simply as a basis for organizing the data in the above review, the relevance of each of the structures for fear and anxiety can be assessed, to some extent, by the microinfusion data reviewed here. The data presented in this review, especially from GABAergic microinfusion studies, overwhelmingly reinforce the involvement in anxiety of all of these structures (PAG, raphe nuclei, hypothalamus, amygdala, septum, hippocampus

and medial prefrontal cortex), except the locus coeruleus, where only NPY receptor ligands have been microinfused. On the other hand, a general look at the above review may form the impression that the bulk of this evidence favored the role of only a few structures in anxiety, (e.g., the amygdala, raphe nuclei), while others, (e.g., the locus coeruleus, medial prefrontal cortex) were less important, or played minor, indirect roles. There are extraneous factors, however, such as the size of the literature on a particular structure, which can artificially inflate the perceived prominence of one structure over the other. While the amygdala is clearly a well-validated component of most biological theories of fear and anxiety (e.g., LeDoux, 1998, 2000; Gray and McNaughton, 2000), the locus coeruleus, for example, also has a prominent place in clinical and neurobiological theories of anxiety and panic, (e.g., Ressler and Nemeroff, 2000; Redmond and Huang, 1979). But clearly the locus coeruleus has received far less attention in the microinfusion literature. Importantly, though, if this historical imbalance of experimental attention were reversed, exactly the same caution would apply. In this light, the inclusive neuroanatomical model proposed by McNaughton and Corr (2004) could serve as a conservative and useful approach to studying the neural substrates of anxiety (even though, in terms of parsimony, it is the least virtuous).

In the same light, but now in terms of the neuropharmacology of anxiety, it is evident, for example, that the serotonin system has received a great deal of attention in the microinfusion literature compared to the catecholamine or neuropeptide systems, even though many times the effects of intracerebral infusions of serotonin-receptor ligands on animals' untrained fear reactions appear to be no more consistent than the effects of catecholamine or peptidergic receptor ligands. Peripheral administration of serotonergic agents leads to equally inconsistent results, except in the ultrasonic vocalization test (Treit et al., 2003).

Several explanations have been offered for the inconsistent behavioral effects of serotonergic ligands in animal models of unconditioned anxiety: e.g.,

complex interactive effects between the dorsal and median raphe nuclei (Lechin et al., 2006); a differential role played by serotonin in brain stem versus forebrain structures (Graeff, 2002); context-dependent changes in presynaptic or postsynaptic regulation of serotonergic neurotransmission (Lowry et al., 2005); 5-HT ligand stimulation of the HPA hormonal stress pathway (de Boer et al., 1990); acute versus chronic drug administration (e.g., Treit et al., 2003); and strain and sex differences (Griebel, 1995). Nevertheless, none of these neurobehavioral variables seems able to account for the significant variation of serotonin effects seen across animal models. For example, central infusion of 5-HT1A compounds have mixed (mostly null) effects in the elevated plus-maze; fairly consistent, dose- dependent anxiolytic like effects in the social interaction test; and uniform (with the exception of ipsapirone) anxiolytic-like effects in the ultrasonic vocalization test. These findings lead to the conclusion that 1) The anxiety-related effects of a compound or family of compounds can be test-specific, 2) The finding that a compound or a family of compounds does not have anxiolytic effects in a certain behavioral test does not grant the conclusion that this compound has no anxiety-related effects.

At this point, it should be emphasized once again that supportive data from receptor knockout studies, immunohistochemistry, electrophysiological and neuroimaging work, and microinfusion data from studies using learned or conditioned fear reactions have not been included in the above review. Thus, this should not be viewed as a definitive or comprehensive review of the neurobiological basis of anxiety. However, the vast size of the above data reveals that the microinfusion literature is extensive and significant, in that it simultaneously integrates the neurochemical, neuroanatomical, and behavioral correlates of anxiety in a whole organism. As a result, I believe that the majority of the conclusions, shortcomings, contradictions and gaps in knowledge noted above can be generalized to the field of neurobiological anxiety research as a whole.

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CHAPTER 2

NEUROSTEROIDS AND NEUROPEPTIDES IN ANXIETY RESEARCH

1. New Directions in Anxiety Research

While many of the findings reviewed in Chapter 1 do not have immediate clinical implications, some reflect the mutual interaction of clinical and experimental research in this area. For example, problems associated with existing anxiolytic drug treatments (e.g., partial efficacy, undesirable side-effects, delayed therapeutic effects, abuse potential; see Enkelmann, 1991; Stevens and Pollack, 2005) have motivated or benefited from basic research into the fundamental properties of $GABA_A$ receptors, neurosteroids and neuropeptides. The following chapters of this thesis are focused on the basic roles of neurosteroids and neuropeptides in the neural circuitry of anxiety, while acknowledging the possible relevance of this research for the development of more selective and effective anxiolytic agents. A brief overview of neurosteroids and neuropeptides precedes the research papers that follow.

2. Neurosteroids

A "steroid" is any compound that is synthesized from cholesterol. In mammals, the first step in the synthesis of steroids is the conversion of cholesterol to pregnenolone through the action of the cytochrome P450 side-chain cleavage enzyme. A number of other steroids are metabolized from pregnenolone, including progesterone, which serves as the precursor to a large group of steroids that modulate neuronal function (Schumacher et al., 2000; see Figure 2.1 for a summary of steroid synthesis pathways in mammals). Steroids that are capable of modifying neuronal function are called "neuroactive steroids". Neuroactive steroids which are synthesized de novo within the nervous system are called "neurosteroids" (Baulieu, 1981).

Neurosteroids bind to and modulate several different membrane receptors and ion channels, including glycine activated chloride channels,

nicotinic acetylcholine receptors, NMDA, AMPA and kaninate receptors, and voltage-activated calcium channels (Dubrovsky, 2005). However, the most extensively studied neurosteroids interact with the $GABA_A$ receptor complex. Oxidized, ring α-reduced pregnanes 3α,5α TH PROG (i.e., allopregnanolone) and TH DOC, positively modulate the $GABA_A$ receptor complex by binding allosterically, and increasing both the frequency and duration of $GABA_A$-gated chloride flux (Harrison and Simmonds, 1984). While this effect is similar to those of benzodiazepines and barbitures, which have their own allosteric binding sites, the neurosteroids appear to bind to a distinct site on the $GABA_A$ receptor (Gee, 1988). Other neurosteroids, such as PREGS, DHEA and its sulphate-conjugated metabolite DHEAS are negative modulators of the $GABA_A$ receptor complex (Majewska, 1992; Rupprecht, 2003). In addition to these rapid, nongenomic effects, some of these neurosteroids can exert longer term, genomic effects on receptors (van Broekhaven and Verkes, 2003).

At the level of the whole organism, neurosteroids exert effects on aggressive, fear-related and sexual behaviors, as well as on learning and memory processes, sleep patterns, depression-like behaviors, and seizure activity (Belelli et al., 1989; Crawley et al., 1986; Damianish et al., 2001; Kavaliers, 1988; Khisti et al., 2000; Reddy and Kulkarni, 1998). Some of these neurosteroids are currently being clinically investigated for possible therapeutic effects (Dubrovsky, 2005). Chapter 3 of this thesis focuses on one of those neurosteroids, allopregnanolone, and its anxiety-related effects following administration into different brain areas.

3. Neuropeptides

Neuropeptides are short-[<50] chains of amino acids (polypeptides) that can act as neurotransmitters and neuromodulators. The early observation that the expression of these peptides was not confined to the median eminence

/ infundibilum of the basal hypothalamus, but was rather observed in different areas of the brain, ignited interest in neuropeptide research, which has now been going on for about 40 years (Hokfelt et al., 2000).

Neuropeptides differ significantly from classical neurotransmitters (i.e., acetylcholine, amino acids or monoamines) in terms of their size, synthesis, release and degradation. These differences, combined with their (co)expression patterns in the brain, have important implications for their functionality. For example, the efficient and fast synthesis and reuptake mechanisms of classical neurotransmitters makes it possible to keep transmitter levels relatively constant, even in the face of continued release. Neuropeptides, on the other hand, are much larger [up to 10 times the molecular weight of classical transmitters], are held together with peptide bonds, degraded with peptidases, and are replaced exclusively through new synthesis, which requires the upregulation of mRNA to guide the process in ribosomes. Thus, a considerable delay takes place before neuropeptide levels in the nerve endings are restored. This leads to the phenomenon of neuropeptide expression plasticity, where the expression of neuropeptides is very sensitive to the environmental or neuronal events that lead to their release (Hokfelt et al., 2000).

Another important difference between neuropeptides and classical neurotransmitters is their release processes. Neuropeptide release can take place as a response to Ca2+ concentrations as low as a tenth of that required for classical neurotransmitter release (Verhage et al., 1991). This high-affinity process with respect to Ca2+ observed with neuropeptides compared to the nonpeptidergic low-affinity process is related to the stimulation-type dependency of intracellular Ca2+. Low frequency stimulation of the neuron results in a Ca2+ increase concentrated only around the active zones where nonpeptidergic neurotransmitters are released (McMahon and Nichols, 1991; Sihra et al., 1992). The Ca2+ levels return to normal rapidly following the cessation of stimulation, which results in the rapid termination of

neurotransmitter release. High frequency stimulation of the neuron, on the other hand, leads to more diffuse changes in Ca2+ levels observed throughout the neuron. The high-Ca2+-affinity nature of peptidergic release results in a release of neuropeptides as a response to high-frequency stimulation, where the incremental increase in local Ca2+ levels may initially be too low to promote nonpeptidergic neurotransmitter release (Sihra and Nichols, 1993). As a result, while the phasic release of classical neurotransmitters is required for ongoing adjustment to environmental events, neuropeptidergic activity is particularly sensitive to unusual, unexpected changes in environmental conditions or in the internal working of the organism (e.g., stressful events, neuronal injury). Furthermore, there is some evidence that their synthesis is upregulated in response to certain environmental stressors (Hokfelt et al., 2000). In principle, this sensitivity to environmental stressors makes neuropeptides ideal candidates for the regulation of anxiety-related processes, where the organism's behaviors (e.g., explorative behavior of a rat) are exquisitely balanced, with both approach to and withdrawal from potentially dangerous aspects of their current environment (e.g., for rats, reduced exploration of a brightly lit area).

In addition to their role as neurotransmitters, neuropeptides act as neuromodulators of other neurotransmitter systems. Neuropeptides almost always coexist with classic neurotransmitters in the same neuron (Hokfelt, 1991). However, the exact combination of classical neurotransmitter and neuropeptide seems to change according to the area of the brain under study. For instance, substance P is colocalized with 5-HT in the most caudal medullary neurons projecting to the spinal cord. In the pons, on the other hand, substance P is colocalized with acetylcholine (Cooper et al., 2003). Thus, co-existance seems to be both specific and flexible, presumably as a function of its role in a particular area of the brain.

The sensitivity of neuropeptides to environmental conditions and the anatomical selectivity they express as neuromodulators of other neurotransmitter systems also make neuropeptides very good candidates as therapeutic alternatives to existing anxiolytic drugs. Thus, a great deal of research has been done on the possible anxiety-related effects of different neuropeptides in the last 20 years (Chaki and Kanuma, 2007; Holmes et al., 2003). So far, 5 neuropeptides (i.e., substance P (SP); vasopressin, neuropeptide Y (NPY), corticotrophin releasing factor (CRF) and galanin) have received extensive attention and their involvement in anxiety-related processes has been quite convincingly demonstrated (Holmes et al., 2003). However, this research is still in early stages and the investigation of the receptor subtypes that mediate the effects of those neurotransmitters is ongoing.

In addition to molecular specification of the anxiety-related effects through search for the specific receptor subtypes that mediate those effects, the specific brain areas where they have their effects is also an area of intense research. As seen in Chapter 1, intracerebral infusion of specific neuropeptide receptor agonists and antagonists seems to be an ideal way of establishing the neuroanatomical and neurochemical co-ordinates of their behavioral effects. While this research has expanded the understanding of neuropeptide actions in the brain significantly, research into the role of neuropeptides in brain anxiety circuits is just beginning (see Chapter 1).

Chapters 4, 5 and 6 of this thesis are concerned with the anxiety-related effects of two different neuropeptides, vasopressin and somatostatin.

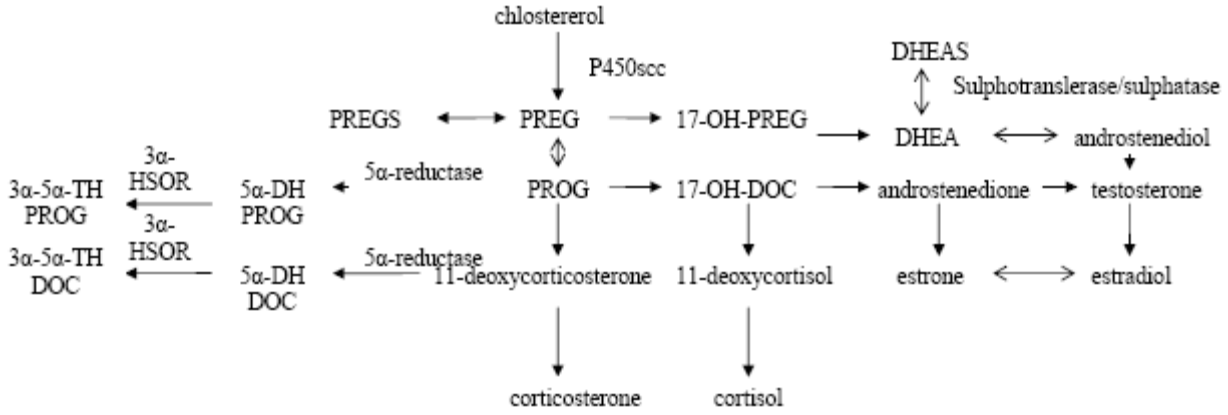
Together with the related peptide oxytocin, vasopressin was one of the first neuropeptides to be discovered and remains to be the most extensively studied (Hokfelt et al., 2000). Thus, our knowledge regarding the behavioral and physiological effects, receptor subtypes and neuroanatomical distribution of vasopressin is quite extensive compared to most other neuropeptides,

although there are still significant gaps in our knowledge of its effects in animal models of anxiety. Furthermore, the exact vasopressin receptor sub-types that mediate the anxiety-related effects of vasopressin remain to be determined. Accordingly, in Chapter 4, the effects of two selective vasopressin ligands are compared and contrasted in the ventral and dorsal regions of the hippocampus.

In contrast to many of neuropeptides such as vasopressin, there is very little known regarding behavioral effects of somatostatin. In particular, the possible role of somatostatin in anxiety-related processes has not been characterized. In Chapters 5 and 6, some initial findings supporting the involvement of somatostatin in anxiety-related behaviors are reported.

FIGURES AND TABLES

Figure 2.1: Stereoidogenesis pathways. (3α,5α –diol: 3α,5α androstanediol, DHEA: dehydroepiandrosterone, DHEAS: dehydroepiandrosterone - sulphate, 5 α -DH DOC: 5 α -dihydrodeoxycorticosterone, 5α -DH PROG: 5α - dihydroprogesterone, DHT: dihydrotestosterone, 3α,5α,20α -HH PROG: 3α,5α,20α-hexahydroprogesterone or allopregnanediol, 3α-HSOR: 3α - hydroxysteroid oxidoreductase, P450scc: P450 side chain cleavage enzyme, PREG: pregnenolone, PREGS: pregnenolone-sulphate, PROG: progesterone, 3α,5α -TH DOC: 3α,5α -tetrahydrodeoxycorticosterone, 3α,5α -TH PROG: 3α,5α -tetrahydroprogesterone or allopregnanolone.


chlostererol
P450scc
DHEAS
Sulphotranslerase/sulphatase
PREGS
PREG
17-OH-PREG
DHEA
androstenediol
3α-
HSOR
5α-reductase
PROG
17-OH-DOC
androstenedione
testosterone
3α-5α-TH
PROG
5α-DH
PROG
3α-
HSOR
5α-reductase
3α-5α-TH
DOC
5α-DH
DOC
11-deoxycorticosterone
11-deoxycortisol
estrone
estradiol
corticosterone
cortisol

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CHAPTER 3

THE ANXIOLYTIC-LIKE EFFECTS OF ALLOPREGNANOLONE MICROINFUSED INTO THE AMYGDALA, MEDIAL PREFRONTAL CORTEX, OR HIPPOCAMPUS

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1. Introduction

Allopregnanolone (5-alpha-hydroxy-3-alpha-pregnan-20-one; 3α,5α – TH PROG) is a neurosteroid derived from progesterone by the sequential action of 5α-reductase and 3α-hydroxysteroidoxidoreductase (See Figure 2.1). The synthesis of allopregnanolone occurs in both the periphery--in ovaries and adrenal gland--and de novo in the brain (Rupprecht, 2003; Belelli and Lambert, 2005). As mentioned in Chapter 2, in addition to its relatively slow genomic effects, allopregnanolone has fast modulatory effects at the $GABA_A$ receptor complex, potentiating GABA-induced chloride conductance and neuronal inhibition (Majewska, 1986; Rupprecht, 2003). Accordingly, allopregnanolone shares many of the pharmacological characteristics of benzodiazepines, barbiturates, and other positive modulators of $GABA_A$ receptors: e.g., sedative (Norberg et al., 1987), hypnotic (Pluchino et al., 2006), anesthetic (Korneyev & Costa, 1996; Turkmen et al., 2008), anticonvulsive (Belelli et al., 1989), pro-appetitive (Chen et al., 1996; Reddy and Kulkarni, 1998; Fudge et al., 2006), amnesic (Backstrom et al., 2008; Johansson et al., 2002; Matthews et al., 2002) and anxiolytic effects (Khisti et al., 2000; Reddy and Kulkarni, 1997; Ugale et al., 2007; Zimmerberg and Kajunski, 2004). In addition, like triazolobenzodiazepines (e.g., alprazolam), there are reports that allopregnanolone also has antidepressant-like effects in animal models of depression (Khisti et al., 2000; van Broekhoven & Verkes, 2003).

As mentioned earlier, the binding site for allopregnanolone on the $GABA_A$ receptor is distinct from that of benzodiazepines or barbiturates, but the exact site has been difficult to identify (Belelli and Lambert, 2005). A recent study, however, has suggested that allopregnanolone produces its effects at the $GABA_A$ receptor by binding to a cavity within the α subunit, and at interfacial residues between the α and β subunits (Hosie et al., 2006). Thus, it is possible that allopregnanolone positively modulates a distinct set of

receptors with a distinct anatomical distribution in the brain as compared to benzodiazepines and barbiturates.

The findings reviewed above, combined with evidence that acute stress increases brain levels of allopregnanolone (Purdy et al., 1991; Higashi et al., 2005), should encourage attempts to localize the anxiety-related effects of allopregnanolone within the brain. Despite the ubiquitous distribution of GABA and its receptors in the brain, a natural target would be those areas that are known to be involved in anxiety processes and those areas that have a particularly high concentration of $GABA_A$ receptors. "Limbic" structures such as the septum, amygdala, hippocampus and certain parts of the "extended" limbic system, such as the medial prefrontal cortex, satisfy both of these criteria. In fact, all of these structures have been previously implicated in the anxiolytic effects of benzodiazepines (Chapter 1; Engin and Treit, 2008). Nevertheless, very few micro-infusion studies have been designed for the purpose of understanding the behavioral effects of allopregnanolone in these areas. Of these, most have infused precursors of allopregnanolone, allopregnanolone synthesis inhibitors, or agonists of mitochondrial-derived allopregnanolone, rather than allopregnanolone itself. Considering the number of unknowns in the process, such as rate-limiting steps and anatomically specific synthesis rates for allopregnanolone in the brain (Stoffel-Wagner, 2003), the findings from those studies make it difficult to reach unequivocal conclusions.

For example, Bitran et al., (1993), injected rats (s.c) with progesterone, a precursor of allopregnanolone, and then tested rats in the elevated plus-maze. Progesterone produced clear anxiolytic-like effects in the plus-maze. In other studies Bitran et al., (1991; 1995) confirmed that the anxiolytic effect of exogenous progesterone was in fact due to its bioconversion to allopregnanolone by 5-alpha reductase, and its subsequent action at the $GABA_A$ receptor complex. Furthermore, micro-infusion of pregnanolone into

the septum or hippocampus produced anxiolytic effects in the plus-maze and shock-probe burying tests (Britan et al., 1999), however, whether this related neurosteroid produces its unique effects is unclear. It is a possibility that the behavioral effects of pregnanolone might be through the changes it poses on allopregnanolone synthesis.

Other researchers found that micro-infusions of progesterone aimed at the medial amygdala produced anxiolytic effects in the open-field, plus-maze and defensive freezing paradigms (Frye and Walf, 2004); however, whether this behavioral change is due to changes in allopregnanolone levels is unclear. Although inhibiting the biotransformation of progesterone to allopregnanolone within the hippocampus produced expected anxiogenic-like effects in the open-field and plus-maze tests, it also produced paradoxical anxiolytic-like effects in the emergence test and the social interaction test (Frye et al., 2000). Furthermore, in one study, intra-hippocampal infusion of allopregnanolone itself did not produce anxiolytic effects (Martin-Garcia and Pallares, 2005). In contrast, allopregnanolone produced reliable anxiolytic effects in the conflict test and plus-maze test after micro-infusion into the central amygdala (Akwa et al., 1999) and in the conflict test after micro-infusion into the lateral septum (Molina-Hernandez et al., 2003).

Bitran et al., (2000) showed that stimulation of intra-hippocampal mitochondrial benzodiazepine receptors increased local allopregnanolone levels and produced anxiolytic effects in the plus-maze and shock probe burying tests. Both the increase in intra-hippocampal allopregnanolone and the concomitant anxiolytic effects could be reversed with a 5α-reductase inhibitor, but not by the benzodiazepine receptor antagonist flumazenil. Finally, micro-infusion of a 5α-reductase inhibitor into the amygdala of progesterone-primed OVX rats produced anxiogenic-like effects in the open-field, plus-maze and defensive freezing tests (Walf et al., 2006).

Overall, these findings clearly suggest that the amygdala mediates some of the anxiolytic-like effects of allopregnanolone. While similar evidence can also be marshaled for the septum, the hippocampus has produced mixed results, some statistically non-significant (Martin-Garcia and Pallares, 2005; figures 1 and 2), some indirectly supportive (Britan et al., 1999), and some contradictory (Rhodes and Frye, 2001). Furthermore, with only one exception (Martin-Garcia and Pallares, 2005), there have been no published studies where allopregnanolone itself has been micro-infused into the hippocampus. A final limitation of these studies is that the putative anxiolytic effects of allopregnanolone have not been corroborated in other brain areas rich in $GABA_A$ receptors and known to be involved in the modulation of anxiolytic-related behaviors, such as the medial prefrontal cortex (mPFC; Shah and Treit, 2004; Shah et al., 2004).

Accordingly, the aim of the present study was to assess the anxiolytic role of allopregnanolone in the hippocampus and MPFC. To this end, we measured the behavioral effects of intra-MPFC and intra-hippocampal micro-infusions of allopregnanolone in two animal models of anxiety: The elevated plus-maze and the shock-probe burying test. We also included a group of rats microinfused with allopregnanolone into the amygdala, to serve as a positive comparison condition.

2. General Methods

2.1. Subjects

Male Sprague - Dawley rats, weighing 250-400 g at the time of surgery, served as subjects. 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. Just prior to surgery, the rats were randomly assigned to surgery conditions (amygdala, mPFC, or hippocampus).

2.2. Surgery

The rats were injected with atropine sulfate (0.1 mg/0.2 ml ip), and anaesthetized with pentobarbital (Nembutal 50 mg/kg, ip). Following hydration with 0.9% saline (3 cc, ip), they were placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA), and an incision was made to expose the skull. The subjects were 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 medial prefrontal cortex (AP:+2.9, ML:-1.9, DV:-1.8, cannulae angled at 15° towards the midline) or the hippocampus (AP:-3.1, ML:-2.2, DV:-2.0, cannulae angled at 15° towards the midline). The cannulae were secured to the skull with four jeweler's screws and cranioplastic cement. A dummy cannula was inserted into each guide cannula in order to keep the cannula tract clear. Following the surgery, the subjects were placed in a warm environment, until they regained consciousness. Rats were then allowed to recover for at least 7 days in their home cages before the start of behavioral testing.

2.3. Infusion procedure

Rats from each surgery group were randomly assigned to either a vehicle control condition or an allopregnanolone condition prior to behavioral testing. Allopregnanolone (Sigma, St Louis, Missouri, USA), was dissolved in a vehicle of 2-hydroxypropyl-β-cyclodextrin (5μg/μl), and infused via an infusion pump (Harvard Apparatus 22) at a rate of 1 μl/min, varying the duration of the infusion according to the desired dose. Allopregnanolone or 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 8mm below the ventral tip of the guide cannula for amygdalar infusions, 5mm below the tip for MPFC infusions and 3.8mm below for hippocampal infusions. 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 similar to those employed in several previous studies (for details see Treit et al., 1993; Treit and Pinel, 2005). The experimenter handled the rats (5 min each), and checked the cannulae tracts for blockage, on each of the 4 consecutive days prior testing. All behavioral testing was recorded on videotape. Testing started 10 min after the end of infusion procedure. The subjects were assigned to the same drug treatment group (allopregnanolone or vehicle) for both behavioral tests. The plus-maze test occurred first, followed seven days later by the shock-probe test. Previous work in our laboratory has shown that this order of testing has little effect on the shock-probe test, whereas the reverse order alters baseline behavior in the plus-maze test, consistent with studies of the effects of "pre-stress" on subsequent plus-maze behavior (e.g., Ebner et al., 2004).

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 (See Figure 3.1). All testing was conducted between 0800 and 1700 h in a quiet and dimly illuminated (<15 lux) 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 arm entries (open-arm entries + closed-arm entries) and the total of closed-arm entries, were used as indexes of general activity (Pellow, 1986; Hogg, 1996).

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 (Figure 2.2). 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 (see Treit et al., 1993, Shah and Treit, 2004). 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, with increased reactivity indicating higher pain sensitivity. All testing took place between 0800 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 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 (between AP coordinates –3.5 and + 3.5) 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. As a result of microscopic examination of the brain sections, 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 were expressed as means and standard errors of the mean (S.E.M.). Behavioral measures from the plus-maze were analyzed with one-way analysis of variance (ANOVA; α=0.05). If the overall F-test was significant, post hoc LSD tests were used to compare the effects of different doses of allopregnanolone to the vehicle control condition (α=0.05). In cases where the behavioral data violated the homogeneity of variance assumption (Levene tests, α=0.05), they were transformed to their base ten log equivalents to satisfy this assumption, and then subjected to the ANOVA. In cases where the log-transformed data violated homogeneity of variance, non-parametric analysis was conducted (Kruskal-Wallis and Mann-Whitney U-tests; α=0.05). The shock-probe burying data most often violate assumptions of normality and homogeneity of variance (e.g., Degroot and Treit, 2004).

3. Results

3.1 Experiment 1: Allopregnanolone microinfusions into the amygdala

Numerous studies have shown that ablation or pharmacological inhibition of the amygdala antagonizes both conditioned and unconditioned fear (e.g., for reviews see Menard and Treit, 1999; LeDoux, 2000). For this reason--and the previous demonstration (Akwa et al., 1999) of anxiolytic-like effects of intra-amygdalar allopregnanolone--we used this site as a positive control condition, to compare with the mPFC and hippocampus. A dose of 8µg per side was based on the positive effects of this dose of allopregnanolone in a previous study (Akwa et al., 1999).

3.1.1. Histology

The approximate infusion sites of the amygdalar cannulae included in the behavioral analysis are depicted in Figure 3.3. Eight animals were excluded from the analyses as a result of misplaced cannulae, and one animal was excluded because the head cap assembly came loose just before the shock-probe burying test. The plus-maze data from this animal was, however, included in the behavioral analyses.

3.1.2. Elevated Plus-Maze

The percentage of open-arm entries and percentage of open-arm time are depicted in the left portion of Figures 3.6.A and 3.6.B, respectively. Allopregnanolone microinfusions into the amygdala (8 μg/side) increased the percentage of open-arm entries ($F(1,16)= 4.32$, $p<0.05$) and the percentage of open-arm time ($F(1,16)=7.81$, $p<0.01$) compared to controls, without affecting general activity (closed arm entries: $F(1,16)=0.50$, $p>0.49$).

3.1.3. Shock-Probe Burying

Total burying time and the number of shock-probe contacts are depicted in the left sections of Table 3.1 and Figure 3.7, respectively. Similar to previous studies using intra-amygdalar midazolam infusions (Pesold and Treit, 1994; 1995), intra-amygdalar microinfusions of allopregnanolone significantly increased the number of probe contacts ($F(1,16)=4.91$, $p<0.04$), without affecting the duration of burying ($F(1,15)=0.002$, $p>.5$), general activity ($F(1,15)=0.42$, $p>0.52$) or shock sensitivity ($F(1,16)=2.09$, $p>0.16$).

3.2. Experiment 2: Allopregnanolone microinfusions into the mPFC

A previous study (Shah and Treit, 2004) has shown that midazolam microinfused into the mPFC produced anxiolytic effects in both the plus-maze and shock-probe burying tests. Similar results were expected after intra-

amygdalar infusions of allopregnanolone. Four different doses of allopregnanolone (5, 8, 14, and 20 μg/side) were used to test this hypothesis.

3.2.1. Histology

The approximate infusion sites of the medial prefrontal cannulae included in the overall analysis are depicted in Figure 3.4. Nine animals were excluded from the analyses as a result of misplaced cannulae, and four animals were excluded because the head cap assembly came loose just before the shock-probe burying test, leaving 50 animals for further analyses in elevated plus-maze test, and 46 for shock-probe burying test.

3.2.2. Elevated Plus-Maze

The vehicle control groups for the four different doses of allopregnanolone (N=4 for 5 μg/side, N=3 for 8 μg/side, N=4 for 14 μg/side, or N= 3 for 20 μg/side) did not differ in the plus-maze (% open arm entries F: (3, 10)=1.86, $p>0.20$; % open arm time: F (3, 10)=1.56, $p>0.26$). Accordingly, the vehicle control groups were combined in the following analyses.

The percentage of open-arm entries and percentage of open-arm time are depicted in the middle sections of Figures 3.6.A and 3.6.B, respectively. Intra-mPFC allopregnanolone increased the percentage of open-arm entries ($F(4,45)=4.28$, $p<0.01$) and the percentage of open-arm time spent ($F(4,45)=4.61$, $p<0.01$), without changing the number of closed arm entries ($F(4,45)=1.73$, $p>0.16$). The LSD test ($\alpha = .05$) confirmed that allopregnanolone significantly increased both the percentage of entries and percentage time in the open arms, compared to the vehicle control group at doses of 8μg/side, 14μg/side, and 20 μg/side (see Figures 3.6.A and 3.6.B).

3.2.3. Shock-Probe Burying

The four vehicle control groups did not differ in the shock-probe burying test (burying time: $F(3,7)=1.15$, $p>0.40$; probe contacts: $F(3,7)=2.05$,

p>0.20), and their data were combined in subsequent analyses. ANOVA showed that shock-probe contacts did not differ significantly between the groups (F = 2.41, p>0.07; Figure 3.7, middle section). The shock-probe burying data showed heterogeneity of variance, even after log data transformations (p<.002). Subsequent non-parametric analysis (Kruskal-Wallis) failed to detect a statistical difference in the burying behavior of the different groups (Chi-Square (3 df) = 3.95, p>0.05; Table 3.1, middle section).

3.3. Experiment 3: Allopregnanolone microinfusions into the hippocampus

Although a previous pilot study in our laboratory failed to demonstrate any anxiolytic-like effect of allopregnanolone infused into the hippocampus (data not shown), we conducted a more extensive study using intra-hippocampal allopregnanolone infusions of 14 μg/side and 20 μg/side, which is reported here.

3.3.1. Histology

The approximate infusion sites of the hippocampal cannulae included in the overall analysis are depicted in Figure 3.5. All included subjects had implants within the dorsal hippocampus. The behavioral data for twelve animals were excluded from the analyses because of misplaced cannulae, leaving the data from 26 animals for further analyses. After the plus-maze test, one more animal was excluded because of the loosening of its head-cap between the two tests.

3.3.2. Elevated Plus-Maze

The vehicle control groups for the two different doses of allopregnanolone did not differ significantly in the plus-maze (percentage of open- arm entries: F (1, 8)=4.45, p>0.06; percentage of open-arm time: F (1,

8)=3.45, p>0.10). Accordingly, the data were combined in the following ANOVAs. Allopregnanolone microinfusions into the dorsal hippocampus did not produce an anxiolytic effect in the plus-maze (% open-arm entries: F (2,23) = 1.87, p>0.17; % open-arm time F (2,23) = 1.26, p> 0.30; see Figures 3.6.A, 3.6.B, right section), replicating the results of our previous pilot study. Although there was a significant decrease in the number of closed-arm entries (F(2,23)=3.66, p<0.04) at doses of 14µg/side and 20µg/side (LSD test α=0.05), there was no difference the total number of arms entered (F(2,23) = 0.45, p>0.05).

3.3.3. Shock-Probe Burying

The two vehicle control groups did not differ significantly in the shock-probe burying test (burying time: F(1,6)=0.77, p>0.4; shock number: F(1,6)=3.37, p>0.11) and were therefore combined. Tests on the combined data indicated significant heterogeneity of variance for both the burying data and still time (Levene test, p<0.05). However, logarithmic transformations of these data satisfied the homogeneity of variance assumption, which allowed statistical evaluation with analysis of variance. This analysis, as in our pilot study, failed to detect a significant effect of allopregnanolone on burying behavior (F(2,23) = 1.30, p>0.28; see Table 3.1, right section), or shock-probe contacts (F (2,23) = 0.009, p > 0.5; Figure 3.7). Also unaffected by intra-hippocampal allopregnanolone were 'still time', the inverse measure of general activity (F(2,23)=0.006, p> 0.5; see Table 3.1), and shock-probe reactivity (F(2,23)= 1.08, p>0.35). Thus, neither effects on general activity nor effects on shock-probe sensitivity could have obscured a true anxiolytic effect of intra-hippocampal allopregnanolone in this experiment. If anything, these potential side effects would have mimicked, not obscured, an anxiolytic effect in the shock-probe burying test.

4. Discussion

Allopregnanolone had full anxiolytic effects when microinfused into the amygdala, partial anxiolytic effects microinfused into the mPFC, and no anxiolytic-like effects when microinfused into the dorsal hippocampus. The anxiolytic effects of intra-amygdalar allopregnanolone were observed in both the elevated plus-maze and shock-probe burying tests, whereas intra-mPFC allopregnanolone resulted in systematic anxiolytic effects that were restricted to the elevated plus-maze. In neither of these cases were anxiolytic effects confounded by changes in general activity or pain sensitivity. In sharp contrast, anxiolytic effects were not observed on any measure in either the plus-maze or shock-probe tests after intra-hippocampal microinfusion of allopregnanolone.

The behavioral results of intra-amygdalar allopregnanolone in the first experiment are consistent with previous findings (Akwa et al., 1999), as well as findings from the benzodiazepine literature (Pesold and Treit, 1994; 1995). This was expected since both compounds act at the $GABA_A$ receptor site after either systemic or central administration, and both compounds are anxiolytic in animal models such as the plus-maze and shock-probe tests. Even more interesting is the similarity of effects found in the shock-probe burying test after intra-amygdalar infusion of allopregnanolone (this study) or the benzodiazepine midazolam (Pesold and Treit, 1994; 1995). Pesold and Treit reported that microinfusion of midazolam into the central amygdala increased the number of shock-probe contacts, without affecting burying behavior, a pattern that is replicated exactly in the current study with allopregnanolone. However, intra-amygdalar allopregnanolone in the present study also produced clear anxiolytic-like effects in the plus-maze, as it did in a previous study in the plus-maze (Akwa et al., 1999), whereas midazolam infused into the central amygdala did not produce anxiolytic effects in the plus-maze (Pesold and Treit, 1995). It is important to note, however, that our amygdalar infusion sites were concentrated in the basolateral and the central nuclei, with scattered sites

in the medial nuclei (see Figure 3.3). In addition, given the high fat solubility of steroidal compounds, it is likely that both the central and the basolateral nuclei could have been activated by allopregnanolone infused into either site. This is significant because $GABA_A$ agonists such as the benzodiazepines produce reliable anxiolytic effects in the elevated plus-maze when infused into basolateral nucleus of the amygdala (e.g., Green & Vale, 1992; Pesold & Treit, 1995). As for the increase in open-arm exploration observed by Akwa et al. (1999) after central amygdalar microinfusions, it seems quite possible this anxiolytic effect was also due to diffusion of allopregnanolone away from the central nucleus, into the nearby basolateral nucleus of the amygdala (see their Figure 2).

Although intra-mPFC allopregnanolone microinfusions produced robust anxiolytic-like effects in elevated plus-maze, at each of three different doses, we were unable to demonstrate an anxiolytic effect of any dose of allopregnanolone in the shock-probe burying test. It might be argued that the plus-maze test is more sensitive to the anxiolytic effects of allopregnanolone than the shock probe burying test, but a previous study of intra-mPFC midazolam showed clear anxiolytic effects in both the plus-maze and shock-probe tests (Shah et al., 2004). One possibility is that allopregnanolone activates fewer $GABA_A$ receptors in this area compared to midazolam, because of its distinct binding pattern. Another explanation is that the very low levels of burying by the vehicle control group in the present study may have obscured an anxiolytic effect of intra-mPFC allopregnanolone (see Table 3.1). Only 3 out of 10 rats in the control group buried the shock-probe at all, producing a 'floor effect' that could have easily obscured an anxiolytic effect. And even if there were no floor effects inherent in these data, there was significant heterogeneity of variance in the probe-burying scores, which forced a less powerful, non-parametric test, which ultimately was non-significant. In contrast to these problems with the burying data, the shock-probe contact data were normally distributed, with equivalent between-group variances, and a

control mean of about two probe-contacts, typical of this parameter. There was no hint of a significant drug effect on shock-probe contacts, consistent with the absence of an allopregnanolone effect on shock-probe burying. Taken together, these results provide only modest and somewhat equivocal support for the conclusion that mPFC allopregnanolone produces anxiolysis in some tests (elevated plus-maze), but not in others (shock-probe burying).

On the other hand, microinfusion of allopregnanolone into either the amygdala or the mPFC produced significant anxiolytic effects in at least one or both of the behavioral tests, supporting the hypothesis that $GABA_A$ receptors in these brain regions are involved in the anxiolytic-like effects of allopregnanolone. This cannot be said about the hippocampus. Allopregnanolone microinfusions into the hippocampus did not affect anxiety-related behaviors in either the elevated plus-maze or the shock-probe burying test, at doses that were comparable to those successfully used in the mPFC and amygdala. The lack of positive effects in the hippocampus was not due to changes in general activity levels. There was no significant difference between drugged and non-drugged animals in general activity in shock-probe burying test or in the elevated plus-maze test. Although 20μg of allopregnanolone did cause a decrease in the number of closed arm entries, no change in total entries was observed. Thus, even at this dose, a sedative effect was not obvious. This overall pattern of results does not support the hypothesis that a non-specific drug effect on general activity had masked a true anxiolytic effect.

A second possibility is that the overall volume of infused allopregnanolone was not great enough to interact with a sufficient number of $GABA_A$ receptors in the hippocampus necessary to mediate significant anxiolytic effects in the plus-maze or shock-probe tests. This seems unlikely for two reasons. First, the overall size of the medial prefrontal cortex is at least as large as the dorsal hippocampus, perhaps larger, but the same volumes of allopregnanolone that were effective in the mPFC (those for 14 and 20 μg),

were ineffective in the hippocampus. Second, our infusion procedure itself was designed to mitigate against large differences in the total size of target structures (e.g., amygdala versus hippocampus): i.e., dose was varied by volume, not by concentration (see methods section). Thus, although it is possible that our infusion volumes may have been large enough to produce diffusion away from target structures, they cannot explain the absence of anxiolytic effects within the hippocampus.

A third, more important, explanation of these negative results is that the hippocampus does not mediate the anxiolytic effects of allopregnanolone in the shock-probe and plus-maze tests. However, several findings suggest the contrary. For example, the activation of peripheral mitochondrial benzodiazepine receptors in the dorsal hippocampus increased allopregnanolone synthesis in the hippocampus and decreased anxiety in the plus-maze and the shock-probe burying test (Bitran et al., 2000). Intra-hippocampal infusion of pregnanolone, a precursor of allopregnanolone, induced clear anxiolysis in both the elevated plus-maze and shock-probe burying paradigms (Bitran et al., 1999). Parallel findings with intra-cerebral benzodiazepines show that the hippocampus is also involved in their anxiolytic effects (Gonzalez et al., 1998; Menard & Treit, 2001). Finally, the hippocampus has been shown to be an important structure for other pharmacological effects of allopregnanolone, such as its anti-depressant and anti-convulsant effects (Finn et al., 2005; Nin et al., 2008; Rodriguez-Landa et al., 2005).

It is important to note, however, that in none of the studies reviewed above was allopregnanolone itself micro-infused into the hippocampus and evaluated in animal models of anxiety. A recent study showed that intra-hippocampal infusions of allopregnanolone did not affect anxiety measures in the open-field test (Martin-Garcia and Pallares, 2008). Another study, designed to evaluate the effects of intra-hippocampal nicotine and neurosteroids in

alcohol-drinking rats, showed that intra-hippocampal allopregnanolone did not, on its own, affect rats' fear behavior in the open-field test (see Figures 1 and 2, Martin-Garcia and Pallares, 2005**)**. However, it should be noted that the dose of allopregnanolone used in both of these studies were very low compared to the doses used in the current studies (0.2μg and 0.5μg, respectively).

In light of these negative results, it is also noteworthy that the general literature on the role of the hippocampus in anxiety is not entirely consistent. Some of this inconsistency seems to hinge on the dorsal and ventral aspects of the hippocampus. For example, several researchers have failed to produce significant anxiolytic-like effects when the dorsal hippocampus is the target of permanent lesions (Kjelstrup et al., 2002; McHugh et al., 2004) or temporary inactivation with sodium channel blockers (Degroot & Treit, 2004; McEown and Treit, 2009), $GABA_A$ agonists (Menard & Treit, 2001) or NMDA antagonists (Padovan et al., 2000; Hackl & Carobrez, 2006). Moreover, several researchers have argued that the dorsal aspect of the hippocampus is more involved in memory-related functions, whereas the ventral aspect of the hippocampus mediates fear and anxiety responses (Bertoglio et al., 2006; Hackl & Carobrez, 2006; Pentkowski et al., 2006). Given these general anatomical and functional considerations, our finding that allopregnanolone in dorsal hippocampus does not modulate anxiety-related behaviors is not entirely unexpected. These earlier findings further pertain to the question of whether the null findings from the current study are comparable to the earlier studies reporting positive findings with allopregnanolone precursor infusions into the hippocampus. It should be noted that none of these studies were designed with the idea of exact anatomical distinctions within the hippocampus in mind and infusions into either section of the hippocampus were deemed acceptable in the final analyses. Moreover, the total volume of infusion becomes an important parameter, as it might impose the difference between infusions into a single (e.g., dorsal) aspect of the hippocampus and diffusion into both (i.e., dorsal and ventral) aspects. Clearly, a study that directly compares the anxiolytic-like

effects of allopregnanolone microinfused into the dorsal or ventral hippocampus is needed to properly assess its potential anxiolytic effects in the hippocampus.

Finally, variations in $GABA_A$ receptor subunits may prove to be critical for understanding between-site variations in the anxiolytic properties of allopregnanolone (Bitran et al., 1999). There is evidence, for example, that the γ1 subunit is correlated with an increased efficacy of allopregnanolone at the $GABA_A$ receptor (Puia et al., 1993), whereas the γ2 subtype and/or the δ subtype decrease the effects of allopregnanolone (Zhu et al., 1996). Coincidentally, the γ2 subtype may also be critical for the anxiolytic effects of benzodiazepines (Horne et al., 1993). Taken together, these sorts of data may ultimately explain variations in the anxiolytic effects of allopregnanolone found in the present experiments, as well as benzodiazepines in previous studies (e.g., Pesold and Treit, 1995). Nevertheless, these conjectures must first be placed in the context of specific brain areas that have distinctive distributions of $GABA_A$ receptor subunits, and then related to the site- and test-specific effects of benzodiazepines and neurosteroids in animal models anxiety.

In summary, our studies provide evidence for a role of the amygdala and the mPFC in the anxiolytic-like effects of allopregnanolone, whereas we found no such evidence for the dorsal hippocampus. A possible dissociation in the anxiolytic effects of intra-mPFC allopregnanolone, which we show is anxiolytic in the plus-maze but not the shock-probe test is also suggested. This apparent site-by-test dissociation, as well as the effects of allopregnanolone in the ventral hippocampus, requires future experimental testing.

FIGURES AND TABLES

Figure 3. 1: Two different elevated plus-maze apparatuses.

Figure 3. 2: Basic components of the burying response in the shock-probe burying test:A) The rat contacts the probe and an electric shock is administered. For a brief period afterwards the rat typically exhibits freezing behaviour. B) and C) These pictures illustrate the burying response. The rat uses alternating movements of the forepaws to spray bedding material onto the shock-probe. D) The probe has been covered completely with the bedding material.

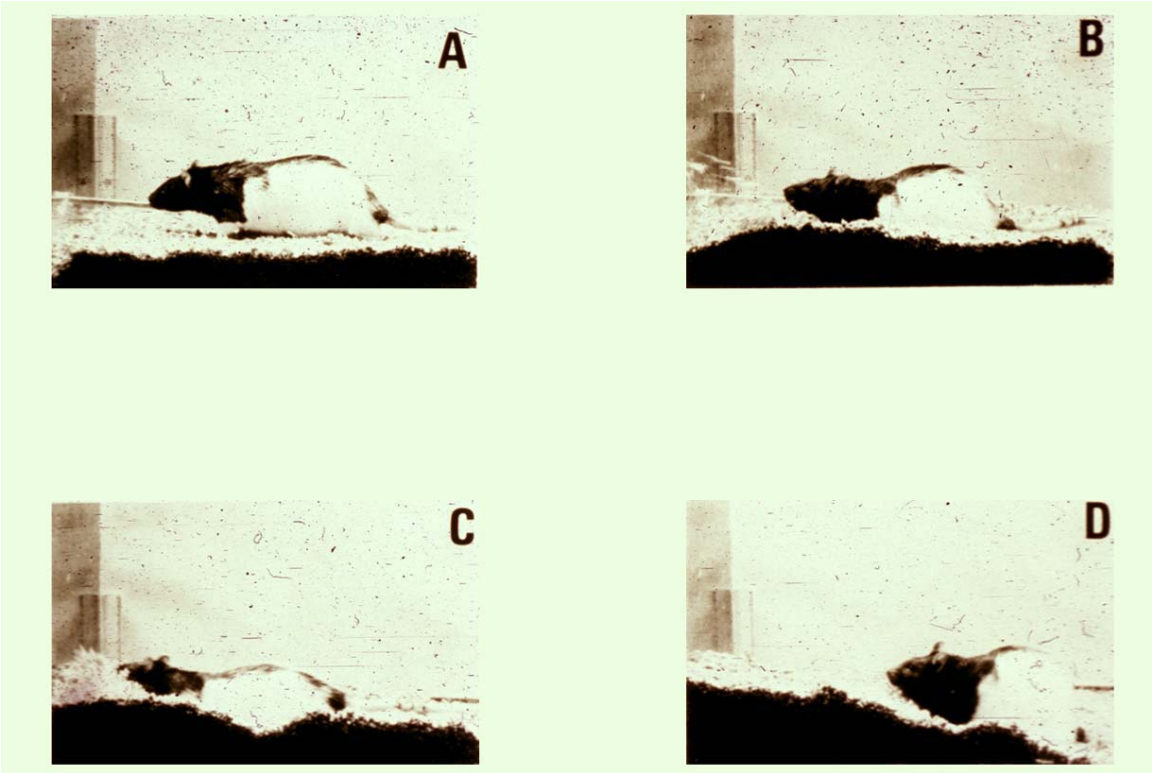
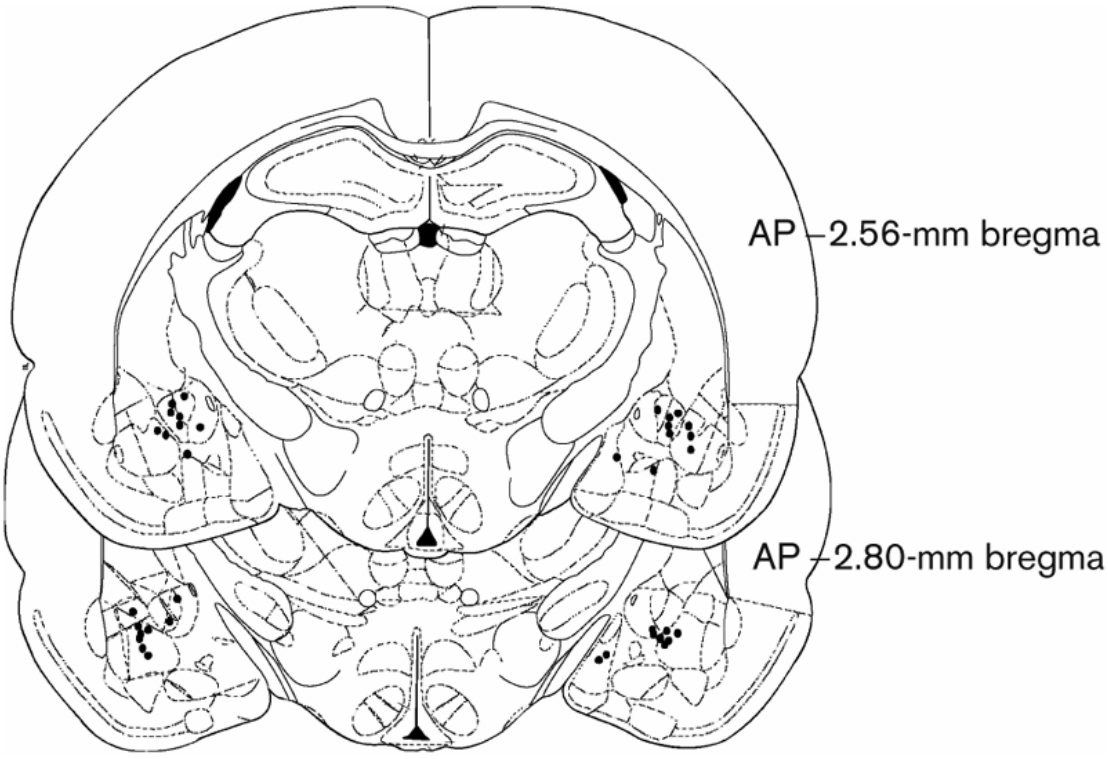
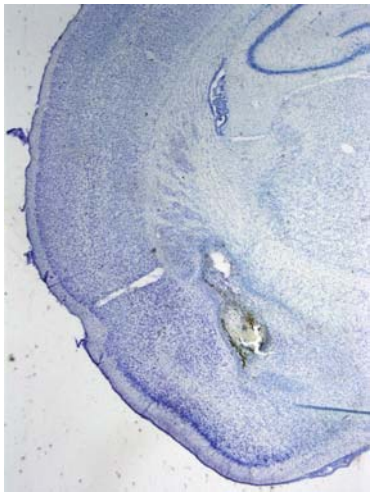


Figure 3. 3: Schematic diagram of coronal rat brain sections illustrating the location of the ventral tip of internal infusion cannulae included in Experiment 1. The atlas plates are adapted from Paxinos and Watson (1986).





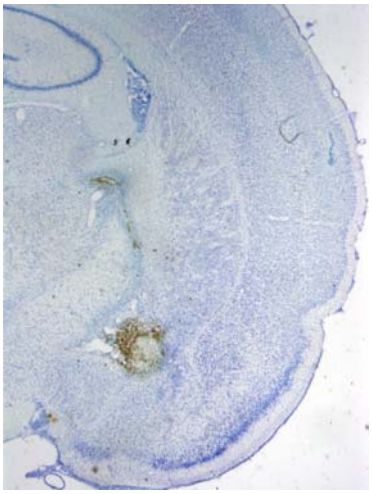
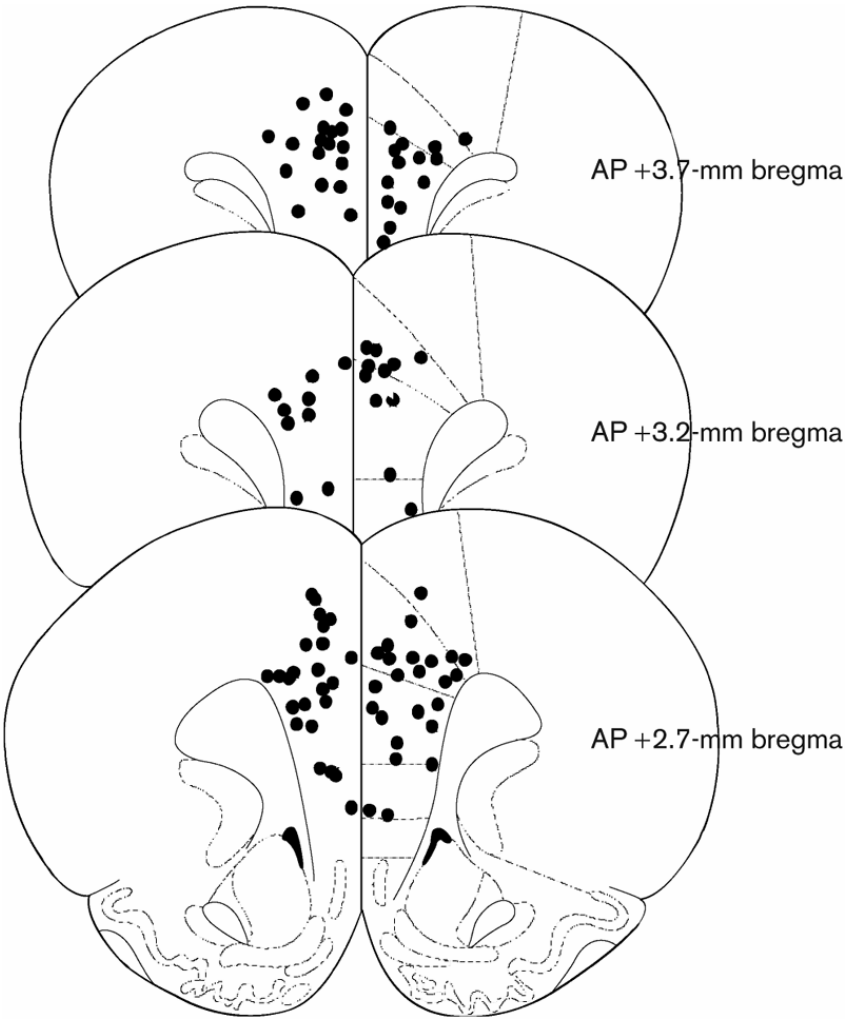


Figure 3. 4: Schematic diagram of coronal rat brain sections illustrating the location of the ventral tip of internal infusion cannulae for rats with mPFC infusions included in the present study. The atlas plates are adapted from Paxinos and Watson (1986). mPFC, medial prefrontal cortex.



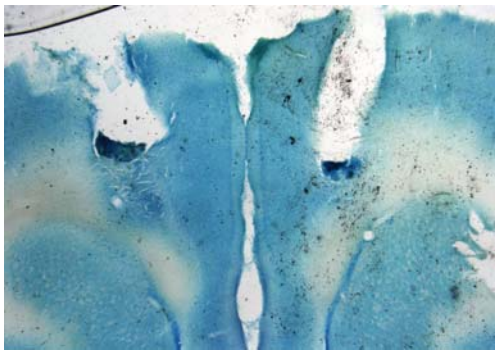
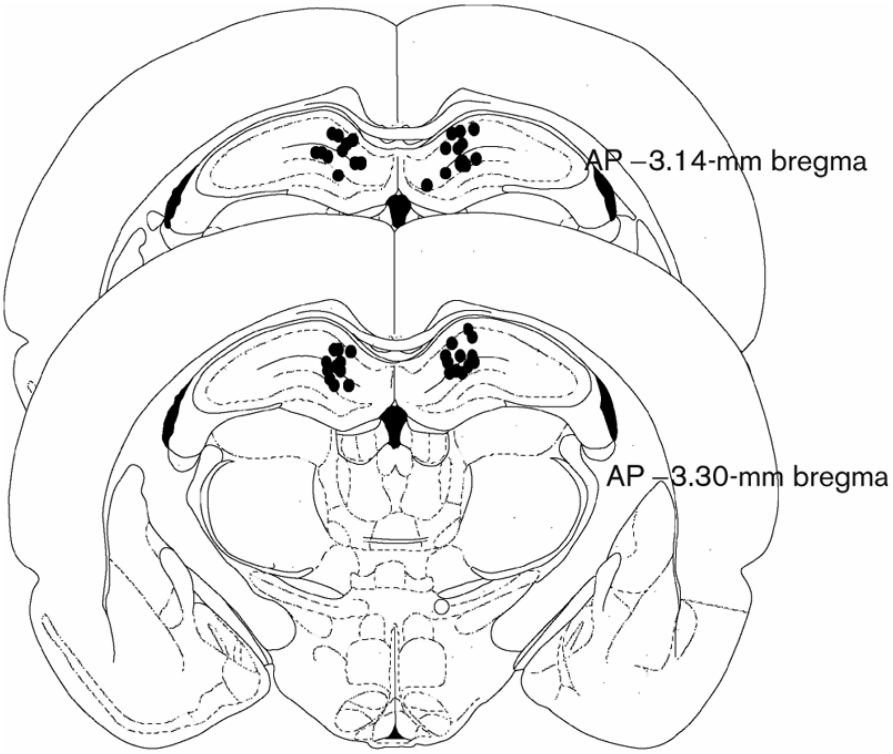


Figure 3. 5: Schematic diagram of coronal rat brain sections illustrating the location of the ventral tip of internal infusion cannulae for rats with hippocampal infusions included in this study. The atlas plates are adapted from Paxinos and Watson (1986).



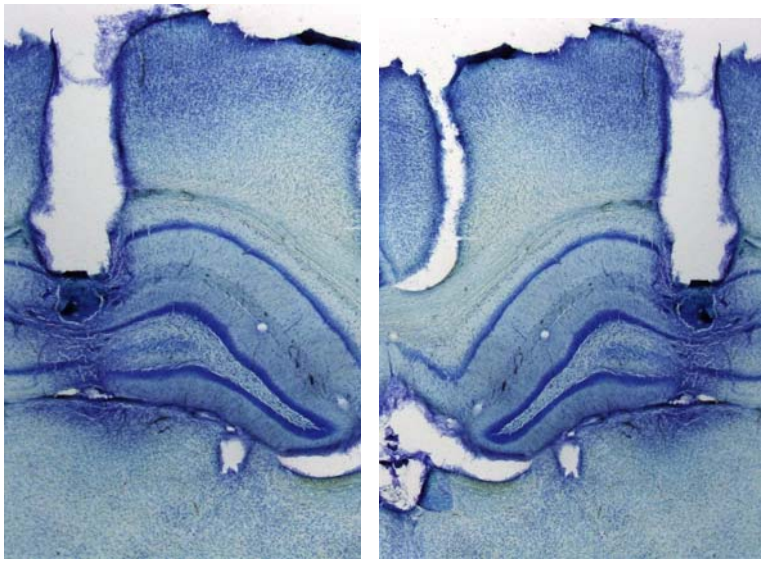


Figure 3. 6: A. The mean percentage of open arm entries (±SEM) in rats microinfused with allopregnanolone into the amygdala, mPFC or dorsal hippocampus. B. The mean percentage of open-arm time (±SEM) in rats microinfused with allopregnanolone into the amygdala, mPFC or dorsal hippocampus, *P<0.05. (Allo, Allopregnanolone); mPFC, medial prefrontal cortex.

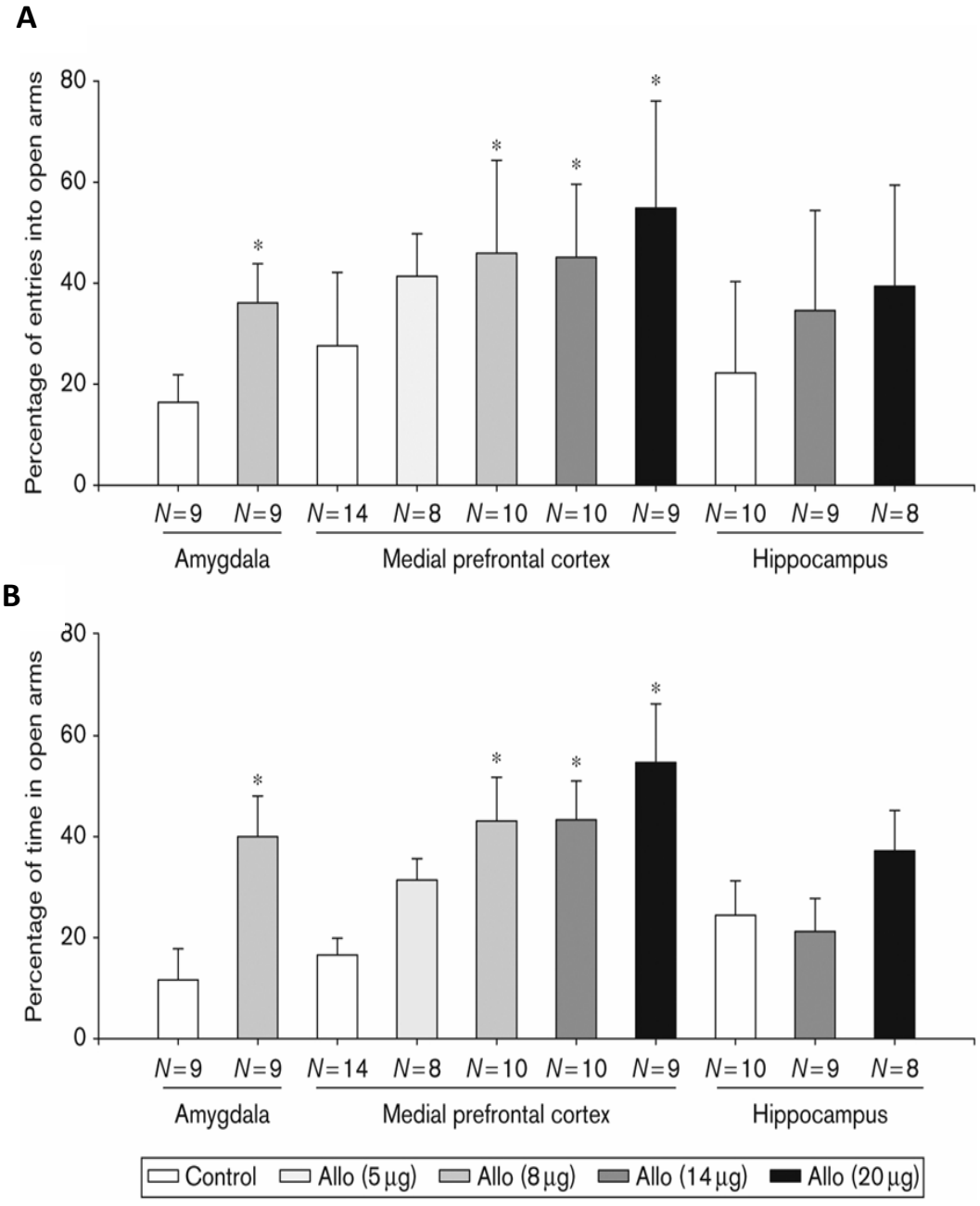


Figure 3. 7: The mean number of probe contacts (±SEM) in rats microinfused with allopregnanolone into the amygdala, mPFC or dorsal hippocampus, *P<0.05. Allo, Allopregnanolone; mPFC, medial prefrontal cortex.

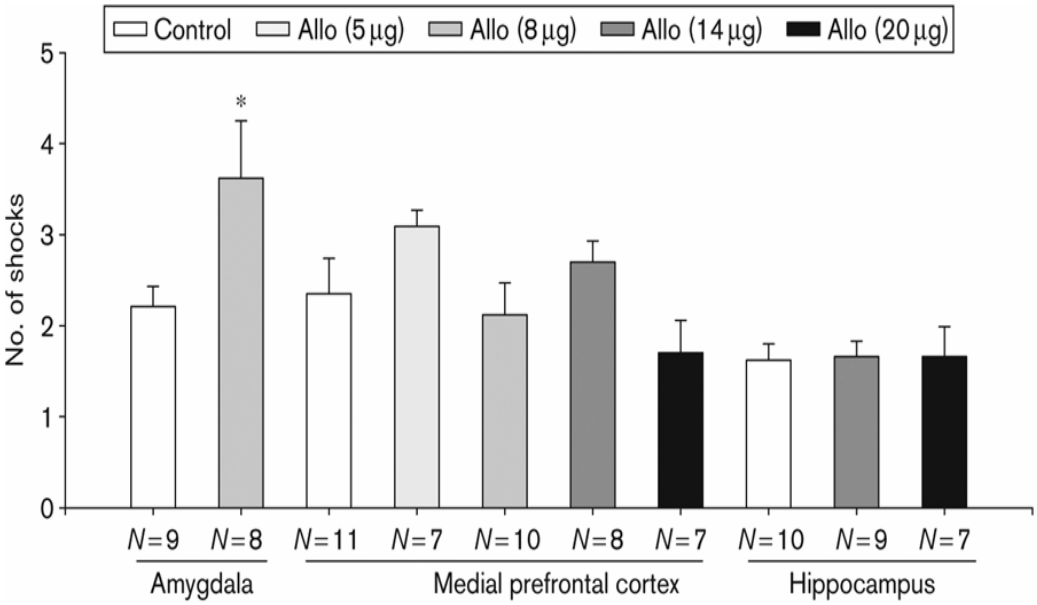


Table 3. 1: Shock-probe burying data for rats microinfused with allopregnanolone into the amygdala, mPFC or dorsal hippocampusAllo, Allopregnanolone; mPFC, medial prefrontal cortex.

	Control	Allo (5 µg)	Allo (8 µg)	Allo (14 µg)	Allo (20 µg)
Amygdala					
Number of participants	9	–	8	–	–
Burying time (s)	49.33 ± 30.28	–	47.38 ± 24.43	–	–
Still time	58.56 ± 22.90	–	34.38 ± 29.57	–	–
Shock reactivity	1.41 ± 0.15	–	1.73 ± 0.17	–	–
Medial prefrontal cortex					
Number of participants	10	7	10	8	7
Burying time (s)	4.82 ± 2.94	1.71 ± 1.41	63.80 ± 44.32	4.88 ± 3.10	0.00 ± 0.00
Still time	127.10 ± 64.60	143.00 ± 34.55	173.70 ± 45.84	149.00 ± 48.35	234.00 ± 95.38
Shock reactivity	1.40 ± 0.20	1.71 ± 0.43	1.80 ± 0.22	1.44 ± 0.12	1.22 ± 0.09
Dorsal hippocampus					
Number of participants	8	–	–	9	9
Burying time (s)	1.63 ± 0.18	–	–	1.67 ± 0.17	1.67 ± 0.33
Still time	17.88 ± 13.72	–	–	62.22 ± 42.15	12.11 ± 4.77
Shock reactivity	1.44 ± 0.20	–	–	2.00 ± 0.34	1.64 ± 0.24

Allo, Allopregnanolone; mPFC, medial prefrontal cortex.

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CHAPTER 4

DISSOCIATION OF THE ANXIOLYTIC-LIKE EFFECTS OF AVPR1A AND AVPR1B RECEPTOR ANTAGONISTS IN THE DORSAL AND VENTRAL HIPPOCAMPUS

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1. Introduction

Arginine-vasopressin (AVP) is a nonapeptide synthesized in the supraoptic and paraventricular nuclei of the hypothalamus. AVP is released into the general circulation through the posterior pituitary, causing effects such as vasoconstriction, increased blood pressure and water reabsorption in the kidneys. In addition to these peripheral effects, AVP has central effects mediated by extrahypothalamic sites (e.g., medial amygdala; bed nucleus of the stria terminalis), which synthesize AVP and project to brain areas such as the lateral septum, hippocampus, olfactory tubercle, locus coeruleus and dorsal raphe nucleus (Cooper et al., 2003; Frank and Landgraaf, 2008; Ring, 2005).

The activities of AVP are mediated through a family of g-protein coupled receptors. Of these, the Avpr2 receptor is expressed mostly in the kidney and has very limited expression elsewhere. Two other vasopressin receptor subtypes are found in various parts of the brain and mediate the central actions of AVP. The Avpr1a receptor is expressed in several areas including the hippocampus, the central nucleus of the amygdala, lateral septum, cerebellum and several hypothalamic nuclei (Ostrowski et al., 1994; Szot et al., 1994). The Avpr1b receptor is also found at many of the same nuclei, as well as the cingulate and frontal cortices (Hernando et al., 2001). Thus, there is considerable anatomical overlap between the Avpr1a and Avpr1b vasopressinergic receptor systems throughout the brain.

Irregularities of the central and peripheral AVP systems have been observed in patients with depressive disorders, and anxiety disorders (Surget and Belzung, 2008) . For instance, there is a decrease in the concentration of AVP in both cerebrospinal fluid (Gjerrnis et al., 1985) and blood plasma in patients with major depression (Laruelle et al., 1990). Similar decreases in cerebrospinal fluid concentrations of AVP have also been observed in patients with obsessive-compulsive disorder (Swedo et al., 1992). On the other hand, post-traumatic stress disorder, manic depression and ‘anxious’ depression are

associated with increased AVP concentrations (deKloet et al., 2008; de Winter et al., 2003; Legros et al., 1993). Finally, postmortem studies of patients with bipolar depression and patients with 'melancholic' depression have revealed elevated levels of AVP-containing neurons in the paraventricular nucleus of the hypothalamus (e.g., Meynen et al., 2006; Purba et al., 1996).

Findings from animal studies show that AVP mRNA expression and release is greater in rats bred for high anxiety than in controls (Landgraf and Wigger, 2002; Wigger et al., 2004), and higher in normal rats after acute stress (Ebner et al., 1999; Engelmann et al., 2000; Veenema et al., 2006; Wotjak et al., 1996). Direct manipulation of AVP in the brain showed that AVP infusion (i.c.v.) increased anxiety-like behavior in animal models of anxiety (Bhattacharya et al., 1998; McCarthy et al., 1996). Conversely, antisense antagonism of Avpr1 receptor binding in the septum decreased anxiety-like behavior (Landgraf et al., 1995). There are also reports, however, that intracerebral infusion of AVP failed to increase anxiety-like behavior in animal models, and in some instances, even decreased anxiety (e.g., Appenrodt et al., 1998; Appenrodt and Schwarzberg, 2000; Liebsch et al., 1996; Winslow and Insel, 1993). These findings are particularly surprising, considering that AVP acts synergistically with corticotrophin releasing factor (CRF) to release adrenocorticotropic hormone (ACTH; Cooper et al., 2003). Thus, it is not entirely clear the manner in which AVP affects anxiety, although it seems likely that some of the behavioral variability may be related to activity at specific AVP receptor subtypes in particular parts of the brain.

The recent introduction of selective ligands for Avpr1a and Avpr1b receptors has made it possible to study the receptor subtypes that mediate the various effects of AVP on anxiety and depression (Lemmens-Gruber and Kamyar, 2006). Peripheral administration of the selective Avpr1b antagonist SSR149415 has anxiolytic and antidepressant effects in several different animal models, after both acute and chronic administration (Griebel et al.,

2002; Iijima and Chaki, 2005; Louis et al., 2006; Serradeil – le Gal et al., 2002; Serradeil –le Gal et al., 2003). In addition, both Avpr1b (Scattoni et al., 2008) and Avpr1a (Bielsky et al., 2004; 2005; Egashira et al., 2007; Lolait et al., 2007) knock-out mice are less reactive to stress stimuli. Avpr1a binding in the brain is positively correlated with the intensity of isolation-potentiated startle, an animal model of anxiety (Nair et al., 2005). Interestingly, individual differences in Avpr1a receptor binding in the thalamic nuclei of prairie voles are strongly associated with anxiety-related behaviors (Hammock and Young, 2005). Although there are no studies to our knowledge that have examined the effects on anxiety-like behavior of intracerebral administration of selective Avpr1a agonists or antagonists, these findings alone suggest that both Avpr1a and Avpr1b receptors mediate at least some aspects of anxiety. Finally, microinfusion of the Avpr1b antagonist SSR149415 into the lateral septum or the amygdala had reliable antidepressant-like effects in animal models, although its anxiolytic-like effects were mixed (Salome et al., 2006; Stemmelin, 2005).

AVP receptors are found in the hippocampus, an area that has been previously implicated in anxiety (Engin and Treit, 2007). Both Avpr1a and Avpr1b receptors are highly expressed in cell bodies of the hippocampal formation (Hernando et al., 2001; Ostrowski et al., 1992; 1994; Szot et al., 1994; Vaccari et al., 1998; Young et al., 2006). However, it should be noted that the hippocampal formation is far from being a uniform, single structure. There is evidence that the dorsal and ventral hippocampus play different roles in the control of anxiety-related behaviors (Engin and Treit, 2007). Studies show that the dorsal aspect of the hippocampus may be more involved in memory-related functions, whereas the ventral aspect may be specialized for anxiety-related functions (Bannerman et al., 2004), however, contradictory findings have also been reported (e.g., Gonzalez et al., 1998; Menard and Treit, 2001) and the overall evidence is far from conclusive. This makes the

hippocampus an ideal target for the investigation of anxiety-related effects of AVP and its two receptor sub-types.

Thus, the purpose of the present study was twofold: first, and more generally, to document the putative roles of centrally-located Avpr1a and Avpr1b receptors in anxiety-related behavior; and second, more specifically, to determine whether the dorsal and ventral hippocampus differentially mediate the anxiolytic-like effects of selective Avpr1a and Avpr1b antagonists.

With these points in mind, we microinfused a selective Avpr1a antagonist ([β-Mercapto-β,β-cyclopentamethylenepropionyl1, O-me-Tyr2, Arg8]-AVP) and a selective Avpr1b antagonist (SSR149415), into either the dorsal or the ventral hippocampus, and observed the effects in two animal models of anxiety: the elevated plus maze test and the shock probe burying test.

2. General Methods

2.1. Subjects

Subjects were male Sprague - Dawley rats, weighing 200-350 g at the time of surgery. The rats were maintained on a 12:12 h light/dark cycle (lights on at 0700 h) and individually housed in polycarbonate cages for the duration of the experiment. 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. The rats were randomly assigned to surgery conditions (dorsal or ventral hippocampus) just prior to surgery.

2.2. Surgery

The general surgical and intracerebral microinfusion procedures were similar to those explained in Chapter 3. The rats were injected with atropine sulfate (0.1 mg/0.2 ml ip), and anaesthetized with sodium pentobarbital (Nembutal 50 mg/kg, ip; Abbot Laboratories, Canada). Following hydration

with 0.9% saline (3 cc, ip), they were placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA), and an incision was made to expose the skull. The rats were bilaterally implanted with Stainless-steel 22-gauge guide cannulae (Plastics One, Roanoke, VA, USA) targeting the dorsal (AP: -3.1, ML:-2.7, DV:-2.6) or ventral (AP:-5.2, ML:-5.2, DV:-5.8) hippocampus. The cannulae were secured to the skull with four jeweler's screws and cranioplastic cement. A dummy cannula was inserted into each guide cannula in order to keep the cannula tract clear. Following the surgery, the subjects were placed in a warm environment, until they regained consciousness. They were then allowed to recover for at least 5 days in their home cages before the start of behavioral testing.

2.3. Infusion procedure

The infusion procedure was similar to that used in Chapter 3. Rats from each surgery group were randomly assigned to a vehicle control condition, an Avpr1a antagonist condition or an Avpr1b antagonist condition. The Avpr1a antagonist, [β-Mercapto-β,β-cyclopentamethylenepropionyl1, O-me-Tyr2, Arg8]-AVP (Sigma, St. Louis, MO, USA), was dissolved in 0.9% saline at concentrations of 10ng / 0.5µl or 200ng / 0.5µl. The Avpr1b antagonist, SSR149415 (Sanofi-Synthelabo, Montpellier, France), was dissolved in a vehicle of 0.9% saline containing 5% dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA) and 5% Cremophor (Sigma, St. Louis, MO, USA) at a concentration of 10 ng / 0.5µl or 200ng / 0.5µl. The DMSO vehicle mixture has no neurotoxic effects (Santos et al., 2003) and has no anxiety-related effects in comparison to saline (Stemmelin et al., 2005). The 10ng and 200ng doses were chosen on the basis of significant behavioral effects found in animal models of depression (Salome et al., 2006; Stemmelin et al., 2005), and on the basis of our own pilot experiments.

The drugs or the vehicles were infused via an infusion pump (Harvard Apparatus 22, MA, U.S.) at a rate of 0.5µl/min. Rats received 0.5 µl/side, through 26-gauge stainless-steel internal cannulae attached to a 10-µl

Hamilton syringe by polyethylene tubing. The internal infusion cannulae extended 5mm below the dorsal tip of the guide cannula for dorsal hippocampal infusions and 7mm below the tip for ventral hippocampal infusions. 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 employed in Chapter 3. Testing started 15 min after the end of infusion procedure. The subjects were assigned to the same drug treatment group (Avpr1a antagonist, Avpr1b antagonist or vehicle) for both behavioral tests. Animals were allowed six days between the plus maze and shock-probe tests.

2.4.1. Elevated plus-maze

The plus-maze apparatus and the testing procedure were the same as those reported in Chapter 3. See section 2.4.1 of Chapter 3 for details.

2.4.2. Shock-probe burying

Two days after the elevated plus-maze test, habituations for the shock probe test started. The shock-probe chamber and the testing procedure were the same as that reported in Chapter 3. See section 2.4.2 of Chapter 3 for details.

2.5. Histology

Following behavioral testing, rats were euthanized with an overdose of sodium pentobarbital (Nembutal) and intracardially perfused 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 (approximately between AP coordinates –2.0 and - 6.0) 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. As a result of microscopic examination of the brain

sections, 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 and shock-probe tests were expressed as means and standard errors of the mean (S.E.M.). Each behavioral measure was analyzed with a between- groups, one-way analysis of variance (ANOVA; α=0.05). If the overall F-test was significant, post hoc LSD tests were used to compare the effects of different doses of the Avpr1a or Avpr1b antagonist to the respective vehicle control condition (α=0.05).

3. Results

3.1 Experiment 1: Avpr1a and Avpr1b antagonist microinfusions into the dorsal hippocampus

3.1.1. Histology

The approximate infusion sites of the dorsal hippocampal cannulae included in the behavioral analysis are depicted in Figure 4.1. Eleven animals were excluded from the analyses as a result of misplaced cannulae.

3.1.2. Elevated Plus-Maze

The percentage of open-arm entries and percentage of open-arm time for the Avpr1a and Avpr1b antagonist infusions are presented in Table 4.1 and Figures 4.3.A – 4.3.B, respectively. As seen in Table 4.1, microinfusion of the Avpr1a antagonist into the dorsal hippocampus did not affect any of the behavioral measures in the elevated plus-maze, regardless of the dose applied. In contrast, as seen in Figures 4.3.A and 4.3.B, microinfusion of the Avpr1b antagonist into the dorsal hippocampus increased both the percentage of open-arm entries ($F(2, 29) = 8.92$; $p<0.01$) and the percentage of open-arm time ($F(2, 29) = 9.49$; $p<0.01$). Post-hoc LSD tests showed that significant increases in both measures occurred at both doses of the drug (10ng/side and 200ng/side, p's<.05). The total number of entries into any of the arms was not affected by

any of the drug infusions ($p>0.35$; Table 4.1). The 10ng/side dose of the Avpr1b antagonist decreased the total number of closed arm entries ($F(2, 29) = 5.33$, $p<0.01$; Table 4.1), although the 200ng/side dose did not (LSD tests, $p=0.05$).

3.1.3. Shock-Probe Burying

The shock-probe burying results are depicted in Table 4.2. The Avpr1a antagonist infusions into the dorsal hippocampus did not affect burying time, general activity levels, or shock reactivity. The Avpr1a antagonist did, however, reduce the number of contact-induced shocks at the high (200ng/side) dose ($F(2,23) = 5.51$, $p<0.01$;LSD, $p<0.05$). Avpr1b antagonist infusions into the dorsal hippocampus did not significantly affect any of the behavioral measures in the shock-probe burying test (Table 4.2).

3.2 Experiment 2: Avpr1a and Avpr1b antagonist microinfusions into the ventral hippocampus

3.2.1. Histology

The approximate infusion sites of the ventral hippocampal cannulae included in the behavioral analysis are depicted in Figure 4.3. Five animals were excluded from the analyses as a result of misplaced cannulae.

3.2.2. Elevated Plus-Maze

The plus-maze data are depicted in Figure 4.4.A-4.4.B and in Table 4.3. In direct contrast to its null effects in the dorsal hippocampus, microinfusion of the Avpr1a antagonist into the ventral hippocampus increased both the percentage of open-arm entries ($F(2, 26) = 3.61$; $p<0.05$; Figure 4.4.A) and the percentage of open-arm time ($F(2, 26) = 3.98$; $p<0.05$; Figure 4.4.B). Significant increases in both of these measures occurred at the 200ng dose, but not at the 10ng dose (LSD tests $p<0.05$). The number of closed arm entries and the total arm entries were not affected by any of the drug treatments ($p>0.38$ and $p>0.11$, respectively; Table 4.3).

As seen in Table 4.3, in contrast to its significant effects in the dorsal hippocampus in Experiment 1, microinfusion of the Avpr1b antagonist into the

ventral hippocampus did not affect any of the anxiety or activity measures in the elevated plus-maze, regardless of the dose applied. Thus, the anxiolytic-like effects of the two AVP antagonists in the plus-maze were directly dependent on infusion site: dorsal versus ventral hippocampus.

3.2.3. Shock-Probe Burying

The findings from the shock-probe burying test are depicted in Table 4.4. Microinfusions of neither the Avpr1a nor the Avpr1b antagonist into the ventral hippocampus affected the anxiety measures in the shock-probe burying test (duration of burying, number of shock-probe contacts). Furthermore, unlike infusions into the dorsal hippocampus, neither antagonist affected general activity levels when infused into the ventral hippocampus. On the other hand, 200ng/side infusion of the Avpr1a antagonist significantly reduced the animal's reactivity to the shocks as compared to the vehicle control group ($F(2, 26) = 3.84$, $p<0.05$; LSD, $p<0.05$). No similar effect was observed with the Avpr1b antagonist (Table 4.4).

4. Discussion

Above results show that antagonism of Avpr1b receptors in the dorsal hippocampus produced significant anxiolytic-like effects in the elevated plus-maze, whereas Avpr1b receptor antagonism in the ventral hippocampus had no significant effect. Conversely, Avpr1a receptor antagonism in the ventral hippocampus produced significant anxiolytic-like effects in the elevated plus-maze, but antagonism of Avpr1a receptors in the dorsal hippocampus had no significant effect. None of these significant effects in the plus-maze were confounded by changes in control measures such as general activity (see tables 4.1-4.4). Overall, the plus-maze data suggest a complete, double dissociation of the anxiolytic effects of Avpr1a and Avpr1b antagonists in the dorsal and ventral hippocampi. Infusion of these antagonists into either site had no significant anxiolytic-like effects in the shock-probe burying test. This is the

first demonstration of 1) the anxiolytic-like effects of selective AVP receptor antagonists in the hippocampus, and 2) the differential mediation of the anxiolytic-like effects of Avpr1a and Avpr1b receptor antagonists by the dorsal and ventral hippocampus.

Because of the relatively large separation between the dorsal and ventral hippocampal targets, it is unlikely that drug diffusion played a significant role in the behavioral effects of Avpr1a and Avpr1b receptor antagonists in the present experiments. Furthermore, the doses of the Avpr1a and Avpr1b antagonists chosen were behaviorally active and sufficient to produce significant anxiolytic-like effects in the elevated plus-maze. No dose of either antagonist produced an anxiogenic-like effect.

Nevertheless, the present results do not rule out possible interactive effects between the dorsal and ventral hippocampus mediated through their extensive interconnections, or the recruitment of outside structures that are also interconnected and involved in anxiety-like behavior, such as the septum or amygdala (Degroot and Treit, 2004). Two studies are important in this regard. First, Stemmelin et al., (2005) found no evidence that the Avpr1b antagonist SSR149415 had anxiolytic-like effects in the plus-maze or punished drinking tests when microinfused into the lateral septum. Thus, the anxiolytic-like effects of SSR149415 found in the dorsal hippocampus were not likely the indirect consequence of vasopressinergic interactions with the lateral septum. Second, Salome et al., (2006) found that SSR149415 was anxiolytic in the plus-maze when microinfused into the basolateral (but not the central or medial) nucleus of the amygdala. In this case, however, the absence of a clearly-defined pathway between the dorsal hippocampus and the amygdala makes it unlikely that interactions between the two could explain the anxiety-related effects seen in the present experiments (Degroot and Treit, 2004; Pikkarainen et al., 1999). A more parsimonious explanation of our results is that the relative densities of the two AVP receptor subtypes differ in such a

way that they map on to the behavioral effects we found (e.g., Avpr1b receptor density is much greater in the dorsal hippocampus and Avpr1a receptor density is much greater in ventral hippocampus). Although this possibility deserves investigation, we are not aware of any significant differences in AVP receptor subtype densities in the two hippocampal regions.

There were no significant anxiolytic effects in the shock-probe burying test, perhaps reflecting the differential sensitivity of these tests to the anxiolytic effects of intra-hippocampal AVP receptor antagonism (for similar examples see Menard and Treit, 1999). Another possibility is that drug effects in the elevated plus-maze carried over to the shock-probe burying test, obscuring anxiolytic effects that would otherwise have occurred in the latter test. On the other hand, we have repeatedly shown that intracerebral infusion of standard anxiolytics such as midazolam have significant anxiolytic-like effects in both tests, using the same experimental design used here (e.g., Pesold, 1994; 1996; Shah and Treit, 2004). There were, however, subtle behavioral changes in the shock-probe test that bear discussion. For example, infusion of the Avpr1a antagonist into the dorsal hippocampus in Experiment 1 did result in greater passive avoidance of the electrified probe, compared to vehicle-infused controls, which resembles an anxiogenic-like effect (Table 4.2). While it is possible that this result was actually due to an increase in pain sensitivity, or to a reduction in general activity, neither pain sensitivity nor general activity was significantly affected in this experiment (see Table 4.2). The Avpr1a antagonist actually *reduced* shock-reactivity in the second experiment, which would indicate a decrease in pain sensitivity (Table 4.4). Another possibility is that the facilitation of shock-probe avoidance by the Avpr1a antagonist in the dorsal hippocampus reflected enhanced learning or memory (Gaffori and De Wied, 1986). It should be noted, however, that AVP itself appears to *facilitate* conditioned avoidance learning (Skopkova et al., 1991). Therefore, if anything AVP receptor antagonists should impair shock-probe avoidance. But in fact, neither of the AVP antagonists impaired shock-probe avoidance in the present

experiments (Tables 4.2 and 4.4). Furthermore, any effect of the Avpr1a antagonist on learning and memory might also be expected to affect burying behavior, which it did not. Thus, it is unlikely that the reduction in the number of shocks received was due to a learning effect. Whether or not this finding represents a genuine "anxiogenic" effect of the Avpr1a antagonist requires further study.

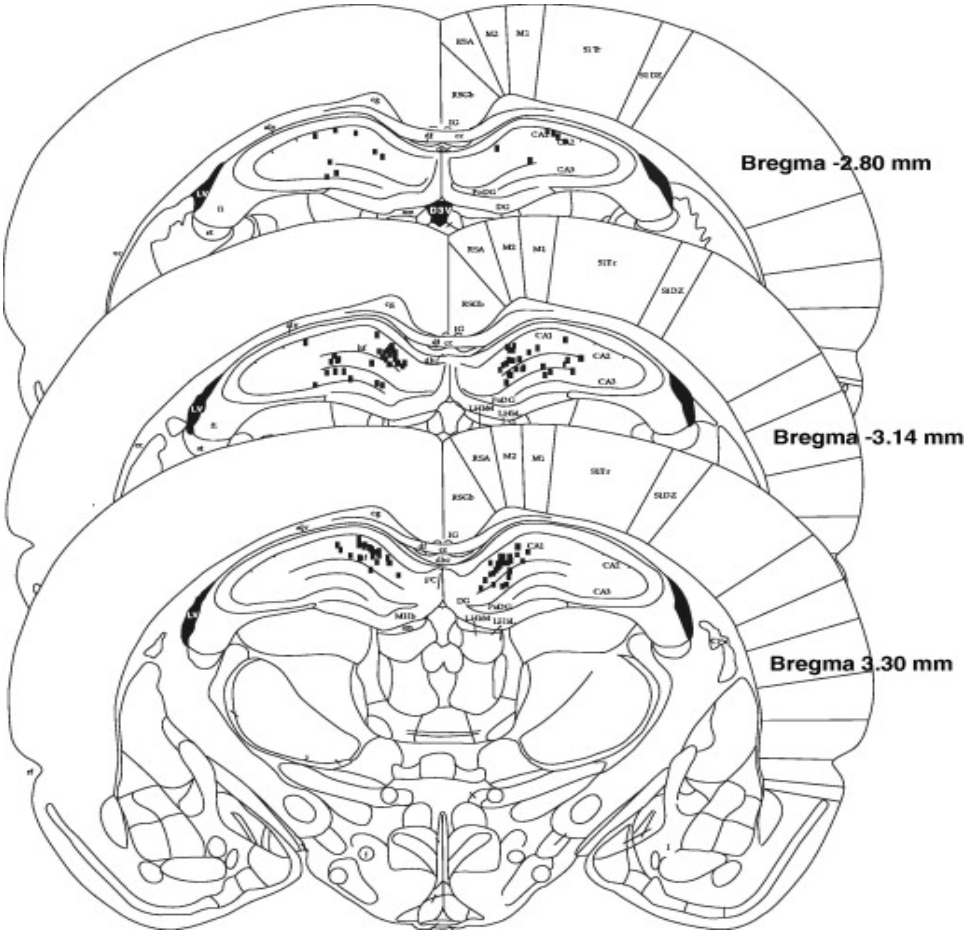
Several reports show that the permanent lesions of the dorsal hippocampus have relatively weak anxiolytic-like effects on animals' untrained anxiety reactions, compared with lesions of the ventral hippocampus, which produce relatively robust anxiolytic-like effects (Kjelstrup et al., 2002; Degroot and Treit, 2004; McHugh et al., 2004; Bertoglio et al., 2006; Pentkowski et al., 2006). Conversely, lesions of the dorsal hippocampus have fairly consistent effects in tasks that involve associative learning and memory, compared with lesions of the ventral hippocampus (Bannerman et al., 2002; McHugh et al., 2004; McNaughton and Corr, 2004; Burman et al., 2006; Pentkowski et al., 2006). This has led to the general hypothesis that the dorsal hippocampus is specialized for memory functions, whereas the ventral hippocampus is dedicated to anxiety functions (e.g. Bannerman et al., 2004). Our results, however, show that both the dorsal and ventral hippocampus are importantly involved in untrained anxiety responses, but the level of this involvement depends critically on specific AVP receptor sub-types (Avpr1a or Avpr1b) located in each of these sub-regions. In addition, our results are consistent with other studies which show that non-AVP related ligands (GABAergic, serotonergic and cholinergic) microinfused into the dorsal hippocampus can also produce reliable, anxiolytic-like effects (Engin and Treit, 2007). Thus, the effects of selective blockade of specific receptor systems within the dorsal hippocampus differ from an overall ablation. While our results in no way undermine the cognitive functions of the dorsal hippocampus, they do add to a growing literature that suggests a variety of peptide and non-peptide receptor

systems within the dorsal hippocampus are involved in the modulation of anxiety-like behavior.

In summary, the present findings extend those of previous studies showing that AVP ligands have anxiety-related effects in animal models when administered peripherally or via the cerebral ventricles (e.g., Bhattacharya et al., 1998). Furthermore, they suggest that the hippocampus may be a nodal point for the anxiety-related effects of AVP. While supporting previous suggestions that the anxiolytic-like effects of AVP antagonists are mediated through Avpr1b receptors (Salome et al., 2006), the present results also demonstrate that Avpr1a antagonists can have centrally-mediated anxiolytic-like effects. Finally, the present study suggests that the anxiolytic effects of both Avpr1a and Avpr1b receptor antagonists depend critically on the sub-region of the hippocampus into which they are infused.

FIGURES AND TABLES

Figure 4. 1: Schematic diagram of coronal brain sections illustrating the location of the ventral tip of the internal infusion cannulae of rats included in the Experiment 1 (Dorsal hippocampus infusions). The atlas plates are adapted from (Paxinos and Watson (1986).



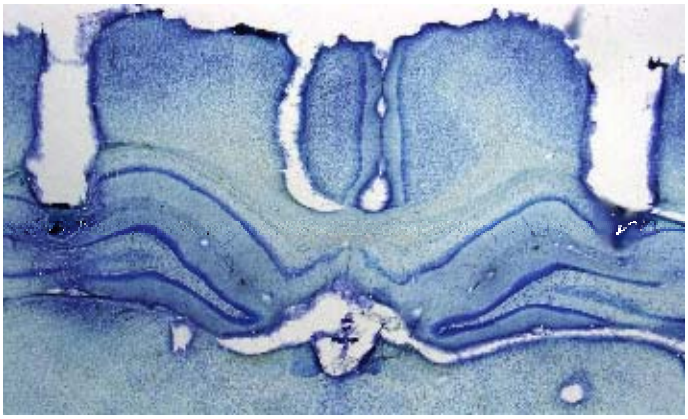


Figure 4. 2: A. The mean percentage of open-arm entries (±SEM) of rats microinfused with the Avpr1b antagonist into the dorsal hippocampus. *Significantly different from the vehicle control group ($p < 0.01$). B. The mean percentage of open-arm time (±SEM) of rats microinfused with the Avpr1b antagonist into the dorsal hippocampus. *Significantly different from the vehicle control group ($p < 0.01$).

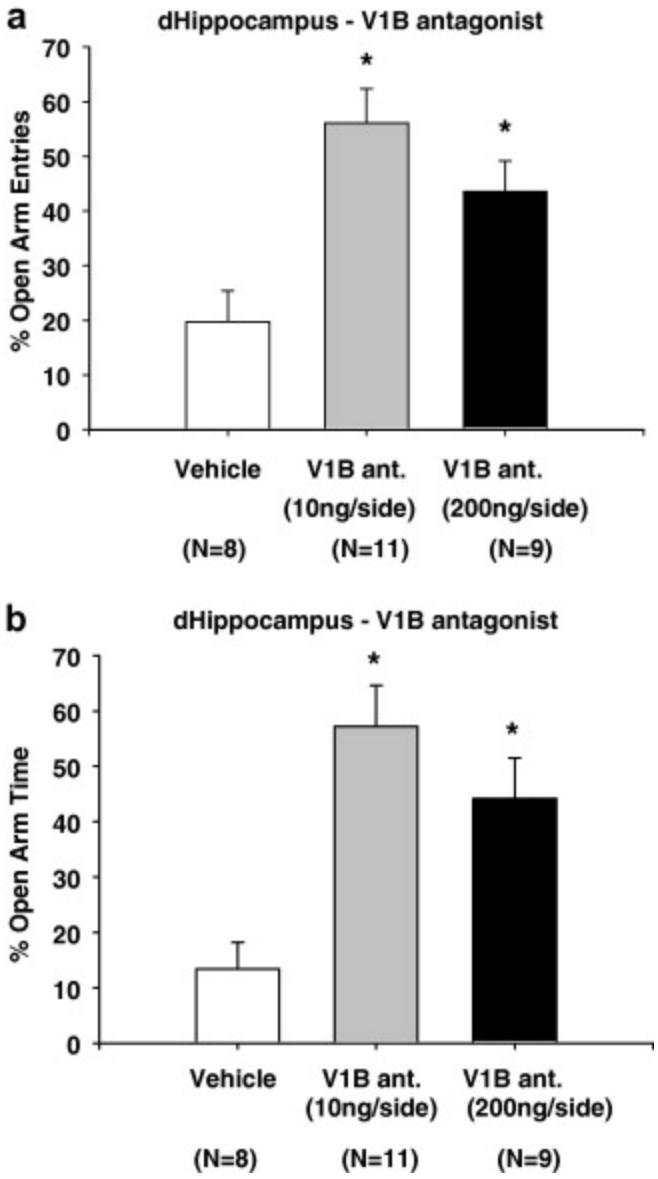
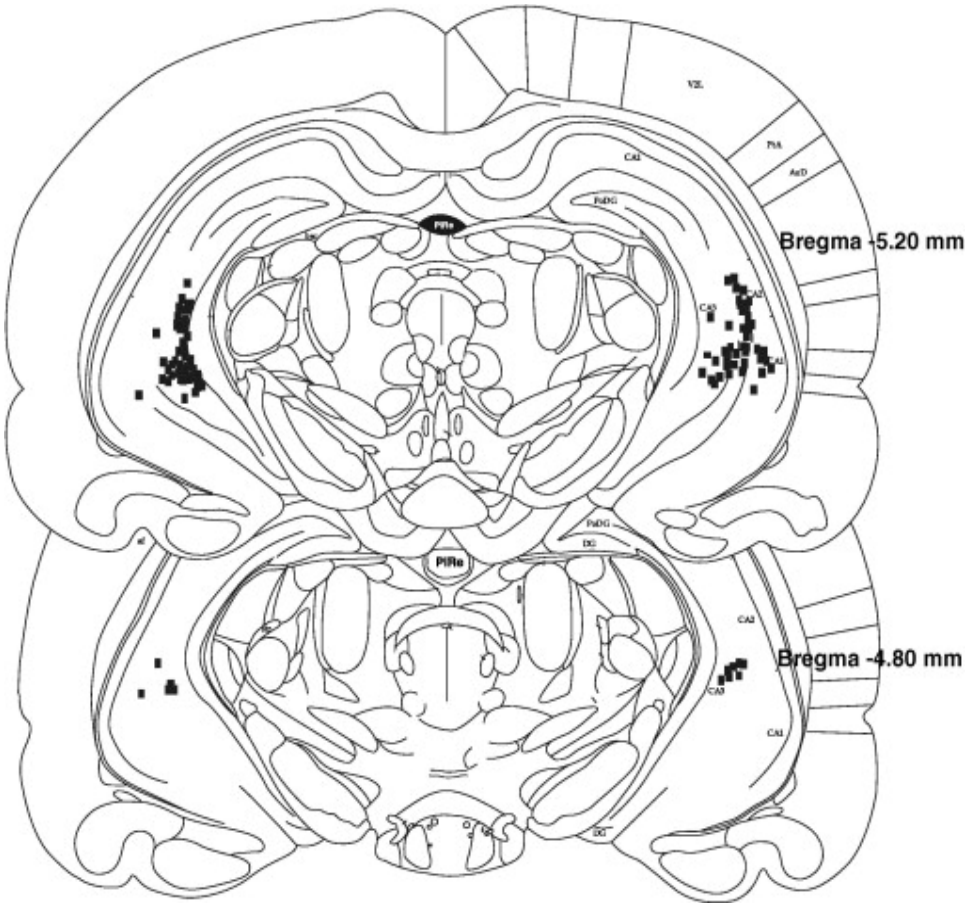


Figure 4. 3: Schematic diagram of coronal brain sections illustrating the location of the ventral tip of internal infusion cannulae of rats included in the Experiment 2 (Ventral hippocampus infusions). The atlas plates are adapted from Paxinos and Watson (1986).



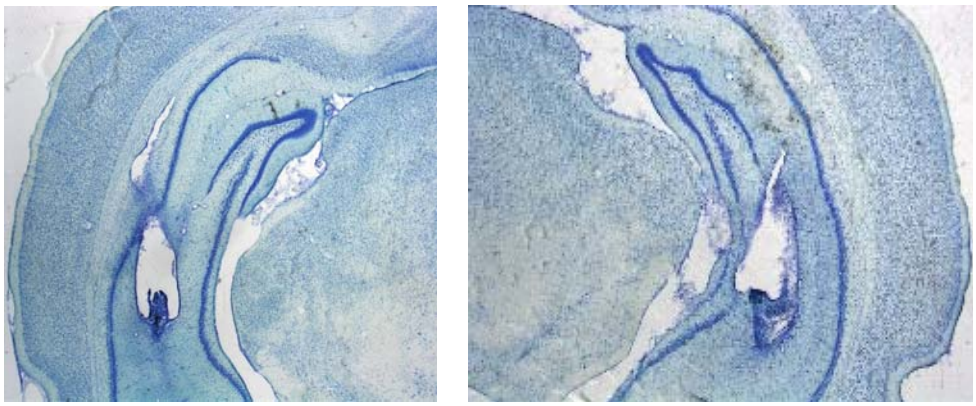


Figure 4. 4: A. The mean percentage of open-arm entries (±SEM) of rats microinfused with the Avpr1a antagonist into the ventral hippocampus. *Significantly different from the vehicle control group ($p < 0.05$). B. The mean percentage of open-arm time (±SEM) in rats microinfused with the Avpr1a antagonist into the ventral hippocampus.*Significantly different from the vehicle control group ($p < 0.05$).

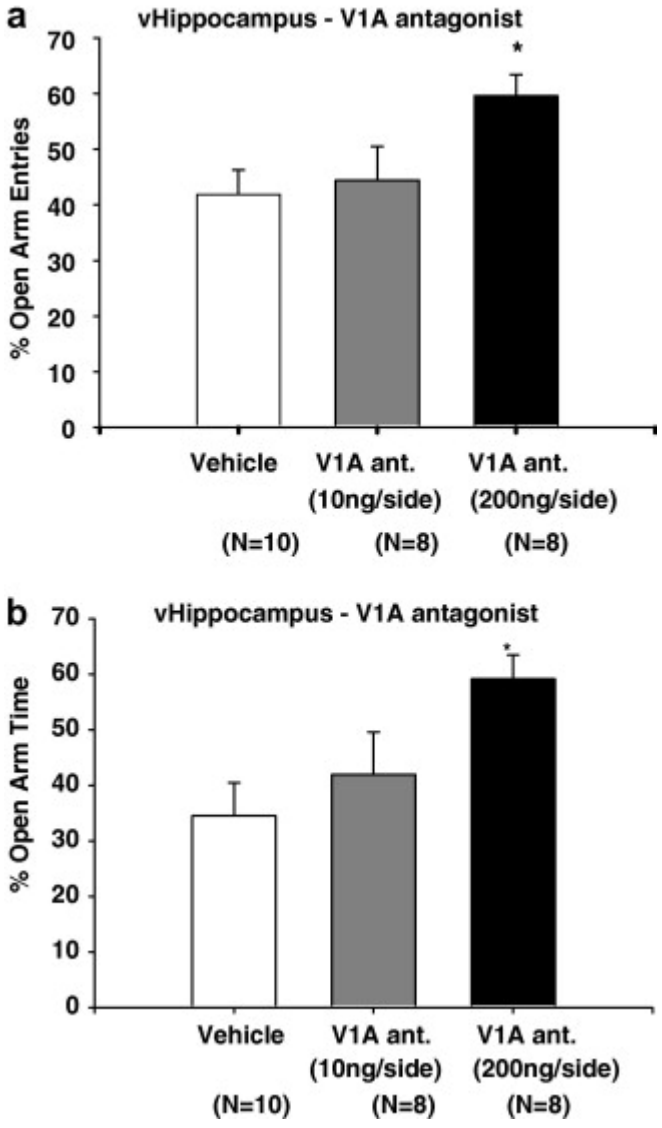


Table 4. 1: Measures of activity and anxiety-like behavior in the plus-maze test following intra-dorsal hippocampal infusions of Avpr1a and Avpr1b antagonists. ** Significantly different from the vehicle control group ($p<.01$) (See Figures 3.3.A, 3.3.B for anxiety measures following Avpr1b antagonist infusion).

	Vehicle (N=9)	Avpr1a ant. (10ng/side) (N=9)	Avpr1a ant. (200ng/side) (N=10)
% Open arm entries	36.21 ± 11.52	44.42 ± 8.21	47.13 ± 6.58
% Open arm time	32.82 ± 12.58	42.91 ± 8.04	50.77 ± 6.56
Number of closed arm	9.78 ± 1.92	8.50 ± 1.33**	8.10 ± 0.94
Number of total entries	16.00 ± 1.96	15.67 ± 1.32	16.00 ± 1.15
	Vehicle (N=8)	Avpr1b ant. (10ng/side) (N=11)	Avpr1b ant. (200ng/side) (N=9)
Number of closed arm	11.50 ± 1.09	7.08 ± 0.92	9.80 ± 0.92
Number of total entries	14.38 ±1.07	16.50 ± 0.99	17.80 ± 1.70

Table 4. 2: Measures of anxiety-like behavior, activity and pain sensitivity in the shock-probe burying test following intra-dorsal hippocampal infusions of Avpr1a and Avpr1b antagonists. **Significantly different from the vehicle control group ($p<0.01$.).

	Vehicle (N=9)	Avpr1a ant. (10ng/side) (N=9)	Avpr1a ant. (200ng/side) (N=10)
Burying time	41.561 ± 18.08	38.58 ± 14.03	56.30 ± 24.58
Still time	0.89 ± 0.68	11.00 ± 5.20	6.58 ± 2.75
Number of probe contacts	3.11 ± 0.42	2.92 ± 0.31	1.60 ± 0.31**
Shock reactivity	1.95 ± 0.25	1.91 ± 0.15	1.70 ± 0.29
	Vehicle (N=8)	Avpr1b ant. (10ng/side) (N=11)	Avpr1b ant. (200ng/side) (N=9)
Burying time	69.13 ± 23.22	123.75 ± 126.41	53.90 ± 18.33
Still time	31.63 ± 14.97	40.58 ± 22.70	8.90 ± 3.93
Number of probe contacts	2.25 ±0.31	2.41 ± 0.26	2.30 ± 0.30
Shock reactivity	1.90 ± 0.26	1.99 ± 0.16	1.68 ± 1.16

Table 4. 3: Measures of anxiety-like behavior and activity in the plus-maze test following intra-ventral hippocampal infusions of Avpr1a and Avpr1b antagonists (See Figures 3.5.A, 3.5.B for anxiety measures following Avpr1a antagonist infusion).

	Vehicle (N=10)	Avpr1a ant. (10ng/side) (N=8)	Avpr1a ant. (200ng/side) (N=8)
Number of closed arm entries	8.20 ± 0.65	8.00 ± 0.80	6.88 ± 0.61
Number of total entries	14.40 ± 1.01	14.67 ± 0.96	17.38 ± 1.12
	Vehicle (N=10)	Avpr1b ant. (10ng/side) (N=11)	Avpr1b ant. (200ng/side) (N=9)
% Open arm entries	27.12 ± 5.57	24.54 ± 4.17	40.09 ± 6.45
% Open arm time	21.91 ± 5.32	19.13 ± 3.48	36.94 ± 8.03
Number of closed arm entries	9.10 ± 0.89	10.60 ± 0.72	8.30 ± 0.94
Number of total entries	12.70 ± 1.15	14.20 ± 0.85	14.40 ± 1.35

Table 4. 4: Measures of anxiety-like behavior, activity and pain sensitivity in the shock-probe burying test following intra-ventral hippocampal infusions of Avpr1a and Avpr1b antagonists. *Significantly different from the vehicle control group ($p<0.05$.)

	Vehicle (N=10)	Avpr1a ant. (10ng/side) (N=8)	Avpr1a ant. (200ng/side) (N=8)
Burying time	80.30 ± 31.65	45.00 ± 28.84	19.13 ± 14.34
Still time	26.30 ± 16.68	75.89 ± 60.73	8.25 ± 6.98
Number of probe contacts	2.00 ± 0.21	2.77 ± 0.32	2.38 ± 0.38
Shock reactivity	2.32 ± 0.26	1.97 ± 0.15	1.49 ± 0.19*
	Vehicle (N=10)	Avpr1b ant. (10ng/side) (N=11)	Avpr1b ant. (200ng/side) (N=9)
Burying time	74.30 ± 26.60	37.30 ± 20.20	40.90 ± 29.91
Still time	0.40 ± 0.27	0.90 ± 0.43	0.60 ± 0.50
Number of probe contacts	2.50 ± 0.17	3.00 ± 0.39	2.20 ± 0.39
Shock reactivity	1.82 ± 0.15	1.95 ± 0.22	1.77 ± 0.24

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CHAPTER 5

ANXIOLYTIC AND ANTIDEPRESSANT EFFECTS OF INTRACEREBROVENTRICULARLY ADMINISTERED SOMATOSTATIN

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1. Introduction

Somatostatin (somatotropin release inhibiting factor; SST) is a cyclic polypeptide with 2 biologically active isoforms (SST-14, SST-28) arising from the same pro-hormone. SST-14 was originally described in the hypothalamus (Brazeau et al. 1973) and the amino-terminally extended SST-28 was discovered later on in the gut (Pradayrol et al. 1980). Initially, SST was thought to have the singular, specialized function of inhibiting growth hormone release (Brazeau et al., 1973). However, further research revealed that it has more general regulatory roles in hormone release (e.g., insulin, glucagons, thyrotropin), gastro-intestinal tract and pancreas function, smooth muscle contractility and cell proliferation (Weckbecker et al., 2003). Moreover, SST is widely distributed within the CNS, acting both as a neurotransmitter by itself and a neuromodulator of other neurotransmitter systems (Meyer et al., 1989; Chesselet and Reisine, 1983).

Because of its diverse roles and potential in the treatment of conditions such as acromegaly, endocrine and gastric/pancreatic tumors, cancer, certain eye diseases, and inflammatory and immune disorders, SST has received wide experimental attention (Weckbecker et al., 2003; van der Hoek et al., 2005). More recently, researchers have started to focus on some of the CNS-related effects of this peptide, especially its effects on sleep profile, seizures and memory-related processes. Reduction of SST function was found to increase REM sleep, whereas SST itself decreased sleep quality and time, especially in the elderly (Frieboes et al., 1997; Toppila et al., 2000). Furthermore, SST administration reduced epileptiform activity both in in vivo (Mazarati and Telegdy, 1992) and in vitro (Tallent and Siggins, 1999) models, suggesting the potential of SST-related compounds as antiepileptic drugs (Vezzani and Hoyer, 1999). SST depletion was found to impair performance in tasks related to memory function such as the Morris water maze (Dournaud et al., 1996; Dyer and Cain, 2007; Matsuoka et al., 1994; 1995). Moreover, SST content in the

cortex reduced with age (Dournaud et al., 1996) and increased with environmental enrichment (Nilsson et al., 1993), supporting the role of somatostatin in cognitive functioning. Other effects of SST administration include a reduction in locomotor activity (Izquierdo-Claros et al., 2001; Tashev et al., 2001; 2004) and analgesia (Tashev et al., 2001).

Despite this wide range of behavioral effects induced by SST, little attention has been paid to the possible role of SST in affective responses. There are, however, several lines of evidence suggesting that such an involvement is likely.

First of all, in several brain areas, SST is colocalized with neurotransmitter systems that are known to be involved in affective and stress-related responses, such as the GABAergic (Esclapez and Houser, 1995; Llorenscortes et al., 1992; McDonald and Mascagni, 2002; Xie and Sastry, 1992), cholinergic (Vandersee et al., 1991) and dopaminergic (Asan, 1992) systems. As is the case for several other neuropeptides (see Chapter 2, Section 3), the co-expression pattern changes anatomically and cellularly, depending on the brain area and type of neuron involved. Secondly, SST receptors are extensively expressed in limbic areas such as the amygdala, the septum and the hippocampus (Shindler et al., 1997; Stroh et al., 1999). In these areas SST is densely colocalized with GABA. Moreover, SST is coreleased from GABAergic neurons in the amygdala (Batten et al., 2002; Brodin et al., 1994), hypothalamus (Arancibia et al., 2000; Benyassi et al., 1993) and the hippocampus (Arancibia et al., 2001) as a response to stressful stimuli. Finally, neurophysiologically, SST invokes an inwardly rectifying K+ current in amygdalar (Meis et al., 2005) and hippocampal (Tallent and Siggins, 1997) neurons, resulting in hyperpolarization of the membrane at rest. The result is a reduction in excitatory post-synaptic currents, without an effect on GABA-mediated post-synaptic inhibitory currents (Tallent et al., 1999; Tallent and Siggins, 1997). Thus, neurophysiological function of SST seems to be similar

to that of GABA: membrane hyperpolarization and inhibition of excitatory post-synaptic currents (Hattori et al., 1995). SST neurons, like GABAergic neurons, play an important role in negative feedback circuits in the brain (Binaschi et al., 2003).

Although these neurophysiological observations indirectly suggest a possible involvement of SST in anxiety, to date there is no clear demonstration that SST causes a reduction in the anxiety-like responses of a behaving organism. Thus, the first purpose of this study (Experiment 1) was to test the effects of intracerebroventricularly (i.c.v.) administered SST in a model of anxiety, the elevated plus-maze, and a model of depression-like behavior, the forced swim test, and to compare these effects to that of diazepam, a benzodiazepine agonist. Moreover, because of the extensive co-localization and co-release of SST with GABA in limbic brain sites, the possible interactions between GABA and SST in the modulation of anxiety- and depression-like behavior were also examined.

The second purpose (Experiment 2) was to examine the effects of SST on hippocampal theta rhythms elicited by reticular stimulation. Although the neurophysiological data reviewed above do suggest that SST has inhibitory effects on neurons, similar to GABA, this does not necessarily mean that SST has anxiolytic-like neurophysiological properties. Clearly, GABAergic anxiolytics do have inhibitory effects on neuronal populations, but not all drugs with anxiolytic efficacy in humans share this effect. Antidepressant drugs, especially SSRIs, for example, have broad efficacy across a range of human anxiety disorders (e.g., Borsini et al., 2002; Rocca et al., 1997). However, their effects on neuronal populations is generally excitatory (Stewart and Reid, 2000). Thus, trying to infer the anxiolytic value of a drug simply by determining its general excitatory or inhibitory effects at the neuronal level is tenuous. Alternatively, McNaughton and his colleagues (McNaughton et al., 2007) have suggested that suppression of reticularly-activated hippocampal

theta rhythm can be used as a neurophysiological index of anxiolytic drug action. According to this model, all anxiolytic drugs (e.g., benzodiazepines, 5-HT1A agonists, SSRIs) reduce the frequency of reticularly-elicited theta, while benzodiazepines also reduce the slope of the function that relates the strength of the reticular stimulation to the frequency of the elicited theta rhythm. Drugs that do not selectively affect anxiety (e.g., antipsychotics such as haloperidol, or procognitive drugs such as donepezil) do not modulate brainstem elicited theta frequency. Suppression of the frequency of reticularly-elicited theta may thus provide a predictive neurophysiological index of the anxiolytic properties of a drug. Thus far there is considerable pharmacological evidence that supports this model (McNaughton et al., 2007). Accordingly, Experiment 2 was designed to examine the putative anxiolytic-like effects of SST on reticularly-activated hippocampal theta rhythm and to compare these to the classical anxiolytic drug diazepam.

2. Materials and Methods

2.1. Experiment 1: Behavioral effects of i.c.v. somatostatin

2.1.1. Subjects

Subjects were 89 male Sprague - Dawley rats, weighing 250-300 g at the time of surgery. The rats were maintained on a 12:12 h light/dark cycle (lights on at 0600 h) and individually housed in polycarbonate cages for the duration of the experiment. 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.

2.1.2. Surgery

Rats were anesthetized with isoflurane (4% induction, 2% maintenance in 30% N_2O and 70% O_2), injected with atropine sulfate (0.1 mg/0.2 ml ip) and marcaine (1.5mg/0.3 ml, sc on the head) and placed in a stereotaxic frame

(Kopf Instruments, Tujunga, CA, USA). Following hydration with 0.9% saline (3 ml, sc), an incision was made to expose the skull. The rats were implanted with Stainless-steel 22-gauge guide cannulae (Plastics One, Roanoke, VA, USA) targeting the right lateral ventricle (AP: -0.8mm, ML:-1.5mm, DV:-3.5mm). The cannulae were 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; 2.5mg / 0.5ml sc on the head). Following the surgery, the subjects were placed in a warm environment, until they regained consciousness. They were then allowed to recover for at least 5 days in their home cages before the start of behavioral testing. At the end of the experimental procedures, the placement of the cannulae in the ventricle was verified by examining a single slice taken from the implantation area with naked eye. The ventral tips of all cannulae were found to be in the intended region.

2.1.3. Infusion procedure

Rats were randomly assigned to a vehicle control, SST, diazepam, SST + diazepam, bicuculline +diazepam or bicuculline + SST condition. SST (Sigma, St. Louis, MO, USA), was dissolved in 0.9% saline at concentrations of 2µg/µl, 4µg/µl, 0.75µg/µl or 1.5µg/µl. Diazepam (Sabex, Sandoz, Boucherville, QC, Canada) was bought as a 5mg/ml injection solution (40% propylene glycol, 10% dehydrated alcohol, 4.25%benzoic acid, 1.5% benzyl alcohol and H_2O) and was diluted with dH_2O to the desired concentrations of 2.5µg/µl, 5µg/µl or 0.25µg/µl. Bicuculline methiodide (Tocris, Ellisville, MO, USA) was dissolved in dH_2O at a concentration of 2µg/2µl. The drugs or the vehicle were infused via an infusion pump (Harvard Apparatus 22, MA, U.S.) at a rate of 4µl/min using 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 cannula. Drug flow was confirmed by displacement of a bubble inside the polyethylene

tubing. In case of single drug infusions (total volume = 4µl per rat), the drug was infused for 1 min and the internal infusion cannulae were left in place for 40 seconds after the end of the infusion period to allow for diffusion. In case of double drug infusions (SST + diazepam, bicuculline + diazepam, bicuculline + SST; total volume = 4µl per rat; 2µl for each drug), the first drug was infused for 30 seconds, the cannula was held in place for 30 seconds to allow for diffusion, and the same procedure was repeated for the second drug immediately after.

2.1.4. Behavioral testing

All testing occurred in a quite room between 0800 h and 1600 h and was recorded on videotape. Testing started 20 min after the end of infusion procedure. Similar to the studies in Chapter 3 and Chapter 4, the subjects were assigned to the same drug treatment group (SST 8µg, SST 3µg, diazepam 10µg, diazepam 1µg, SST 3µg + diazepam 1µg, bicuculline 2µg + diazepam 10µg, bicuculline 2µg+ SST 8µg, vehicle control) for both behavioral tests. The plus-maze test occurred first, followed six days later by the forced swim test. The behavioral data were subsequently analyzed with ANOVA and followed, where significant (α =0.05), with pair-wise comparisons (LSD post hoc tests, α =0.05).

2.1.4.1. Elevated plus-maze

The apparatus and the procedure employed in the elevated plus maze test was the same as those in Chapter 3. See section 2.4.1 of Chapter 3 for details.

2.1.4.2. Forced swim test

The forced swim test occurred over 2 days, the first a 15 min 'pretest' swim session, followed one day later by a 5 min 'test' swim session (Porsolt et al., 1978). The injection was given prior to the second, 'test' session. Testing was done under normal light conditions. In both sessions, each animal was forced to swim in a cylindrical plexiglass tank (46 cm high × 20 cm in diameter) containing 30 cm of water (Figure 5.1). The water temperature was

maintained at approximately 25 °C. Three measures were taken during the test session: 1) the amount of time the animal spent swimming, 2) the amount of time the animal spent trying to escape (i.e., trying to climb to the walls of the tank), and 3) the amount of time the animal spent 'immobile' (i.e., floating in the water, making only the movements necessary to keep its head above water). Longer immobility times were taken as a measure of increased 'depression'. After each session, the rats were immediately removed from the cylinder, dried with a towel and kept under a heating lamp until completely dry, before being returned to their home cages.

2.2. Experiment 2: Effects of i.c.v. somatostatin on reticularly-elicited hippocampal theta

2.2.1. Subjects

Subjects were 14 male Sprague-Dawley rats weighing 250-300 g at the time of testing. The rats were maintained on a 12:12 h light/dark cycle (lights on at 0600 h) and group-housed in polycarbonate cages (3 rats per cage). Food and water were available ad libitum. All methods used conformed to the guidelines established by the Canadian Council on Animal Care and the Society for Neuroscience and were approved by the Biosciences Animal Policy and Welfare Committee of the University of Alberta.

2.2.2. Anesthesia, surgery and recording

Animals were initially anesthetized with isoflurane (4% induction, 2% maintenance in O_2) and were implanted with a jugular catheter. Isoflurane was discontinued, and general anesthesia was achieved through slow intravenous administration of urethane (0.8 g/ml; final dose 1.3 ± 0.01 g/kg) via the jugular vein. Body temperature was maintained at 37°C using a servo-driven system connected to a heating pad and rectal probe (TR-100; Fine Science Tools, Vancouver, BC, Canada) for the remainder of the surgical and recording procedures. Level of anesthesia was assessed throughout the experiment by

monitoring the withdrawal reflex to a hind paw pinch. A supplemental dose of urethane (0.01ml) was administered whenever a visible withdrawal was observed.

Each rat was implanted with a monopolar recording electrode at the level of the hippocampal fissure in the dorsal hippocampus (AP -3.3mm, ML +2.2mm, DV -3.0mm) and a bipolar stimulating electrode in the brain stem (AP -8.0mm, ML -1.6mm, DV -7.8mm). Both electrodes were secured to the skull with jeweler's screws and cranioplastic cement. Recordings were made by referencing to ground (in this case, the ear bars of the stereotaxic apparatus), amplifying at a gain of 1000 and filtering between 0.1 to 500Hz using a differential AC amplifier (Model 1700, A-M Systems Inc.). Signals were digitized on-line (sampling frequency 1kHz) with a Digidata 1322A A-D board connected to a Pentium PC running the AxoScope acquisition program (Axon Instruments; Union City, CA). The reticular formation was stimulated with 0.1ms biphasic pulses at 100Hz for 5 s. As reported earlier (McNaughton and Sedgwick, 1978) the threshold intensity of stimulation to elicit theta differed from animal to animal. Therefore, a threshold was established separately for each animal and the stimulation intensities used in the experiment were 1x, 2x, 3x and 4x this threshold level for each animal. Different intensities were applied in a random order and 5 readings were taken at each level. A second reading from the same intensity was not taken until the first reading for all intensities had been obtained.

Once the baseline readings were finished, a 26-gauge stainless steel internal cannula was lowered into the right lateral ventricle (AP: -0.8mm, ML:-1.5mm, DV:-3.5mm) and the animal was infused with either 0.9% saline, SST (Sigma, St. Louis, MO, USA, dissolved in 0.9% saline at a concentration of 8µg/4µl) or diazepam (Sabex, Sandoz, Boucherville, QC, Canada, 5mg/ml injection solution diluted with 0.9% saline to a concentration of 10µg/4µl) over a time period of one minute (Total volume = 4µl; Harvard Apparatus 22

infusion pump, MA, U.S.). The stimulation-recording procedures outlined above were repeated at 5min, 25min, 45min and 60min following the infusion.

2.2.3. Histology

At the end of the experiment, a small lesion was made at the tip of the recording and stimulating electrodes by passing 1 mA of DC current for 5 s using an isolated constant current pulse generator (Model 2100; A-M Systems).The rats were sacrificed with an overdose of urethane and perfused intracardially with 0.9% (w/v) saline followed by 4% (v/v) formaldehyde.

After 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 in dry ice and cut into 60-μm sections with a sliding microtome (Model 860; American Optical Company, Buffalo, New York, USA). Every second section was collected and mounted onto a gelatin-coated microscope slide and later stained with thionin. Microscopic inspection of stained slices was used to verify recording and stimulation loci.

2.2.4. Data analysis

Evoked theta activity was analyzed during each stimulation epoch by spectral (FFT) analysis in Clampfit (Axon Instruments). In brief, the epoch was windowed using a Hamming function, and the maximum number of samples constituting a power of 2 within this sample (4096) was used in the FFT algorithm. This yielded a spectral resolution of 0.24Hz. The peak frequency and power at peak frequency were extracted from the resulting spectrum and plotted as a function of stimulation intensity. A least squares regression curve was fitted to the frequency data from the recordings for each time point (baseline and 5min, 25min, 45min and 60min post-infusion), employing the intensity of stimulation in threshold units as the X-axis and the frequency of theta rhythm as the dependent variable (see Figure 5.2 for the baseline recording and the subsequent regression curve from one animal). The slopes and intercepts of the resulting regression lines were employed as raw data for subsequent analyses. The slopes and intercepts of the post-infusion curves

were compared to those of the baseline curve in a within-subjects general linear model design separately for each group employing the timing of the recording (pre-infusion, 5min, 25min, 45min or 60min post-infusion) as the within-subjects factor. The power data were averaged for all recordings of the same stimulation intensity, and then were analyzed separately for each intensity in a mixed within (time point)-between (drug group)-subjects ANOVA design.

3. Results

3.1. Experiment 1: Behavioral effects of i.c.v. somatostatin

3.1.1. Elevated plus-maze

The percentage of open arm entries ($F(7, 83) = 5.12$, $p<0.01$; Figure 5.3.A) and the percentage of open arm time ($F(7, 83) = 4.33$, $p<0.01$; Figure 5.3.B) were significantly different between the groups, whereas the general activity measures number of closed arm entries and the number of total entries did not differ ($p>0.05$; Table 5.1).

An LSD post hoc test revealed that the rats that received an 8μg infusion of SST ($p<0.01$), 10μg infusion of diazepam ($p<0.01$) or a combination of 3μg SST with 1μg diazepam ($p<0.05$) displayed more open arm entries compared to the vehicle controls. The increase in open arm entries seen after 8μg SST or 10μg diazepam was reversed completely when preceded by 1μg bicuculline infusion ($p>0.05$). The groups that received 3μg SST or 1μg diazepam alone did not differ from the vehicle controls in terms of open arm entries ($p>0.05$).

An increase in time spent in the open arms was observed in groups that received 10μg diazepam ($p<0.01$) or 3μg SST in combination with 1μg diazepam ($p<0.01$). While 8μg SST infusion imposed a trend towards increased open arm time, the effect was not statistically significant ($p>0.05$).

Similar to percentage of open arm entries, 1μg diazepam, 3μg SST and diazepam or SST infusions preceded by bicuculline did not affect the percentage of time spent in open arms.

3.1.2. Forced swim test

Total mobility ($F(7, 81) = 4.54$, $p<0.01$; Figure 5.4) in the forced swim test was significantly increased by the administration of 8μg SST alone (LSD, $p<0.01$) or 3μg SST in combination with 1μg diazepam (LSD, $p<0.01$) in comparison to the vehicle control group. The drug effect observed with 8μg SST was not reversed by pre-administration of 1μg bicuculline (LSD, $p<0.05$). Administration of diazepam by itself or in combination with bicuculline did not produce any changes (LSD, $p>0.05$). The differences in total mobility were attributable to a change in swimming behavior ($F(7, 81) = 3.82$, $p<0.01$), whereas climbing behavior was not significantly different between the groups ($p>0.05$).

3.2. Experiment 2: Effects of i.c.v. somatostatin on reticularly-elicited hippocampal theta

3.2.1. Histology

The recording and stimulation loci were confirmed through examination of the thionin-stained brain slices. The approximate sites of stimulation and recording can be seen in Figure 5.5. Most hippocampal recording placements were at the level of the hippocampal fissure, between area CA1 and the dentate gyrus and most reticular stimulation placements were in the vicinity of the oral part of the pontine reticular nucleus and the pedunculopontine tegmental nucleus.

3.2.2. Recordings

The baseline and post-infusion regression lines relating stimulus intensity to the peak theta frequency evoked for a single subject are displayed in Figure 5.6. As can be seen the slope and the intercept value of this function diminished after infusions. Following the fitting of similar regression lines for

each subject, the intercepts and slopes of the regression lines were analyzed in a within-between subjects general linear model design. As expected, the evoked theta frequency averaged over all stimulation intensities changed significantly between different time points in diazepam-infused animals (Mauchly's W =0 .01, $p>0.05$; Sphericity assumed $F(4,4) = 4.80$, $p<0.01$), with a significant reduction following drug infusion. A similar pattern was observed following SST infusions (Mauchly's W = 0.08, $p>0.05$; Sphericity assumed $F(4,4) = 3.59$, $p<0.05$), while saline infusion did not cause any significant change in theta frequency ($F(4,4) = 0.07$; $p>0.05$). The time course of the changes in theta frequency is plotted separately for the saline, diazepam and SST groups in Figure 5.7. As shown in the figure, the greatest reduction in theta frequency was observed at 5min post-infusion with both diazepam ($F(1,4) = 13.00$, $p<0.05$) and SST ($F(1,4) = 16.62$, $p<0.05$).

The slope of the stimulation intensity – theta frequency regression curve was reduced following both diazepam (Mauchly's W = 0.01, $p>0.05$; Sphericity assumed $F(4,4) = 3.59$, $p<0.05$) and SST (Mauchly's W =0 .002, $p>0.05$; Sphericity assumed $F(4,4) = 2.84$, $p<0.06$) infusions, while saline controls did not show such an effect ($F(4,4) = 2.37$, $p>0.05$). The maximum effect was observed 25min post-infusion with diazepam ($F(1,4) = 9.96$, $p<0.05$), whereas the effect was maximum at 45min post-infusion for animals infused with SST ($F(1,4) = 15.70$, $p<0.05$; see Figure 5.8).

The changes in theta power were also investigated in a mixed within-between subjects design. The changes in amplitude through time were significant only for the 4xThreshold stimulation intensity recordings ($F(4,13) = 3.15$, $p<0.05$). Moreover, the drug group x Amplitude interaction was statistically significant ($F(8,13) = 3.87$, $p<0.01$), indicating a different amplitude change pattern for different drugs. As seen in Figure 4.9, SST caused an increase in theta amplitude ($F(4,4) = 5.02$, $p<0.01$), while diazepam caused a trend towards reduced amplitude ($F(4,4) = 3.11$, $p<0.06$). Saline infusion did not change theta amplitude ($F(4,3) = 0.38$, $p>0.05$).

4. Discussion

The behavioral data from our experiments suggest that somatostatin has anxiolytic and antidepressant properties. In the plus-maze test, i.c.v. infusion of SST at a dose of 8μg/rat resulted in increased exploration of the open arms, compared to vehicle controls. Importantly, the magnitude of this anxiolytic-like effect was comparable to that of 10μg of the anxiolytic drug diazepam. Moreover, the co-infusion of sub-effective doses of diazepam (1μg) and SST (3μg) summated to produce significant anxiolytic effects in the elevated plus-maze. Bicuculline, a $GABA_A$ receptor antagonist, reversed the anxiolytic effect of the supra-threshold doses of diazepam and SST on open-arm entries, further suggesting a common mechanism of anxiolytic drug action. None of these effects were confounded by nonspecific changes in general activity.

Our electrophysiological data show that the behavioral effects of SST and diazepam in the plus-maze share a common electrophysiological signature: the suppression of the frequency of reticularly-driven hippocampal theta. Like benzodiazepines, 5-HT_{1A} agonists, and SSRIs, SST decreased the frequency of reticularly-elicited hippocampal theta. Moreover, like benzodiazepines, SST reduced the slope of the function relating the intensity of reticular stimulation to the frequency of hippocampal theta rhythm (See Figure 5.5). Furthermore, the effects of diazepam and SST on this frequency/intensity slope were statistically identical.

In the forced swim test, the immobility of rats (often called "behavioral despair") was abolished by i.c.v. administration of SST. Diazepam, as expected, was devoid of any antidepressant-like effects by itself, however, a summation effect similar to that observed in the plus maze was produced by the co-administration of low doses of SST and diazepam. The antidepressant-like effect of SST was not reversed by pretreatment with bicuculline.

There are a number of issues that can obscure the interpretation of the behavioral effects of drugs, one of which is non-specific changes in general activity, and another of which is drug tolerance. However, in our experiments there were no significant differences in any measure of general activity in the elevated plus-maze test (see Table 5.1). Although "immobility" in the swim test is the target behavior against which antidepressant drugs are tested, there was little evidence, either in the plus-maze or in the swim test, that SST produced non-specific behavioral stimulation, which could be mistaken for an antidepressant effect.

While some of the rats were tested twice with somatostatin, once in the plus-maze and once in the swim test, it is unlikely that drug tolerance or sensitization affected the results in the latter test, for two reasons. First, the six day interval between drug microinfusions was likely long enough for any neuronal changes induced by the first dose to have dissipated by the time the next dose was infused. Second, the drug doses themselves were relatively small compared to the total volume of cerebral ventricular fluid into which they were infused, so that the actual drug concentration at functional receptor sites would have been much smaller than the nominal doses administered.

Furthermore, our behavioral findings are supported by the neurophysiological findings in the current study. According to J.A. Gray's "Neuropsychological Theory of Anxiety," the hippocampus and the septum work in concert to control anxiety (Gray 1982). Over the years, our laboratory has gathered important empirical confirmation of this general model; specifically, we showed that the effects of known anxiolytic drugs are very similar to the effects of the septo-hippocampal lesions in pharmacologically validated animal models of anxiety (e.g., Degroot and Treit, 2003; 2004; Menard and Treit, 2001; Treit and Menard 2000, 1997; Pesold and Treit, 1996). One of the most important functional aspects of septo-hippocampal system is the generation of hippocampal theta. According to the theory,

hippocampal theta should be enhanced by anxiogenic stimuli, and inhibited by anxiolytic stimuli. As predicted, McNaughton et al. (2007) and others have shown that hippocampal theta is inhibited by anxiolytic drugs and enhanced by anxiogenic drugs. The correspondence of our electrophysiological data and our behavioral data provide strong, convergent evidence of the anxiolytic-like effects of SST.

The specific neural mechanisms by which somatostatin produced its behavioral effects are not entirely clear. Knockout studies have implicated the sst_{2A} receptor sub-type in the fear responses of mice (Viollet et al., 2000). Our studies suggest that SST may also act through $GABA_A$ receptors, since bicuculline, a $GABA_A$ receptor antagonist, reversed the anxiolytic effects of somatostatin. Furthermore, diazepam, an allosteric $GABA_A$ agonist, summated with somatostatin to produce significant anxiolytic effects, at doses that were too low to be effective by themselves. While our plus-maze data suggest that the mechanism of the anxiolytic-like effects of SST is primarily GABAergic, the antidepressant-like effect found in the swim test seems to be more complex. While the sub-threshold doses of SST summated with sub-threshold doses of diazepam to produce an antidepressant effect, the antidepressant effect produced by 8μg SST was not reversed by bicuculline. Overall, these different findings suggest that the antidepressant-like effect of SST may be mediated by a number of different mechanisms. It is possible, for example, that SST produces its effects through an interaction with NPY or CCK, neuropeptides that have been implicated in anxiety and depression, and which are often co-localized with somatostatin (Moore and Black, 1991). Somatostatin is also co-localized with norepinephrine (Moore and Black, 1991), a neurotransmitter that is also thought to play a role in anxiety and depression (Ressler and Nemeroff, 2000). On the other hand, it should be noted that the reduction in immobility we observed in the forced swim test following SST administration was characterized by an increase in swimming behavior, without an effect on climbing behavior. This type of behavior change is typical of serotonergic

activity, while noradrenergic activity results in increased climbing behavior (Lucki, 1997). Future studies need to explore the many possible receptor mechanisms by which somatostatin could produce its anxiolytic and antidepressant-like effects.

Also unknown are the specific brain areas where somatostatin might produce its effects. Areas of the limbic system, specifically the amygdala, hippocampus and septum, have long been implicated in anxiety (Engin and Treit, 2008), and somatostatin receptors are found in relatively high densities in these structures (Reubi and Maurer, 1985). The highest concentrations of somatostatin receptors are found in the basal and basolateral amygdalar nuclei and in the ventral and dorsal subiculum (Leroux et al., 1993). In addition, there has been an unconfirmed conference report that somatostatin microinfused in the ventral tegmentum, another area involved in motivation and emotion, produced antidepressant-like effects in the swim test (Pallis et al., 2007). Moreover, there is evidence that increased SST in the pontine reticular nucleus can interfere with the fear potentiation of the acoustic startle response, implicating the role of SST in the fear conditioning circuits in the brain (Fendt et al., 1996). Certainly, these are areas that should be the targets of future studies that examine the specific brain sites where somatostatin acts to produce its effects on anxiety and depression.

While SST itself is not appropriate for clinical use because of its short half-life and diverse range of effects (Pinter et al., 2006), a closely related SST derivative may have some potential for the pharmacological treatment of human anxiety and depression. Treatment of both disorders with one drug would be beneficial for patients co-morbid for both disorders, especially as some common anxiolytics (e.g., diazepam) exacerbate depressive symptoms. Currently used anxiolytics and antidepressants, which include the benzodiazepines, tricyclic antidepressants, SSRI's and MAOI's, all have a variety of unpleasant and sometimes dangerous side effects, which work

against patient compliance, increasing the chance of relapse. Benzodiazepines, for example, in addition to being potent anxiolytic drugs, can produce learning and memory impairments (Cain, 1997; Lader, 1994). SST, on the other hand, has pro-cognitive effects (e.g., Dournaud et al., 1996; Matsuoka et al, 1994) and thus, has potential for being the first drug that has both pro-cognitive and anxiolytic effects. This paradoxical profile is supported by our findings that SST reduces theta frequency (i.e., a characteristic of all known anxiolytic drugs), stimulation intensity – theta frequency curve slope (i.e., a characteristic of benzodiazepine anxiolytics) while at the same time increasing the power of elicited theta (i.e., a characteristic of pro-cognitive drugs such as cholinergic agonists; e.g., Kinney et al., 1999). Our findings indicate that benzodiazepine diazepam attenuates theta power, as would be expected from its amnesic profile. SST, on the other hand, shows a very similar profile to diazepam in terms of its effects on theta frequency and on behavior in the elevated plus maze test, while increasing theta amplitude in contrast to diazepam's suppressive effect. SST's pro-cognitive, anxiolytic and antidepressant effects suggest that its safety and efficacy as a treatment of human anxiety and depression should be perused seriously.

In summary, our studies demonstrate that somatostatin has anxiolytic and anti-depressant-like effects in animal models. We found evidence that somatostatin produces these effects partly though $GABA_A$ receptors. Bicuculline, a $GABA_A$ receptor antagonist, reversed the anxiolytic-like effects of somatostatin. Also supporting a $GABA_A$ receptor mechanism was the observation that a sub-threshold dose of diazepam, which is a $GABA_A$ agonist, summated with a sub-threshold dose of somatostatin to produce anxiolytic effects in the elevated plus-maze. These findings warrant further studies of the neural mechanisms of action of somatostatin, and its anxiolytic and antidepressant potential.

FIGURES AND TABLES

Figure 5. 1: An animal during the typical “floating” behavior in the forced swim test.

Figure 5. 2: Baseline recording from an individual rat receiving different intensities of stimulation (100Hz, 0.1ms pulse width for 5 sec; Threshold stimulation = 60µA). Left panel: Raw EEG records. Right panel: Scatter graph of the readings taken during baseline recording session and the regression line fitted to these data.

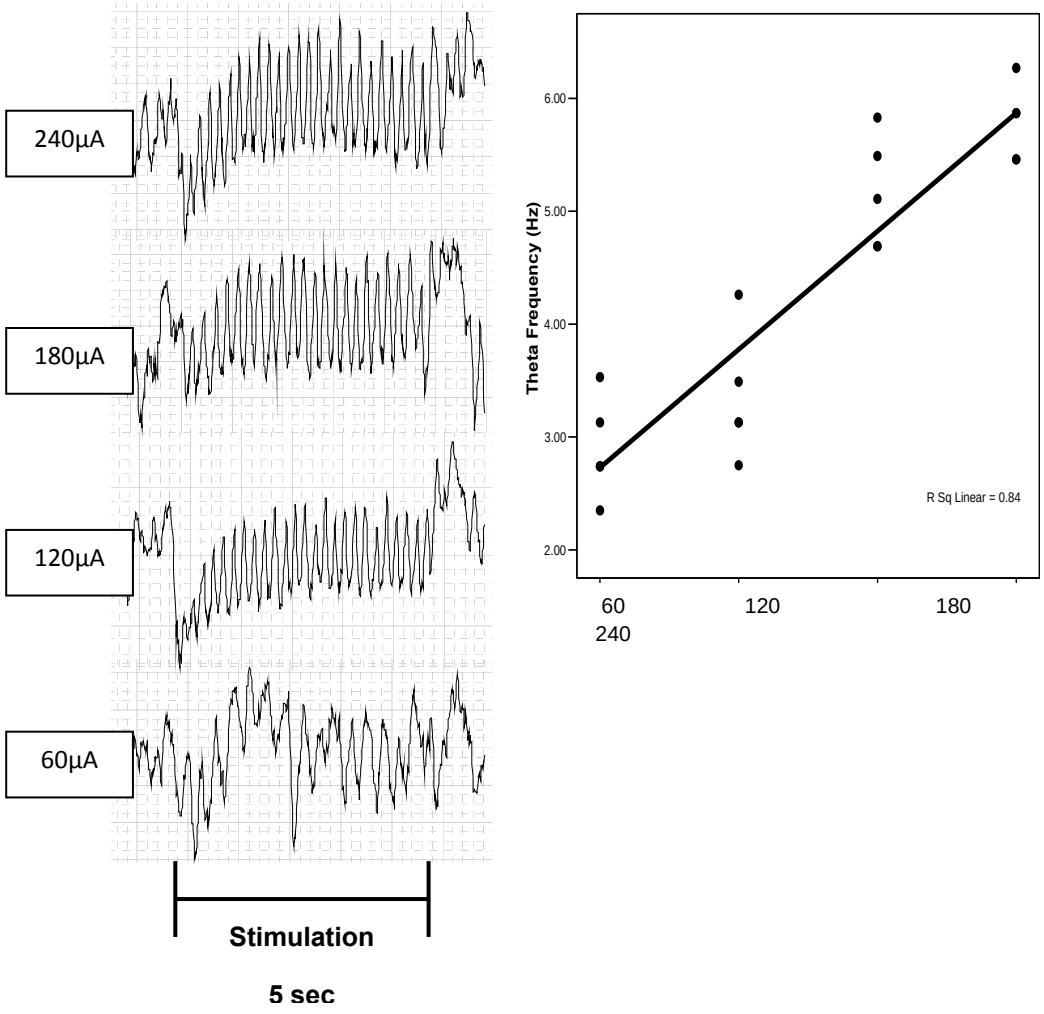

Figure 5. 3: Open arm activity in the elevated plus-maze. A. The mean percentage of open-arm entries (± S.E.M.) B. The mean percentage of open-arm time (± S.E.M.). *Significantly different from the vehicle control group ($p<0.05$).

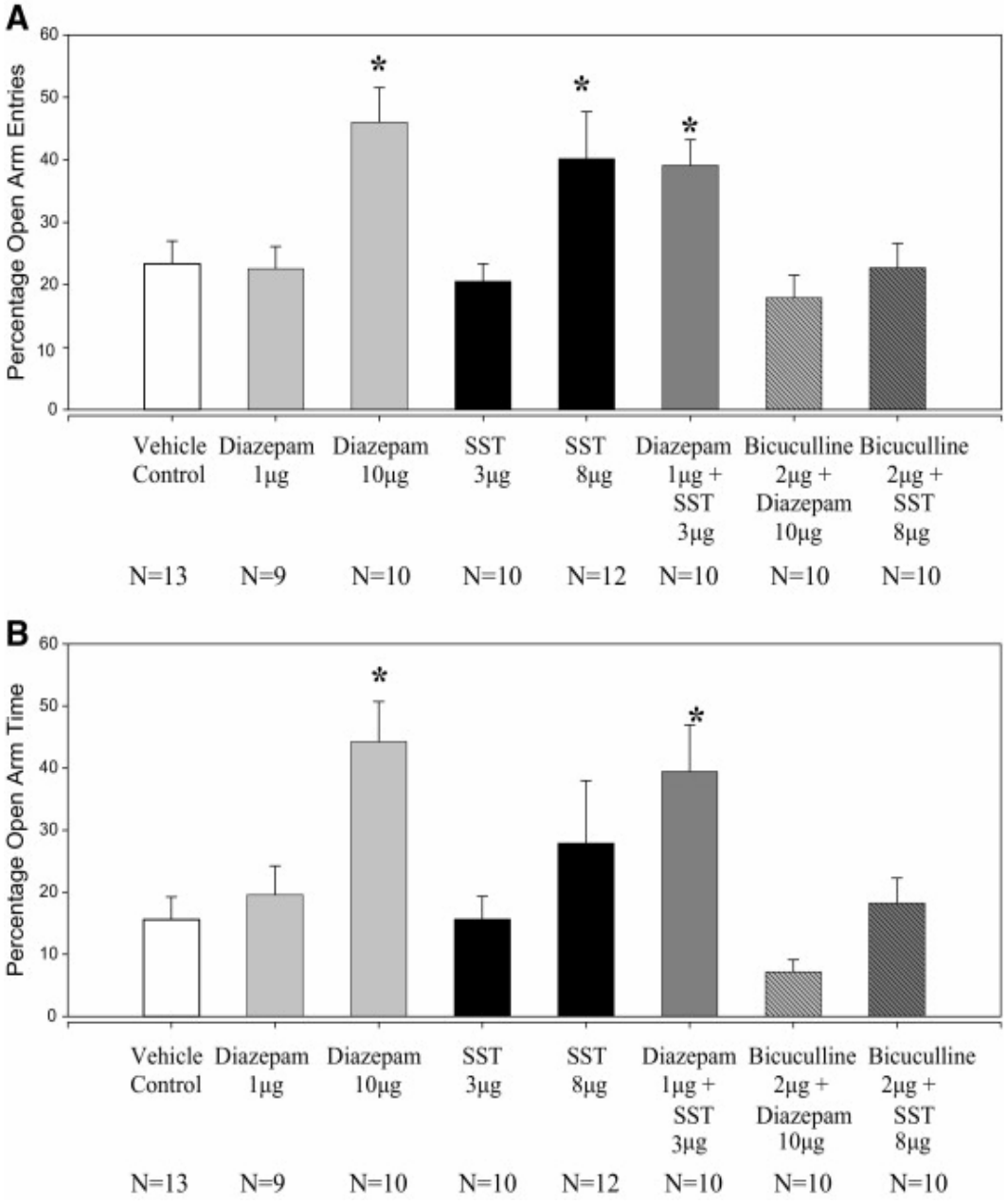

Figure 5. 4: The mean amount of time spent mobile (i.e., swimming or climbing) in seconds (± S.E.M.) in the forced swim test. *Significantly different from the vehicle control group ($p<0.05$).

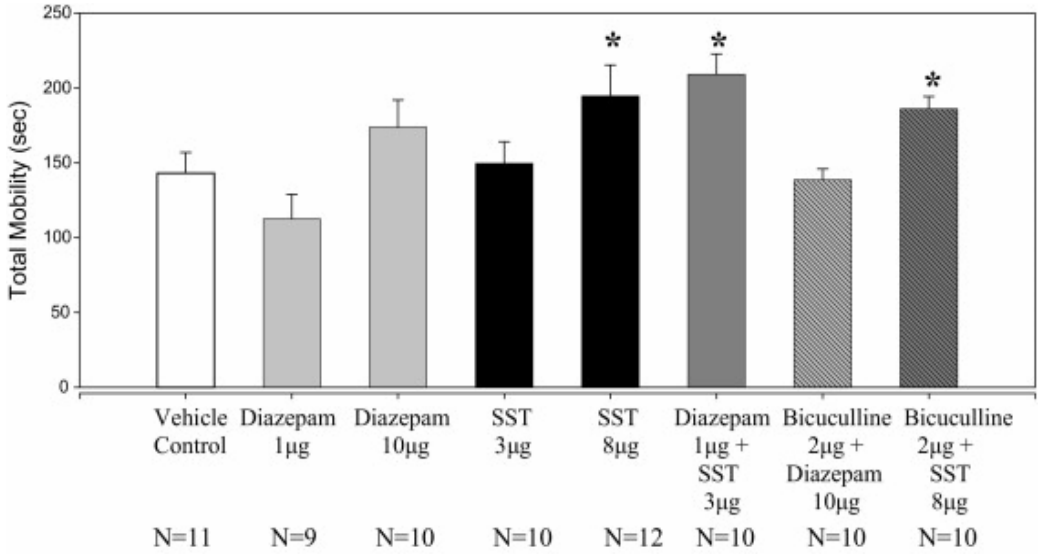

Figure 5. 5: Approximate recording (left) and stimulation (right) loci in experiment 2.

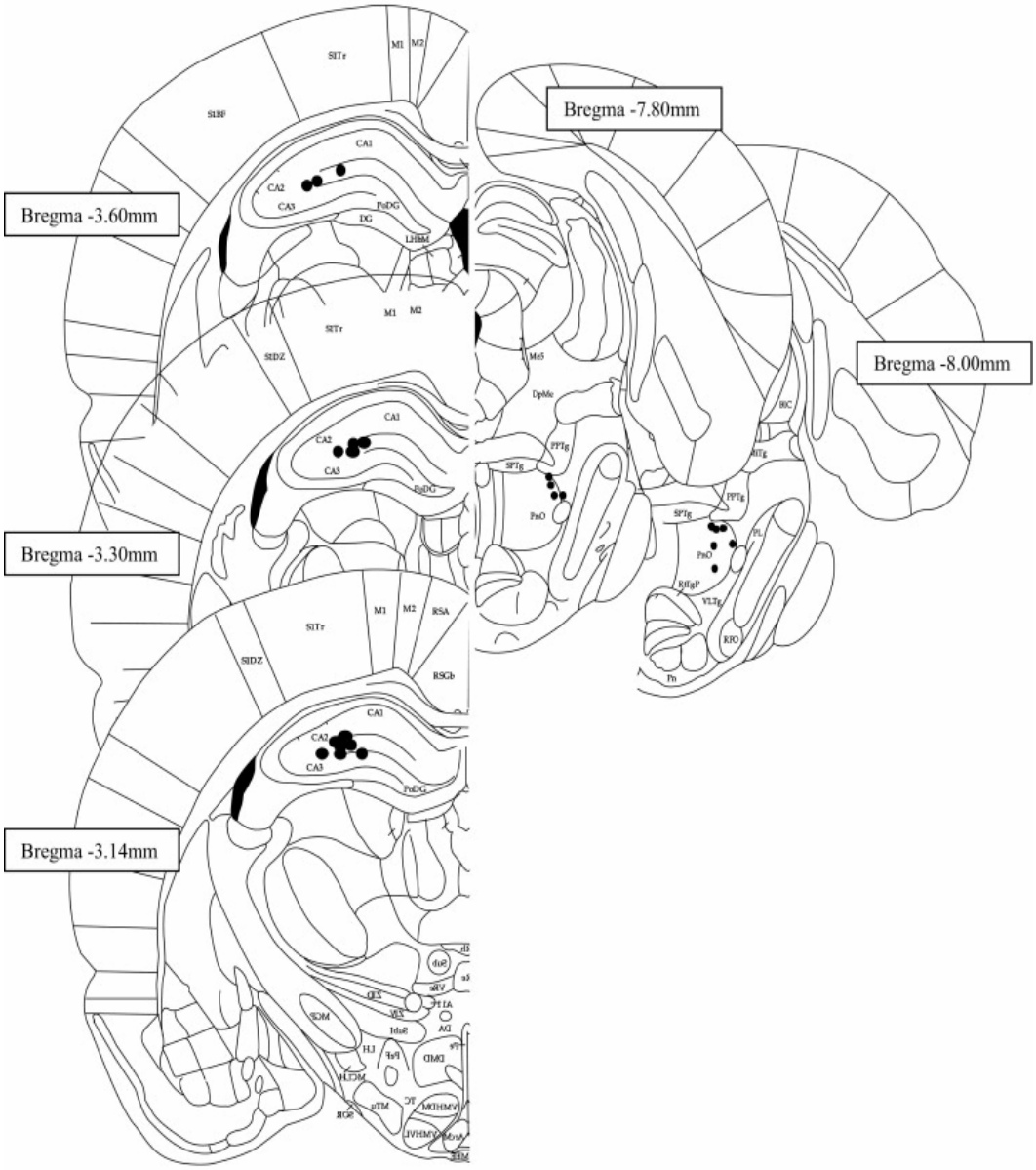

Figure 5. 6: Regression lines fitted to pre-infusion (i.e., baseline) and 5min, 25min, 45min and 60min post-SST-infusion readings from a single rat.

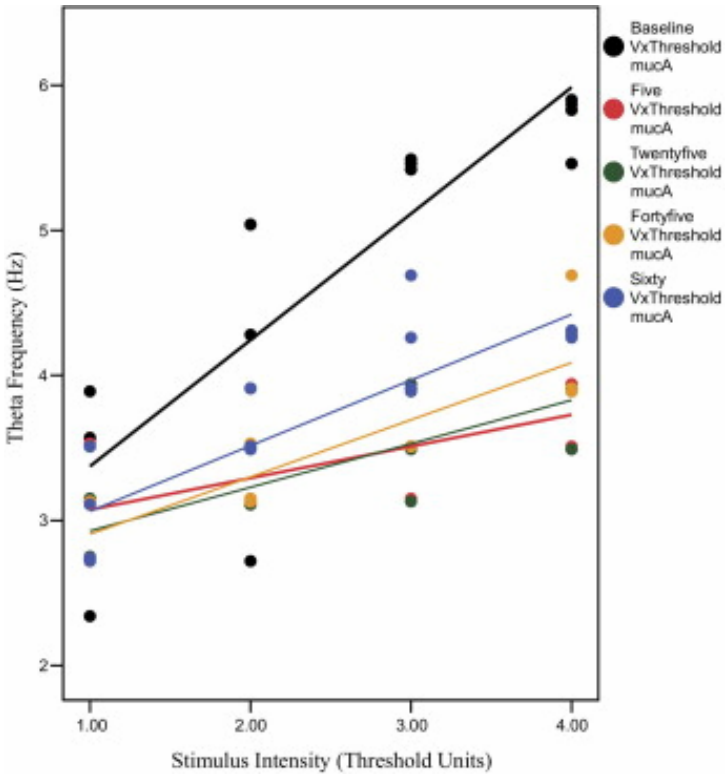

Figure 5. 7: Mean evoked theta frequency averaged over all stimulation intensities expressed as a ratio of baseline frequency at different time points for the saline-, SST- and diazepam-infused animals.

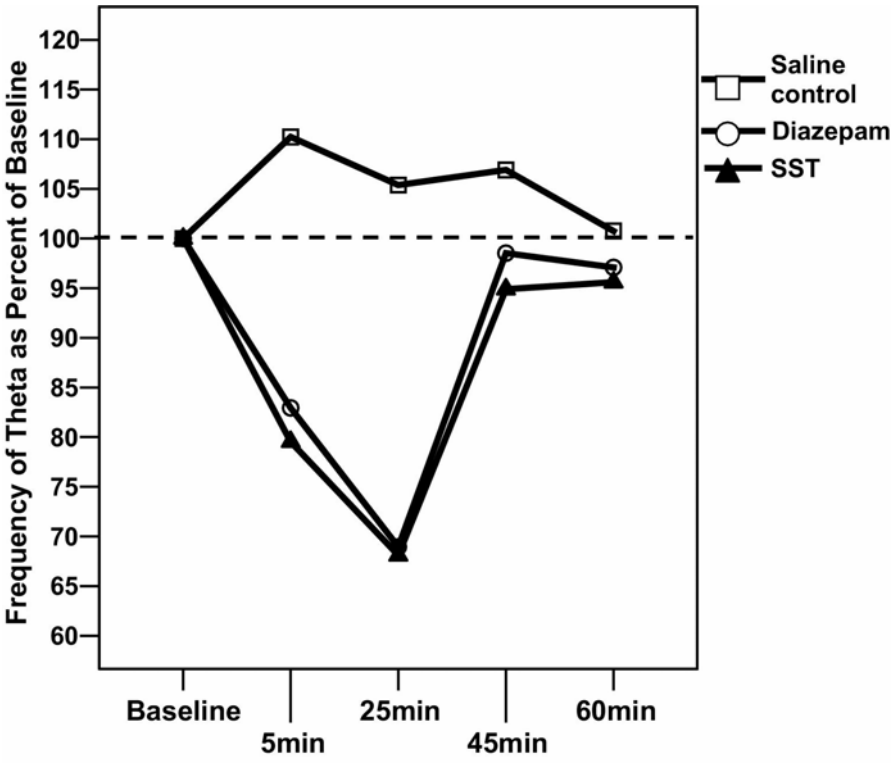

Figure 5. 8: Mean slopes of the regression lines expressed as a ratio of baseline slopes at different time points for the saline-, SST- and diazepam-infused animals.

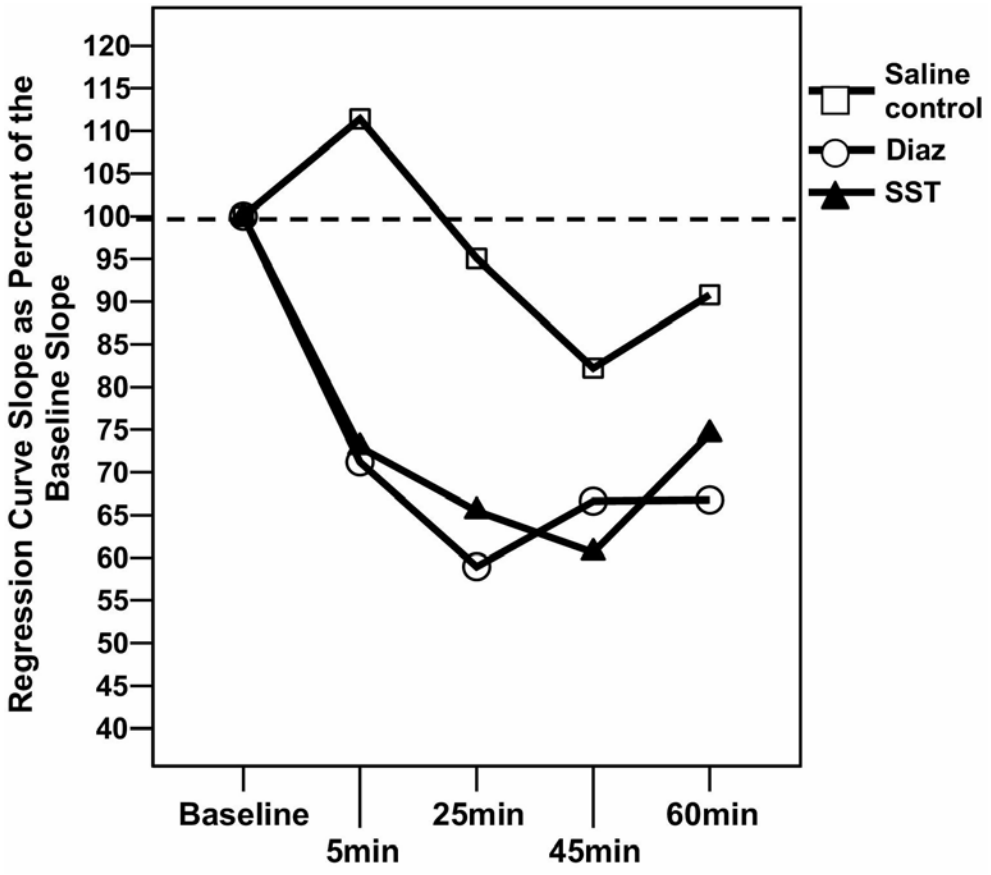

Figure 5. 9: Mean theta power following 4x(Threshold intensity) reticular stimulation expressed as a ratio of baseline amplitude at different time points for the saline-, SST- and diazepam-infused animals.

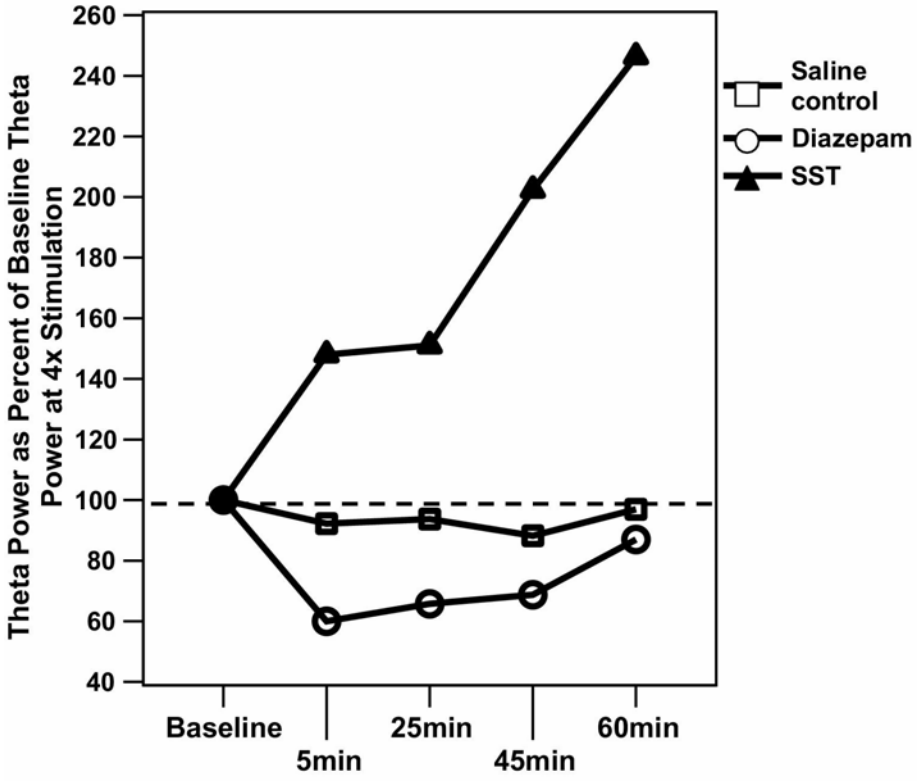

Table 5. 1: General activity measures from plus maze test (Mean ± S.E.M.).

	Number of Closed Arm Entries	Number of Total Entries
Vehicle control	11.69 ± 1.25	15.38 ± 5.42
Diazepam (1µg)	9.67 ± 0.67	12.56 ± 2.24
Diazepam (10µg)	9.40 ± 0.87	17.80 ± 0.95
SST (3µg)	12.70 ± 1.31	15.90 ± 1.69
SST (8µg)	7.83 ± 1.59	11.75 ± 1.91
Diazepam (1µg) + SST (3µg)	9.40 ± 0.64	16.10 ± 1.62
Bicuculline (2µg) + Diazepam (10µg)	10.70 ± 0.60	13.30 ± 0.89
Bicuculline (2µg) + SST (8µg)	10.60 ± 0.58	13.90 ± 0.81

Table 5. 2: Time (seconds) spent swimming and climbing in the forced swim test (Mean ± S.E.M.; *Different from vehicle control group, p<.05).

	Swimming Time	Climbing Time
Vehicle control	73.91 ± 12.69	69.18 ± 11.25
Diazepam (1µg)	53.22 ± 12.93	59.33 ± 8.79
Diazepam (10µg)	95.30 ± 14.18	78.50 ± 16.30
SST (3µg)	91.90 ± 10.60	57.80 ± 13.00
SST (8µg)	95.33 ± 22.40*	99.33 ± 24.89
Diazepam (1µg) + SST (3µg)	151.40 ± 15.14*	57.80 ± 8.32
Bicuculline (2µg) + Diazepam (10µg)	80.70 ± 5.88	58.00 ± 8.26
Bicuculline (2µg) + SST (8µg)	102.80 ± 6.96*	83.30 ± 5.01

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CHAPTER 6

SOMATOSTATIN RECEPTOR SUBTYPES INVOLVED IN THE ANXIOLYTIC- AND ANTIDEPRESSANT-LIKE EFFECTS OF SOMATOSTATIN

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1. Introduction

As mentioned in chapter 5, somatostatin is widely distributed within the CNS, acting both as a neurotransmitter and a modulator of other neurotransmitter systems (Meyer et al., 1989; Chesselet and Reisine, 1983). Five G-protein coupled receptors (sst1-5) mediate the neurotransmitter actions of somatostatin in the brain, with the sst1 receptor acting primarily as an autoreceptor (Roosterman et al., 1999; Thermos et al., 2006). The sst2 receptor is found as two splice variants, $sst2_A$ and $sst2_B$, which differ only in length and composition of their respective carboxyl-terminal domains. Both variants are expressed in the brain, though in different densities across brain areas (Schindler et al., 1999; Yamada et al., 1992).

While the sst2 receptor is the most abundant form in the brain, all 5 receptor subtypes are expressed here, and their distributions have been mapped using in situ hybridization and immunohistochemistry (Dournaud et al., 2000; Selmer et al., 2000). The effects of somatostatin binding at its G-protein coupled receptors are generally inhibitory, either through increasing K^+ currents or through decreasing Ca^{2+} currents (Baraban and Tallent, 2004; Cervia et al., 2008; Meis et al., 2005; Tallent and Siggins, 1997). Somatostatin neurons are thought to play an important role in negative feedback circuits (Binaschi et al., 2003).

There is evidence that central somatostatin is involved in several processes, such as sleep architecture (Beranek et al., 1997; Danguir, 1986; Frieboes et al., 1997; Hajdu et al., 2003; Obal et al., 2003; Steiger et al., 1992; Toppila et al., 2000; Ziegenbein et al., 2004), epileptiform activity (Binaschi et al., 2003; Buckmaster et al., 2002; Mazarati and Telegdy, 1992; Moneta et al., 2002; Tallent and Qiu, 2008; Tallent and Siggins, 1999; Vezzani and Hoyer, 1999), memory formation and retention (Dournaud et al., 1996; Dutar et al., 2002; Dyer and Cain, 2007; Gastambide et al., 2009; Justino et al., 1997; Kluge et al., 2008; Lamirault et al., 2001; Low et al., 1998; Matsuoka et al.,

1994; 1995; Nilsson et al., 1993; Tashev and Belcheva, 2008; Tokita et al., 2005; Zeyda et al., 2001), locomotor activity (Hathway et al., 2004; Izquierdo-Claros et al., 2001; Marazioti et al., 2005; 2006; 2008; Raynor et al., 1993; Santis et al., 2009; Tashev et al., 2001; 2004), and nociception (Betoin et al., 1994; Carlton et al., 2001; Morton et al., 1989; Pinter et al., 2006; Schindler et al., 1998; Tashev et al., 2001).

There is also indirect evidence that somatostatin may be involved in emotional processes such as anxiety and depression (e.g., Viollet et al., 2000; Fendt et al., 1996, Gheorvassaki et al., 1992; Pallis et al., 2006; 2007; 2009; Zhang et al., 1999), although none these studies has directly stimulated somatostatin function in the brain and documented the outcome in pharmacologically validated animal models of anxiety or depression (see general discussion). The studies reported in chapter 5 showed that the intracerebroventricular (i.c.v.) microinfusions of somatostatin produced clear, anxiolytic- and antidepressant-like effects in the elevated plus-maze model of anxiety and the forced swim model of depression (Engin et al., 2008). Specifically, rats infused with somatostatin increased their open- arm activity in the plus-maze and spent more time swimming in the swim test, compared to vehicle-infused controls. Moreover, i.c.v.-infused somatostatin reduced the frequency of reticularly-evoked hippocampal theta rhythm in urethane-anesthetized rats, an effect common to all classes of anxiolytic drugs (McNaughton et al., 2007). While these findings point to an anxiolytic and antidepressant action of somatostatin, the somatostatin receptor subtypes that mediate these effects are unknown.

The purpose of the present study was to begin characterizing the somatostatin receptor subtypes involved in the anxiolytic and antidepressant-like effects we found after microinfusion of the endogenous agonist, somatostatin. Accordingly, five synthetic somatostatin agonists, each selective for one of the somatostatin receptor subtypes (sst1 through sst5), were

administered i.c.v. to different groups of rats. The behavioral effects were evaluated in the elevated plus- maze, and the forced swim test.

2. Methods

2.1. Subjects

Subjects were 107 male Sprague - Dawley rats, weighing 170 - 230 g at the time of surgery. The rats were individually housed in polycarbonate cages and maintained on a 12:12 h light/dark cycle (lights on at 0600 h) for the duration of the experiment. 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. The elevated plus-maze data for one animal that received an sst5 agonist infusion were eliminated because it fell off the maze more than two times. Data from the same animal, however, were used in the statistical analysis of behavior in the forced swim test.

2.2. Surgery

Rats were anesthetized with isoflurane (4% induction, 2% maintenance in 30% N_2O and 70% O_2), injected with atropine sulfate (0.1 mg/0.2 ml ip) and marcaine (1.5mg/0.3 ml, sc on the head), placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA) and hydrated with 0.9% saline (3 cc, ip). An incision was made to expose the skull. The rats were implanted with Stainless-steel 22-gauge guide cannulae (Plastics One, Roanoke, VA, USA) targeting the right lateral ventricle (AP: -0.8mm, ML:-1.5mm, DV:-3.5mm). The cannulae were secured to the skull with three jeweler's screws and cranioplastic cement. A dummy cannula was inserted into each guide cannula in order to keep the cannula tract clear. The surgical wound was treated with 2.5 mg carprofen (Rimadyl©, Pfizer; 2.5mg / 0.5ml sc on the head) at the end of the surgery. Following the surgery, the subjects were placed in a warm environment, until they regained consciousness. They were then allowed to recover for at least 6

days in their home cages before the start of behavioral testing. At the end of the experiments, rats were given an overdose of sodium pentobarbital, their brains extracted and sectioned, and cannula placements confirmed with infusions of a 2.5% Chicago blue solution (4 µl) . All placements were within the lateral cerebral ventricles.

2.3. Infusion procedure

The sst1 agonist L-797,591, sst2 agonist L-779,976, sst3 agonist L-796,778, sst4 agonist L-803,087 and sst5 agonist L-817,818 were kindly provided by Merck Pharmaceuticals Research Laboratories, Rahway, NJ. All drugs were dissolved in DMSO at concentrations of 0.75 µg/µl, 2.25 µg/µl, and 6.75 µg/µl. Total infusion volume was kept constant at 4µl per rat. Rats were randomly assigned to a DMSO control condition or to one of the drug conditions before the start of the experiment. DMSO has no known neurotoxic effects in the amounts administered in our experiments (Santos et al., 2003), and the behavioral patterns observed with DMSO are comparable to those observed with saline (e.g., Engin and Treit, 2008a; Engin et al., 2008). L -797,591, L-779,976, and L-796,778 were administered at doses of 3µg, 9µg, and 27µg per rat. L-803,087 and L-817,818, however, caused seizure-like behavioral responses at the 27µg and 9µg doses, so that only the 3µg dose of these two drugs could be tested. All compounds were administered via an infusion pump (Harvard Apparatus 22, MA, U.S.), at a rate of 4µl/min, using a 26-gauge stainless-steel internal cannulae, attached by polyethylene tubing to a 10-µl Hamilton syringe. The internal infusion cannulae extended 0.5mm below the ventral tip of the guide cannula. Drug flow was confirmed by displacement of a bubble inside the polyethylene tubing. The internal infusion cannula was left in place for 40 seconds after the end of the infusion period, to allow for diffusion.

2.4. Behavioral testing

All testing occurred in a quiet room between 0800 h and 1600 h. Testing started 20 min after the end of infusion period. The subjects received

the same drug treatment or vehicle treatment for both behavioral tests. The plus-maze test occurred first, followed six days later by the forced swim test. This order of testing does not affect performance in swim test, although the reverse order can significantly affect plus-maze performance (see Korte and deBoer, 2003; Walf and Frye, 2008; Wu and Lin, 2008). There is no evidence of drug-carryover effects in our laboratory when several days separate the two tests (e.g., Menard and Treit, 1998; Treit et al., 1993). All testing was recorded on videotape. The behavioral data were subsequently analyzed with ANOVA and followed, where significant (α =0.05), with pair-wise comparisons (LSD post hoc tests, α =0.05).

2.4.1. Elevated plus-maze

The plus-maze apparatus and the testing procedure were the same as those reported in Chapter 3. See section 2.4.1 of Chapter 3 for details.

2.4.2. Forced swim test

The forced swim test apparatus and the testing procedure were the same as those reported in Chapter 5. See section 3.1.2 of Chapter 5 for details.

3. Results

Both the percentage of open- arm entries ($F(11, 105) = 2.98$, $p<0.01$; Fig. 6.1.A) and the percentage of open- arm time ($F(11, 105) = 2.30$, $p<0.02$; Fig. 6.1.B) were significantly different among the drug and control groups, whereas the general activity measures did not differ across groups (Table 6.1; $p>0.05$).

An LSD post hoc test revealed that rats that received a 27µg infusion of the sst2 agonist L-779,976 made more entries into the open arms and spent more time in the open arms than the DMSO controls (LSD, $p<0.01$). None of the other drug treatments produced significant changes in plus-maze behavior.

In the forced swim test, total mobility ($F(11, 106) = 1.87$, $p<0.05$; Fig. 6.2.A) was significantly increased by the 27µg dose of the sst2 agonist L-

779,976 (LSD, $p<0.01$), as well as by the 27μg dose of the sst3 agonist L-796,778 (LSD, $p<0.02$). No other drug group was significantly different from the vehicle control group. In the case of the sst2 group (27μg), the increase in mobility corresponded to an increase in swimming behavior (Fig. 6.2.B; LSD, $p<0.01$), whereas for the sst3 group (27ug), the increase in mobility was accompanied by an increase in both swimming (LSD, $p<0.05$) and climbing (LSD, $p<0.06$) behaviors (Fig. 6.2.B and 6.2.C).

4. Discussion

These findings taken together suggest that the anxiolytic-like effects of somatostatin we previously found were due to activation of sst2 receptors: While the administration of a selective sst2 agonist increased open- arm activity significantly above control levels, none of the other somatostatin receptor agonists displayed this effect. The increase in open-arm activity following sst2 agonist administration was comparable to that observed following administration of standard anxiolytic drugs (e.g., diazepam) in our laboratory and others (e.g., Engin et al., 2008; Engin and Treit, 2008a; for reviews of methods and findings, see Treit, 1985; Treit et al., 2003; Engin and Treit, 2008b). On the other hand, the antidepressant-like effect of somatostatin seemed to involve both the sst2 and sst3 receptor sub-types, stimulation of which increased total mobility time in the forced swim test, an index of antidepressant drug action. The increase in mobility observed following sst2 and sst3 agonists was comparable to that observed following standard antidepressant drugs such as the selective serotonin reuptake inhibitor (SSRI) fluoxetine (e.g., Porsolt et al., 1991; Engin et al., 2009). In summary, the behavioral effects observed in the elevated plus-maze and forced swim tests following sst2 and/or sst3 agonists were equivalent to those of reference anxiolytic (i.e., diazepam) and antidepressant (i.e., fluoxetine) compounds, respectively.

Both the increase in open-arm activity in the plus-maze after i.c.v. infusion of the sst2 receptor agonist, and the increased mobility in the swim test after i.c.v. infusion of the sst2 and the sst3 agonists, are not readily explained by nonspecific changes in general activity. In the elevated plus-maze, none of these compounds significantly increased either measure of general activity (total-arm entries, or closed-arm entries), at any dose. This suggests that the changes produced by the sst2 and sst3 agonists were behaviorally selective.

The receptor-specificity of the behavioral effects found in the present study, most of which appeared to be mediated by the sst2 receptor subtype, need to be confirmed by the reversal of these effects with somatostatin receptor antagonists. Be this as it may, the binding affinity of the sst2 agonist L-779,976 for the sst2 receptor is 6000- to 85000-fold greater than its affinity for any other somatostatin receptor sub-type (Rohrer et al., 1998). In comparison, the selectivity of the somatostatin antagonist, CYN154805, is far less (see van der Hoek et al., 2005). Although it is likely that somatostatin receptor antagonists will be developed that are more ideal for characterizing the receptor specificity of our agonist effects, we believe that the selectivity of sst2 agonist L-779,976 is indicated—if not proven-- by its high relative affinity for the sst2 receptor sub-type and by its corresponding behavioral selectivity in animal models of anxiety and depression (present data).

Sst3 mRNA expression as well as immunohistochemical staining for the sst3 receptor itself indicates that it is widely distributed in the rat brain, most notably in the hippocampus and dentate gyrus, the amygdala, several hypothalamic nuclei, frontal and parietal cortices, the olfactory system, the cerebellum, and in brain stem nuclei such as locus coeruleus and raphe nuclei (Breder et al, 1992; Haendel et al., 1999; Hervieu and Emson, 1999; Kong et al, 1994; Perez et al., 1994. See Selmer et al., 2000 for a review and comparison to other sst receptor subtypes). Given the fairly broad distribution

of sst3 receptors throughout the brain, it is not too surprising that they might play a role in behavior, and more specifically, a role in the antidepressant effects of somatostatin. However, the possibility remains that the sst3 receptor agonist used here actually produced its antidepressant-like effect by a non-specific, partial activation of the sst2 receptor, which also produced antidepressant-like effects. While this possibility cannot be ruled out until a suitable antagonist study is conducted, it should be noted here that the binding affinity of L-796-778 for the sst3 receptor is very high compared to its affinity for sst2 receptors (Ki in nM for sst3 = 24, for sst2 > 10,000). Combined with the very high relative affinity of the sst2 agonist used in this study, it seems more likely that the sst3 receptor agonist independently mediated at least some of the antidepressant-like effects reported here.

While our findings suggest that the sst2 and sst3 receptor agonists do not produce significant behavioral effects at doses below 27μg, we were reluctant to test doses higher than 27μg, because our sst4 and sst5 agonists appeared to produce seizures in some animals at doses of 27μg, and even 9μg (non-systematic observations). This seizure-like activity seems counterintuitive considering that somatostatin itself has anticonvulsant-like actions (see Tallent and Qiu, 2008 for a review). However, it should be noted that in rats, the anticonvulsant activity of somatostatin is mediated by sst2 receptors (Perez et al., 1995; Stragier et al., 2006; Vezzani et al., 1991; see Qiu et al., 2005; 2008 for different findings in mice). Moreover, the convulsive-like behavioral activity observed in the current experiment did not show complete correspondence with typical epileptic-like convulsions observed in rats after electroconvulsant shock or pentylenetetrozole (e.g., Pinel et al., 1977). It is possible that in the case of the sst4 and sst5 receptor agonists, we observed tremors resulting from striatal dopamine release. Somatostatin is known to increase dopaminergic activity in the striatum (Hathway et al., 2004; Marazioti

et al., 2008; Mitchell et al., 2000), although the specific receptors that mediate this effect are not known. In the case of sst4 agonists, it is also possible that the presynaptic facilitation of glutamate release caused by an activation of sst4 receptors resulted in seizure-like activity in the brain, and the consequent convulsive-like behavior.

One of the remarkable aspects of somatostatin, in contrast to other anxiolytics, such as the benzodiazepines and other $GABA_A$ agonists, is that somatostatin has both anxiolytic and *pro*-cognitive effects (Engin et al., 2008). If both of these effects of somatostatin are specifically mediated by agonism of sst2 receptors, one might expect that the anxiolytic effect would be reversed by selective antagonism of the sst2 receptors, and that the pro-cognitive effects of somatostatin would also be reversed. While the first expectation has not yet been tested, previous studies have shown that antagonism of the sst2 receptor actually *facilitates* cognitive function (Dutar et al., 2002). Therefore, the specific activation of sst2 receptors may in fact have effects (anxiolysis and anterograde amnesia) similar to those of traditional $GABA_A$ anxiolytics (e.g., diazepam). Specific agonism of sst4 receptor function, on the other hand, results in an enhancement of at least some forms of memory function (Gastambide et al., 2009; Moneta et al., 2002). Thus, it is possible that the anxiolytic effects of somatostatin are mediated by sst2 receptors, while its pro-cognitive actions are mediated by sst4 receptors. Systematic studies involving the agonism and antagonism of specific somatostatin receptors need to be carried out to test these predictions, both in animal models of anxiety as well as models of learning and memory (e.g., McEown and Treit, 2009).

The mechanism of the anxiolytic and antidepressant effects of sst2 receptor activation also needs experimental elaboration. Somatostatin has inhibitory effects in the brain (increased K+ conductance, decreased Ca+ conductance), which are thought to be partly mediated by sst2 receptors (Jiang et al., 2003; Meis et al., 2005). The inhibitory role of somatostatin

interneurons in limbic areas such as the hippocampus, the co-localization of somatostatin within GABAergic terminals in several brain areas (Esclapez and Houser, 1995; Llorenscortes et al., 1992; McDonald and Mascagni, 2002; Xie and Sastry, 1992), and the inhibition of glutamate release following sst2 receptor activation (Baraban and Tallent, 2004; Lanneau et al., 2000), could all contribute to the anxiolytic-like effects of somatostatin (see Engin and Treit, 2008b). In addition, the dense distribution of sst2 receptors in anxiety-related structures such as the amygdala, the septum, the hippocampus, the hypothalamus and the periaqueductal gray (Holloway et al., 1996) also supports a role for sst2 receptors in anxiety.

The antidepressant effect, on the other hand, may involve somatostatin's positive modulatory effects on serotonin release (Munozacedo et al., 1992; Popova et al., 1991). While the somatostatin receptor subtypes that mediate this facilitation of serotonin activity are unknown, the pattern of behavior we observed in the forced swim test following sst2 agonist administration may be informative. The sst2 agonist produced increased swimming behavior with no change in climbing behavior, a pattern that closely matches that observed following specific facilitation of serotonergic neurotransmission (Detke et al., 1995). Both the sst2 and sst3 receptor subtypes seemed to be involved in the antidepressant actions of somatostatin in our study, and it is noteworthy that these subtypes are expressed in the raphe nucleus and locus coeruleus (Selmer et al., 2000), the main sources of serotonergic and noradrenergic innervation of the forebrain, respectively. Thus, it is possible that the modulation of these monoamine systems by sst2 and sst3 receptors leads to the behavioral effects observed in the forced swim test. Experimental characterization of the interactions between the somatostatin, serotonin and norepinephrine neurotransmitter systems may further contribute to, or refine, monoamine theories of depression.

Finally, it seems possible that somatostatin--or more likely one of its synthetic analogues--may ultimately represent a novel and effective treatment of both anxiety and depression. Somatostatin itself has a wide range of effects in both the CNS and the periphery, some of which may not be desirable. When combined with its relatively short plasma half-life, and lack of receptor selectivity, somatostatin may be less attractive as a candidate for clinical use than its synthetic analogues (Pinter et al., 2006). However, while more stable analogs such as octreotide and lanreotide have been used in clinical settings to treat disorders such as inflammation, pain, tumor formation and growth (Carlton et al., 2004; Chrubasik and Ziegler, 1996; de Jong et al., 1999; Hofland et al., 1992; Pinter et al., 2006), and bind with high affinity to sst-2 receptors (Pinter et al., 2006), they do not show high receptor selectivity (Pawlikowski and Melen-Mucha, 2003). Thus, these agents still do not resolve the issue of somatostatin's broad systemic and central actions. Nevertheless, it is reasonable to expect that further preclinical exploration will ultimately yield analogues with considerable receptor subtype selectivity. sst-2 receptor-specific agonists that are safe, and have reasonably long half lives, may be particularly promising candidates for the clinical treatment of anxiety and depression, especially when these disorders are comorbid.

FIGURES AND TABLES

Figure 6. 1. Elevated plus-maze findings following i.c.v. administration of vehicle (DMSO) or different doses of the five somatostatin agonists: a. Mean (±S.E.M.) percentage of open arm entries, b. Mean (±S.E.M.) percentage of open arm time. *Significantly different from the vehicle control group at $p<0.05$, **Significantly different from the vehicle control group at $p<0.01$ in a post hoc LSD test.

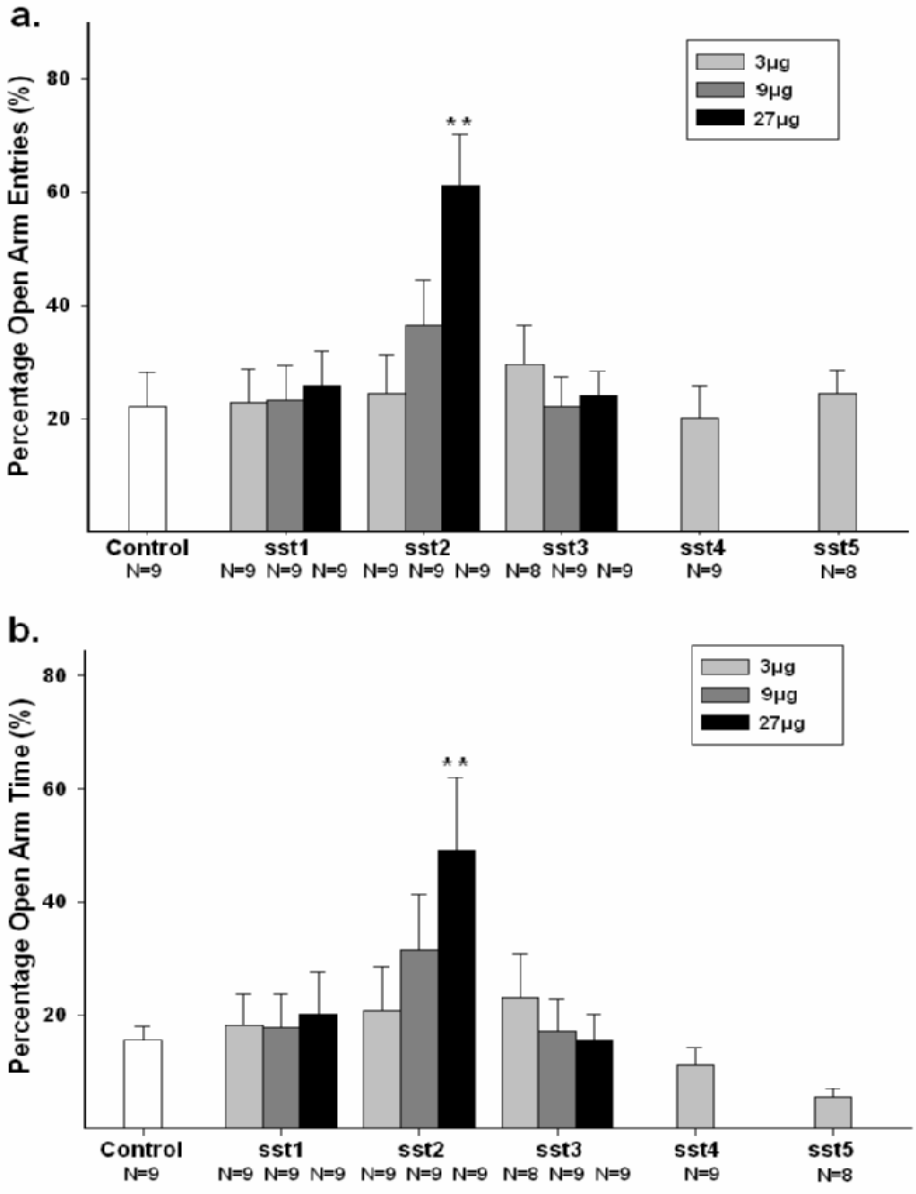

Figure 6. 2. Forced swim test findings following i.c.v. administration of vehicle (DMSO) or different doses of the five somatostatin agonists: a. Mean (±S.E.M.) time spent immobile in seconds, b. Mean (±S.E.M.) time spent swimming in seconds, c. Mean (±S.E.M.) time spent climbing in seconds. †Significantly different from the vehicle control group at $p<0.06$, *Significantly different from the vehicle control group at $p<0.05$, **Significantly different from the vehicle control group at $p<0.01$ in a post hoc LSD test.

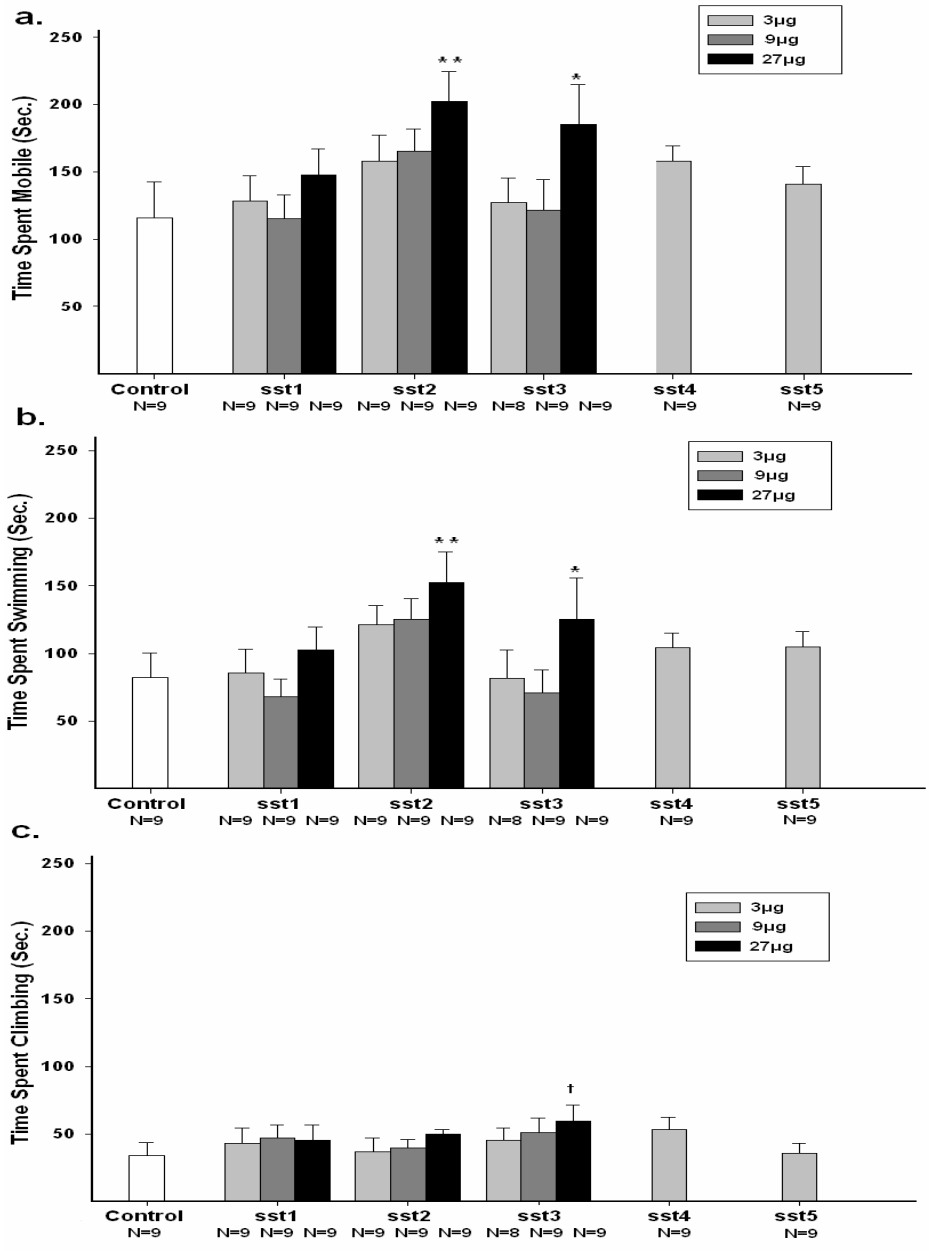

Table 6. 1. Activity measures from the elevated plus maze following the i.c.v. administration of vehicle (DMSO) or different doses of the five somatostatin agonists: Mean (±S.E.M.) number of closed arm entries, Mean (±S.E.M.) number of total arm entries.

Drug	Dose	Closed arm entries	Total arm entries
Vehicle	-	8.89 ± (1.29)	11.33 ± (1.31)
Sst1	3µg	8.89 ± (1.21)	11.22 ± (1.32)
	9µg	9.00 ± (0.69)	12.56 ± (1.56)
	27µg	8.00 ± (0.85)	11.00 ± (0.91)
Sst2	3µg	7.89 ± (1.17)	10.67 ± (1.51)
	9µg	7.11 ± (1.62)	10.56 ± (1.52)
	27µg	4.11 ± (1.14)	9.67 ± (1.31)
Sst3	3µg	9.13 ± (1.23)	13.63 ± (2.01)
	9µg	8.78 ± (0.85)	11.22 ± (0.70)
	27µg	8.44 ± (1.43)	11.00 ± (1.61)
Sst4	3µg	7.56 ± (1.12)	9.11 ± (1.05)
Sst5	3µg	6.75 ± (0.96)	8.75 ± (1.03)

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

1. Overview of the Findings and Future Directions

1.1. Allopregnanolone

Several studies have shown that allopregnanolone has an anxiolytic-like function through the positive modulation of the $GABA_A$ receptor complex (vanBroekhaven and Verkes, 2003). A summary of the current findings is depicted in Table 5.1 together with findings from earlier studies. As seen in the table, several structures such as the septum, the amygdala, ventral tegmental area (VTA) and the medial prefrontal cortex (MPFC) seem to be involved in the anxiolytic actions of allopregnanolone. These findings are consistent with studies that have microinfused $GABA_A$ receptor agonists in these brain areas and found anxiolytic-like effects (e.g., Degroot et al., 2001; Higgins et al., 1991; Menard and Treit, 2001; Pesold and Treit, 1994; 1996; Shah and Treit, 2004).

As seen in Chapter 3, while the amygdala and the MPFC seem to be involved in the anxiolytic actions of allopregnanolone, infusions of allopregnanolone into the dorsal hippocampus did not affect anxiety-related behavior. This finding was unexpected as anxiolytic effects have been reported following the infusion of benzodiazepines in this area, at least in certain behavioral paradigms (Gonzalez et al., 1998; Menard and Treit, 2001). Thus, while allopregnanolone seems to have similar effects to benzodiazepines, current findings suggest that the effects may not be identical.

One explanation for these distinct behavioral effects of benzodiazepines and allopregnanolone following intra-hippocampal infusions might be their different patterns of molecular effects on the $GABA_A$ receptor complex. There is evidence that the allopregnanolone binding site in the α and β subunits of the $GABA_A$ receptor are preserved throughout the α and β subunit families, and thus exist in all $GABA_A$ receptors in the brain (Hosie et al., 2007). Thus, the α_4, and β_1 subunit containing $GABA_A$ receptors, which make up 13% and 20-30% of all $GABA_A$ receptors in the hippocampus respectively

(Sieghart, 2003), cannot be positively modulated by benzodiazepines, while these receptors can potentially be affected by allopregnanolone. Thus, from a molecular point of view, it is possible that benzodiazepines and allopregnanolone have distinct patterns of modulatory actions on different sets of hippocampal $GABA_A$ receptor populations, which may translate into differences in behavioral effects.

Another possibility is that, the dorsal aspect of the hippocampal formation is not directly involved in the expression of unconditioned anxiety responses. Thus, an inhibition of the dorsal hippocampal activity through the positive modulation of the $GABA_A$ receptors in the area may have no effect in the observed anxiety responses. The hypothesis that the dorsal section of the hippocampus is mainly involved in the learning- and memory-related functions of the hippocampus, whereas the ventral section is involved in unconditioned anxiety responses has been discussed in Chapter 1, Section 2.2.6. As mentioned there, there is some evidence supporting the hypothesis (see, for instance, Hackl and Carobrez, 2007), however, the data so far are far from conclusive. A comparative study involving the microinfusion of allopregnanolone into the ventral section of the hippocampal formation could help clarify the issue, at least in terms of the anxiety-related effects of intra-hippocampal allopregnanolone.

Finally, a comparison of Table 7.1 to the brain structures discussed in Chapter 1 shows that there are several brain areas which are known to be involved in anxiety-related processes, where the effects of allopregnanolone have not yet been tested. These include the pariaqueductal gray, hypothalamus, locus coereleus and the raphe nuclei. Future microinfusion studies investigating the effects of allopregnanolone in these areas can provide a full anatomical map of the allopregnanolone effects on anxiety.

1.2. Vasopressin

Several lines of evidence from both clinical and animal studies suggest that the brain vasopressin system is involved in affective responses (Surget and Belzung, 2008; Caldwell et al., 2008). However, few studies have elucidated the role of the Avpr1a and Avpr1b receptor sub-types in emotional responses. The findings from the current study suggest that the role of these different receptor subtypes in anxiety is highly dependent on the brain site in question and the behavioral task employed. More specifically, our studies showed that the antagonism of Avpr1a receptors in the ventral hippocampus reduced anxiety responses in the elevated plus maze, while the same manipulation in the dorsal hippocampus was without effect. The reverse was true for Avpr1b receptors, with antagonism in the dorsal hippocampus reducing anxiety in the same paradigm and antagonism in the ventral hippocampus having no effect. None of these manipulations caused changes in behavior in the shock-probe paradigm (See Table 7.2 for a summary).

This complex pattern may arise from the anatomically distinct distribution of the Avpr1a and Avpr1b receptors in the brain. Even in areas where both receptors are expressed, the receptor densities are generally different for the two receptors (Ring, 2005). Moreover, vasopressin is colocalized with different neurotransmitters in different brain areas, so that modulation of neurotransmitter release and receptor binding may be specific to each of these brain areas. For instance, in the hypothalamus, vasopressin is colocalized with different neuropeptides in different hypothalamic sub-nuclei, as well as with GABAergic neurons in some nuclei but not others (Buijs et al., 1995; Mezey and Kiss, 1991; Walker et al., 2001 Wu et al., 2006), so that predictions about the role of vasopressin in even a single structure such as the hypothalamus is extremely difficult. In contrast, in the frontal cortex and the hippocampus, vasopressin is only colocalized with norepinephrine in some sections but they interact in a complex and selective manner, determining each others' release patterns (Beyer et al., 2004; Brinton et al., 2000). In the bed

nucleus of stria terminalis and the amygdala, a similar interaction is seen between vasopressin and galanin (Miller et al., 1993). As a result of these complex and anatomically-specific interactions, the overall behavioral effects of vasopressin agonists and antagonists are going to be particularly difficult to predict.

In summary, a better understanding of the role of vasopressin and its receptors in anxiety requires the systematical study of the effects of receptor-specific vasopressin ligands in different brain structures. A suitable start for such research would be filling out the blank cells in Table 7.2. However, as mentioned in Section 1.1 a full understanding of the role of this neuropeptide in the brain anxiety circuits will require a much more extensive neurochemical and neuroanatomical analysis. Several brain structures in the hierarchical circuit discussed in Section 1.1, such as the locus coereleus, dorsal raphe nucleus, arcuate, paraventricular and supraoptic nuclei of the hypothalamus are characterized by extensive distributions of vasopressin receptors (Ring, 2005), and thus provide ideal starting targets for this analysis.

1.3. Somatostatin

Several neurophysiological findings suggest that somatostatin has a similar inhibitory profile to GABA (e.g., Arancibia et al., 2001; Batten et al., 2002; Meis et al., 2005; Tallent and Siggins, 1997). However, the involvement of somatostatin in anxiety-related behavior had never before been directly investigated. In the current study, it was shown that somatostatin has anxiolytic actions in the elevated plus maze test when administered intracerebroventricularly (i.c.v.). Moreover, this anxiolytic effect seemed to be a consequence of the interaction of somatostatin with the GABAergic system. Electrophysiologically, somatostatin shared the same characteristics as all classes of anxiolytic drugs (i.e., benzodiazepines, 5-HT_{1A} agonists, SSRIs) suppressing reticularly-evoked hippocampal theta rhythm. Somatostatin also

showed electrophysiological properties that are specific to benzodiazepine anxiolytics. The experiments in Chapter 6 revealed that the anxiolytic actions of somatostatin are mediated by sst2 receptors.

In addition to its anxiolytic effects, i.c.v. administered somatostatin had antidepressant-like effects in the forced swim test. While a GABAergic mechanism seemed to be important for the anxiolytic effect of somatostatin, the antidepressant effect appeared to be GABA-independent. Moreover, sst3 receptors, in addition to sst2, seemed to be involved in the antidepressant actions of somatostatin.

Finally, in contrast to benzodiazepines, somatostatin increased the power of reticularly-evoked hippocampal theta rhythm, an effect common to pro-cognitive drugs. This is in line with earlier studies suggesting an enhancement of memory functions following somatostatin treatment (e.g., Dournaud et al., 1996; Matsuoka et al, 1994). This finding, separates somatostatin from all other known anxiolytic and antidepressant agents, many of which produce cognitive impairments.

In addition to adding to our understanding of the neurochemical mechanisms of anxiety and depression, the above findings suggest that selective sst2 receptor agonists might prove to be useful therapeutic agents in the treatment of anxiety disorders, especially in cases where the disorders are comorbid with depression.

2. Limitations of the present studies

As noted in Chapter 1, the intracerebral microinfusion technique is the most powerful tool currently available for determining the site of anxiolytic drug action in the brain, providing both anatomical and neurochemical specificity. However, like any other technique, it is not without its pitfalls.

One possible problem with microinfusion techniques is the possibility of drug diffusion away from the target area (Engin and Treit, 2008; Menard and Treit, 1999). If drug diffusion to non-target areas of the brain cannot be ruled out, the results may actually reflect drug action outside of the target structure. The risk of such diffusion increases as cannulae targets near the cerebral ventricles. Diffusion or leakage into the ventricles can result in the drug being carried in the circulating cerebrospinal fluid to almost any area in the brain, making the determination of the drug's site-specificity almost impossible. One approach to the general problem of drug diffusion is to infuse the drug in very small fluid volumes (e.g., 0.5µl), as in the current studies. The general usefulness of this tactic, however, also depends on the relative solubility of the compound being infused, since less soluble compounds require larger fluid volumes in which to dissolve. Another important variable is the relative fat solubility of the drug. Compounds that are highly fat-soluble are more likely to diffuse farther than less fat-soluble compounds because they pass through the phospholipid bilayers of cell membranes more easily.

To mitigate these sorts of uncertainties, site- specificity can be independently assessed by infusing the drug into areas of the brain that are just outside of the true target area. If behavioral effects in this "negative" control condition do not mimic those seen in the true target area, then the site-specificity of drug effects within the true target is more firmly supported. Although the design of the present experiments did not include a formal, negative control condition, site-specificity was supported in a post hoc analysis, by examining behavioral effects that occurred after infusions into "misplaced" cannulae. The behavioral effects of drugs infused into the target area were not seen in any of the studies described in this thesis when the same drug was infused into "misplaced" cannulae. It should also be noted that the importance of site-specificity is inversely proportional to the size of the target structures, since the risk of significant diffusion away from a relatively large structure is low compared to diffusion away from a relatively small structure.

In this regard it is important to note that the effects of amygdalar infusions described in Chapter 2 did not occur in the 8 rats with misplaced cannulae. These findings were also consistent with those of an earlier study (Akwa et al., 1999). Finally, neuroanatomical specificity is not an issue when drug infusions are intended to reach all areas of the brain (e.g. i.c.v. infusions; Chapter 4). On the contrary, it is important to use a relatively large fluid volume (e.g., 4µl) so that the drug will be distributed more broadly in the CNS (see Chapter 4).

In almost all of the studies reported in this thesis, two different beahvioral models were used in order to provide converging evidence of anxiolytic-like effects: The elevated plus maze and the shock-probe burying test. However, there were a number of instances when drug effects in the two models sometimes failed to converge (e.g., Chapter 3, intra-MPFC allopregnanolone infusions; Chapter 4, intra-dorsal-hippocampal Avpr1b infusions, intra-ventral-hippocampal Avpr1a infusions). There may be several reasons for these inconsistencies. For one, the shock-probe burying and elevated plus maze models involve fearful stimuli that differ in nature: While the fearful stimulus in the elevated plus maze (i.e., open space) is not painful, the shock probe burying test involves a painful fear stimulus (i.e., electric shock). It is possible that different neural systems in different brain areas are recruited to deal with painful and non-painful fear stimuli, certainly at the level of primary receptors. In fact, many of the earlier studies from this laboratory have demonstrated reliable dissociations between particular infusion sites (e.g., the central amygdala versus the basolateral amygdala) and particular fear responses (e.g., shock-probe avoidances versus open-arm avoidance; Pesold and Treit, 1995). Nevertheless, it seems likely that the plus-maze results demonstrated in this thesis should be replicated in other exploration-based models, such as the light/dark box (Crawley, 1981).

3. Theoretical Implications and Conclusions

The effects of neurosteroids and neuropeptides in unconditioned animal models of anxiety may have theoretical implications for the neural mechanisms of anxiety itself, especially when the neurochemical effects of those compounds are anatomically specified, as in this thesis. The following section places the present results in the context of current neurobiological theories of anxiety.

An early attempt to construct a neuropsychological theory of anxiety was published by Jeffrey Gray in 1982. At the core of Gray's (1982) theory was the correspondence he and McNaughton (Gray and McNaughton, 1983) documented between the effects of hippocampal lesions and the effects of anti-anxiety drugs on conditioned avoidance behaviors (e.g., one-way avoidance). Based on these observations, and the massive neural communication pathways shared between the septum and hippocampus, Gray argued that a "septo-hippocampal system" lay at the heart of the neural bases of anxiety. At about the same time, other researchers, using tests based on Pavlovian fear conditioning, argued that it was the amygdala that played a central role in the control of anxiety, as well as "extended" amygdalar structures such as the bed nucleus of stria terminalis (e.g., e.g., Blanchard and Blanchard, 1972; Davis, 1997; LeDoux, 1998; Walker et al., 2003). Other brain areas that have been implicated in anxiety, either because of their amygdalar connections or because of their independent roles in anxiety, include they periaqueductal gray (e.g., Behbehani, 1995; Graeff, 2004), hypothalamic nuclei (e.g., Muller et al., 2004; Sullivan and Gratton, 2002) and the prefrontal cortex (e.g., Deacon et al., 2003, Shah and Treit, 2004). Monoaminergic systems originating in brain stem areas such as the raphe nuclei and the locus coeruleus have also been thought to modulate anxiety, as emphasized first by Gray (1982), and then by many other researchers (e.g., Graeff, 2002; Lowry et al., 2005; Bremner et al., 1996a,b). Also implicated in anxiety are glutamatergic (e.g., Bergink et al., 2004),

GABAergic (e.g., Kalueff and Nutt, 2007) and opioid (Drolet et al., 2001) neurotransmitter systems. Along with these "classical" neurotransmitter systems, the present thesis encourages the inclusion and study of various neuropeptides and their roles in anxiety.

McNaughton and his colleagues have developed a neural model of anxiety that aims to accommodate these different views of the neural bases of anxiety, and combine them into one, hierarchically-organized and interactive system in the brain, which controls a behavioral hierarchy of animal defense reactions, which themselves serve as a proxy for anxiety disorders (Gray and McNaughton, 2000; McNaughton and Corr, 2004; see Figure 1). As seen in Figure 1, the model can be broken down into a number of separate and, at least in principle, empirically testable hypotheses:

1. *Anxiety/fear reactions can be arranged hierarchically in terms of defensive distance. Short defensive distances (i.e., immediate threats) require the recruitment of lower level anatomical structures (e.g., periaqueductal gray), while long defensive distances (i.e., distant or implied threats) require behaviors organized by higher level structures (e.g., prefrontal cortex).* This proposition is based on the observations of Blanchard et al. (1986) regarding the differences in the behaviors of rats against threats of different proximity. According to these observations, rats engaged in three different levels of defensive behaviors. The first level involves "risk-assessment" behaviors (e.g., stretched-attend) and is observed when the rat is placed in either a novel environment or a context that has been associated with a threat in the past. The second level behavior, characterized by "freezing" (i.e., total immobility except for breathing), is observed when a predator is present, but located at a safe distance, where it poses no immediate threat to the rat (i.e., distal threat). The third level of behavior, which involves flight and defensive attacks, is observed when the predator is sufficiently close to initiate an attack (i.e., proximal threat). According to this idea, behaviors in models that involve an

immediate threat, such as the presence of a predator or a pain-causing object (e.g., a shock probe) as the aversive stimulus should be more readily affected by manipulations targeting lower level brain structures. Behaviors in the presence of an implied threat (e.g., the open spaces in the elevated plus maze) would be controlled by higher level structures. There is some earlier anatomical/behavioral evidence that suggests that risk-assessment behaviors are readily affected by manipulations to the septo-hippocampal system (Graeff, 1994; 2004), whereas the manipulation of the amygdala more readily affects freezing-like responses (Davis, 1986). Flight behavior, on the other hand, seems to be controlled mainly by the dorsal section of the periaqueductal gray (Graeff, 2004). There is also evidence that these different behavioral levels are pharmacologically distinct, as panicolytic and panicogenic agents consistently reduce and increase flight behavior respectively, while having minimal effects on risk-assessment behaviors (Blanchard et al., 2003; Griebel et al., 1995).

In Chapter 3, we reported that manipulation of the amygdalar (i.e., a middle level structure) allopregnanolone system changes behavior in the shock probe burying test (i.e., a distal threat situation, where a stationary threat is present but avoidance of the threat is under subject's control), while the same manipulation in two higher level structures, the hippocampus and the medial prefrontal cortex, had no effect. Similarly, in Chapter 4, vasopressin antagonism in the hippocampus affected behavior in the elevated plus-maze (i.e., longer defensive distance), but not in the shock-probe test (i.e., shorter defensive distance). It should be noted that the assignment of defensive distance to different tests in the above reasoning is somewhat arbitrary, and that only through systematic manipulation of defensive distance in an experiment designed for this purpose can unequivocal evidence can be gathered. Here, we can only report that our findings are not contradictory to the claims of the model, while they also cannot provide strong support.

2. *Defensive approach and defensive avoidance behaviors are controlled by different structures at each level of the hierarchy.* Shock-probe burying test provides a good empirical model for this issue, as noted by McNaughton and Corr (2004) themselves, as it provides separate measures for defensive approach (i.e., burying) and avoidance (i.e., avoidance of probe contacts) in the same test. In Chapter 3, we demonstrated a case of this separation between avoidance and approach, where allopregnanolone infusions into the central amygdala affected avoidance (i.e., increased probe contacts) without changing approach behaviors (see Pesold and Treit, 1994; 1995 for similar findings). In contrast, earlier studies have shown that intra-septal infusions of benzodiazepines reduce approach behaviors (i.e., decreased burying) without affecting avoidance (Pesold and Treit, 1994). A systematic test of approach-avoidance behaviors at different levels of the hierarchy was beyond the scope and aims of the current studies; however, our findings lend support to the idea that approach and avoidance behaviors may be controlled by anatomically distinct structures.

3. *Serotonergic and noradrenergic systems provide diffuse modulatory input to the entire defense system, the nature of which depends on the hierarchical level in question (i.e., lowest level is suppressed by input that activates the highest levels). While serotonin and noradrenalin systems are at the center of the control of the defensive system, GABAergic, glutamatergic and cholinergic systems are also involved in the control of anxiety.* As mentioned in Chapter 1, the involvement of the serotonergic, noradrenergic, cholinergic, GABAergic and glutamatergic systems in anxiety-related behavior has been shown repeatedly (see Engin and Treit, 2008 for a review). However, this seems to be an oversimplification of the neurochemistry of anxiety. For example, there is evidence for the involvement of the dopaminergic system, at least at certain levels of the hierarchy (e.g., medial prefrontal cortex; Shah et al., 2004; Wall et al., 2003). Moreover, the neuropeptide CRF causes a consistent increase in anxiety-like reactions at the low to middle levels of the

anatomical hierarchy (e.g., Kask et al., 2001; Martins et al., 1997; Sahuque et al., 2006; Sajdyk et al., 1999). The findings reported in Chapters 4, 5 and 6 of this thesis suggest further that the McNaughton and Corr model is incomplete in its neurochemical view of the brain anxiety system. As mentioned in the corresponding chapters, the vasopressin and somatostatin systems modulate the activity of other neurotransmitter systems in different brain areas, as well as having direct effects on neuronal function through their own g-protein coupled receptors, and should be included in any complete neurobiological theory of anxiety.

4. *Dysfunction of different levels of the defense system leads to different forms of anxiety disorders.* While the current studies did not distinguish different forms of anxiety, a neurochemically- and neuroanatomically-specific mapping of behavioral effects, as was performed in Chapters 3 and 4, is necessary to address this hypothesis. For example, an overactivation of the vasopressin system in a certain brain structure may increase freezing behavior, which is often employed as an animal model of panic (Brandao et al., 2008). In current studies, we have shown that an inhibition of the vasopressin system in the hippocampus may lead to a disinhibition of exploratory behavior, which may indicate a decrease in generalized anxiety (i.e., the disorder is characterized by aversion) in McNaughton-Corr model.

McNaughton and Corr's (2004) two-dimensional model of defense is the most recent and most inclusive model of anxiety, accommodating a great deal of current empirical knowledge of the neural bases of anxiety. The studies reported in the current thesis seems to lend at least partial support for some aspects of this model, while making it clear that the model is incomplete with respect to the neurochemical control of anxiety. In particular, the role of neuropeptide neurotransmitters in anxiety has been omitted from the model, in spite of the fact that the current studies, as well as earlier work, provide clear

evidence that at least some neuropeptides (e.g., CRF, substance P, vasopressin and somatostatin) play important roles in the control of anxiety states. The exact nature of the involvement of these neuropeptides, as well as that of neuromodulators such as neurosteroids, should be clarified in future studies and the theoretical models should be modified to accommodate these findings.

FIGURES AND TABLES

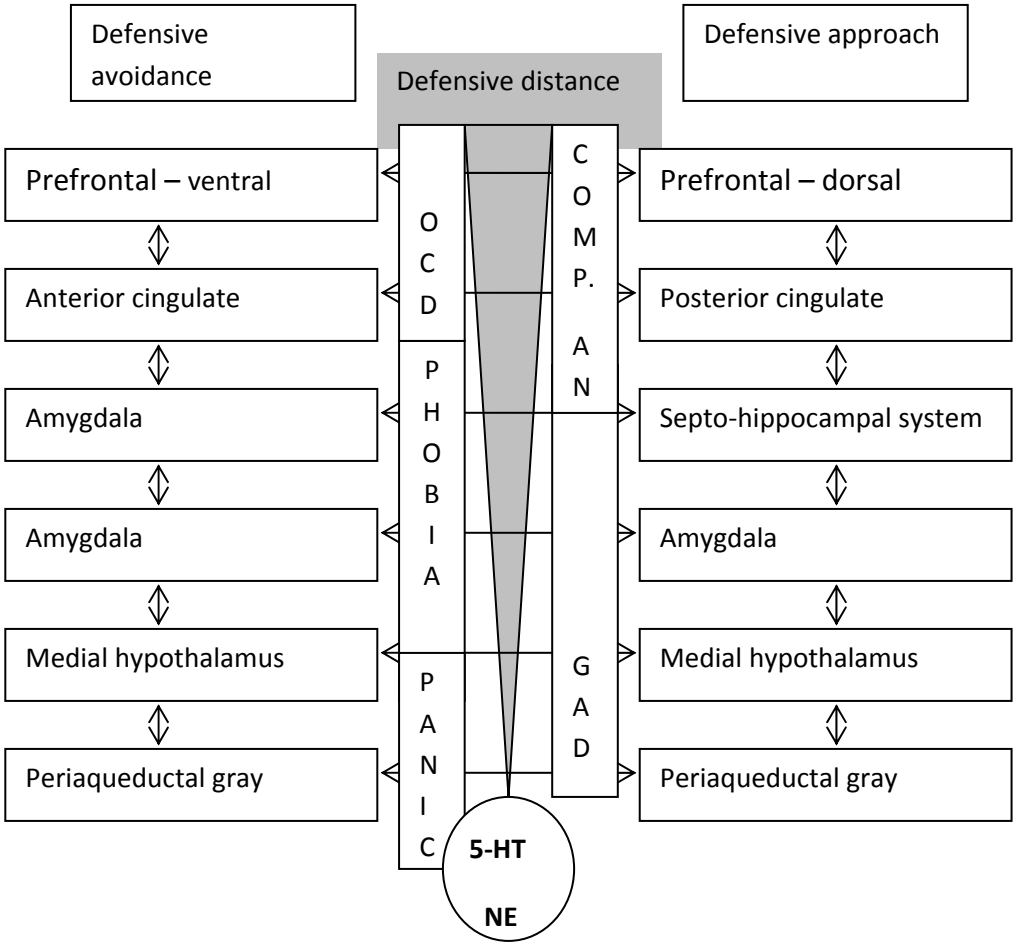
Table 7. 1: Summary of findings from Chapter 2 in the context of earlier studies. EPM: elevated plus maze, MPFC: medial prefrontal cortex, OF: open field test, SPB: shock-probe burying test, VC: Vogel conflict test, VTA: ventral tegmental area.

Brain Area	Dose	Model	Finding	Reference
Amygdala	8µg/side	EPM	(-)	Akwa et al., 1999
		VC	(-)	
	8µg/side	EPM	(-)	Current study
		SPB	(-)	
Septum	1µg/side	VC	(-)	Molina-Hernandez et al., 2003
Hippocampus	0.2µg/side	OF	(0)	Martin-Garcia and Palares, 2005; 2008
	14µg, 20µg/side	EPM	(0)	Current study
		SPB	(0)	
VTA	0.1µg/side	EPM	(-)	Frye and Rhodes, 2008
		OF	(-)	
MPFC	8, 14, 20µg/side	EPM	(-)	Current study
		SPB	(0)	

Table 7. 2: Summary of findings from Chapter 3 in the context of earlier studies that employed microinfusions of different Avpr1a and Avpr1b antagonists. EPM: Elevated plus maze, SPB: shock-probe burying test.

Brain Area		Infused compound	Model	Finding	Reference
Amygdala	Central	Avpr1b antagonist	EPM	(0)	Salome et al., 2006
	Basolateral	Avpr1b antagonist	EPM	(-)	Salome et al., 2006
	Medial	Avpr1b antagonist	EPM	(0)	Salome et al., 2006
Septum	Lateral	Avpr1b antagonist	EPM	(0)	Stemmelin et al., 2005
		Avpr1a antagonist	EPM	(+)	Everts and Koolhaas, 1999
			SPB	(0)	
	Medial	No data	No data	No data	
Hippocampus	Dorsal	Avpr1a antagonist	EPM	(0)	Current study
			SPB	(0)	
		Avpr1b antagonist	EPM	(-)	Current study
			SPB	(0)	
	Ventral	Avpr1a antagonist	EPM	(-)	Current study
			SPB	(0)	
		Avpr1b antagonist	EPM	(0)	Current study
			SPB	(0)	

Figure 7. 1: A summary of the McNaughton and Corr model of defensive behaviors. 5-HT: serotonin, Comp. An.: Complex anxiety (e.g., social anxiety), GAD: general anxiety disorder, NE: norepinephrine, OCD: obsessive-compulsive disorder.



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