NATURALLY OCCURRING VARIATIONS IN DEFENSIVE BURYING BEHAVIOR ARE ASSOCIATED WITH DIFFERENCES IN CENTRAL NEUROPEPTIDE EXPRESSION IN THE MALE RAT

by

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ABSTRACT

The shock prod defensive burying test has proven incredibly reliable and instrumental in determining the underpinnings of normal anxiety in rodents. Largely ignored in tests of defensive burying, however, is the capacity for individual animals to display marked variations in active and passive coping behaviors. To unmask the neurobiological correlates of this behavioral differentiation, rats were exposed to a mousetrap that was remotely triggered upon approach to remove the quality of pain. This design invited striking variations in defensive burying behavior levels, in which some rats either buried robustly or showed little to no levels of defensive burying. Furthermore, differences in burying behavior were associated with marked differences in the central expression of arginine vasopressin (AVP) and oxytocin (OT). Thus, relative to animals showing no significant levels of defensive burying activity, rats showing sustained elevations in defensive burying expressed higher levels of AVP mRNA and increased numbers of androgen receptor positive cells in the medial amygdala and posterior bed nuclei of the stria terminalis, brain regions that integrate emotional appraisal and sensory information. In contrast, animals showing little to no defensive burying responses expressed relatively higher levels of AVP and OT mRNA within the supraoptic nucleus and subregions of the paraventricular nucleus of the hypothalamus responsible for neuroendocrine and autonomic function. CRH mRNA levels did not vary as a function of burying activity in the central nucleus of the amygdala, the anterior division of the bed nuclei of the stria terminalis, nor in the paraventricular nucleus. These findings suggest a role for central AVP and OT in mediating differential defensive behaviors, and demonstrate the utility of using a pain free test of conditioned defensive burying as a framework for exploring individual differences in behavioral coping and neuroendocrine capacity.
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1. Introduction

In our daily grind we are constantly bombarded with situations that take us outside our comfort level. There is pressure put on us from our surrounding environment: school, work, relationships, and family. This pressure materializes in our bodies as stress. Stress does not always entail distress; thrill rides, such as bungee jumping or skydiving, or even an exam may be exhilarating for some yet cause fear and anxiety for others. Increased stress increases productivity to a point, after which there is a rapid deterioration. It is this point where marked differences appear between individuals. How we deal with stressful situations shapes our future, that is, we are capable of learning from stress. Animals, like humans, live in a dynamic and complex environment in which they have to find food, deal with conspecifics, and react to environmental changes. Animals, also like humans, probably experience some form of stress brought about by these challenges. As stress is subjective, however, we can only assume stress in animals based on putative behavioral and physiological correlates. Whether good or bad, stress permits us to deal with extraordinary situations in our ordinary lives.

1.1 Stress

The concept of stress is first discussed to provide a general background for understanding the basis for individual differences in stress responsiveness and predisposition to disease, and to provide a framework for implicating central neuropeptides on individual differences in neuroendocrine and behavioral responses to stress. Hans Selye coined the term "stress" in 1936, defined as "the non-specific response of the body to any demand for change". Selye noted, in numerous experiments, that laboratory rodents subjected to acute noxious physical and emotional stimuli (for example, blaring light, deafening noise, extremes of heat or cold, perpetual frustration) all caused the same pathologic changes, which includes stomach ulcerations, shrinkage of lymphoid tissue, and enlargement of the adrenal glands. Thus, persistent stress could cause these animals to develop various diseases, believed at the time to be caused by specific, but different, pathogens. Selye postulated that these responses could be broken into three stages defined as the “General Adaptation Syndrome”. Stage one: Alarm brings about the fight or flight response and activation of the HPA axis to produce elevations in glucocorticoids and mobilize energy stores. Stage two: As the stressor persists, it becomes necessary to attempt some means of coping, in which resources become gradually depleted. Stage three: Resources
are depleted and the body is unable to maintain normal function and homeostasis. If the body’s defenses are exhausted, long-term damage may result manifesting in obvious illnesses such as ulcers, cardiovascular problems, and depression along with other mental disorders. While far different from modern definitions of stress, Selye’s stages of stress remind us that stress is a very natural and normal process that should only be thought of as detrimental when it comes at the expense of normal body function. In other words, without experiencing stress, organisms would not be capable of adapting to and interacting with their environment.

Most understand that stress is a natural component of our every day lives, yet find it stressful to define the term stress. Understandably, there is considerable ambiguity when it comes to its meaning, as stress is such a highly subjective phenomenon that it defies definition. Traditionally, stress is thought of as a real or perceived threat to homeostasis, where homeostasis is the dynamic equilibrium that sustains a constant internal environment. Actual threats to homeostasis activate immediate responses, while perceived threats act in anticipation of a threat. Beyond attempting to define the term stress, what is most important is the realization that under normal conditions the brain is responsible for assessing the quality of an aversive stimulus and processing the appropriate response, be it behavioral and/or physiological.

What also deserves emphasis is that the brain is also a target of stress along with many other organ systems in the body, including the immune, metabolic, cardiovascular and reproductive systems. So-called “stress” hormones (glucocorticoids, discussed below) are capable of mediating both adaptive and maladaptive responses. They are protective over the short term and yet destructive over the long term when they are allowed to persist at elevated levels. The brain is capable of adjusting to anticipated and repeated stress by adapting, including morphological and functional changes in neuronal activity, ultimately involving changes in neurotransmitter release centrally as well changes in hormone release in the periphery (McEwen, 2000). The latter neuroendocrine response can feedback on the brain to produce behavioral effects, including effects on arousal, alertness, vigilance, focused attention, and cognitive processing (De Kloet et al., 2005). In summary, stress is essentially an adaptive response that serves to induce changes within the central nervous system, but left unchecked can produce effects detrimental to long-term health.
1.2 Anxiety and fear

Fear and anxiety are heavily intertwined with the body's response to stress, and are equally difficult to define. Emotion has been defined as feelings in humans that are consciously perceived. In animals we can only infer and study variables that may represent components of this human trait (Kabbaj, 2004). The emotion of fear has often been equated with studies of stress and anxiety in laboratory settings. We experience fear automatically when confronted with natural threats, however most of the things we fear are learnt through experience. Studies of fear conditioning in laboratory animals suggest that emotionality is a process of stimulus appraisal that is mediated by or impacts limbic and limbic associated areas in the brain (Le Doux, 2000). A state of stress is thought to be a product of fear, in which the quality or severity of the stressor is determined by the extent to which a stimulus source is cognitively appraised as aversive.

Anxiety is thought to be a form of fear that persists in the absence of any direct or immediate threat. As such, these terms are often used as one in the same. Anxiety is thought to be an essential emotion, which is highly conserved during evolution and which acts to coordinate response systems to face general distress (Gross and Hen, 2004). Like stress, anxiety is adaptive if and when it is appropriately used to prepare the individual for imminent danger (Rosen and Schulkin, 1998). And just like stress, anxiety may be pathological if it continues to persist in the absence of actual threats and/or is so overwhelming that the individual cannot function normally. Why stress is capable of overwhelming some individuals and not others remains poorly understood, however differences in an animals coping behavior may provide insight.

1.3 Coping style

A wide variety of studies in humans and animals demonstrate that individual subjects can differ considerably in their vulnerability to stress related disorders. Differences in coping style may form an important basis for this differentiation. The term coping is used to define the efforts made by the organism to successfully manage the demands of stress or a perceived source of danger. The type or form of coping executed by the individual likely involves some component of cognitive appraisal and the degree to which an aversive stimulus source is deemed controllable. Physiological and behavioral responses to stress are thought to occur in parallel, and can be coupled into distinct suites of correlated traits or stress coping styles. Coping styles
have been defined as “a coherent set of behavioral and physiological stress responses, which is consistent over time and which is characteristic to a certain group of individuals” (Koolhaas et al, 1998). Coping, if successful, is thought to be shaped by evolution and to be an adaptive response. Moreover, recent studies are beginning to show that coping style has a profound influence on the capacity of the individual to adapt to subsequent challenges (Borta et al., 2006; Kabbaj et al., 2000; Ray and Hansen 2004, Walker et al., 2007). Understanding these individual differences in coping ability has become a prominent task in biological psychiatry and stress research (Korte et al., 2005).

Stress and anxiety are normal and adaptive, although they have the capacity to overwhelm. Individual animals may respond differently to a similar stress due to differences in appraisal, which may very well depend on genetics, previous experience, or the animal’s current arousal level. Responses are activated in two ways by real or perceived threats. Real threats, such as visceral, somatic, and pain, for example, represent challenges to homeostasis that directly activate sensory pathways that initiate autonomic and hypothalamic-pituitary-adrenal (HPA) responses. Perceived threats generate a response in anticipation or recognition of danger, either innate or learnt. In these cases it is not the physical aspect of a stressor but the cognitive appraisal of the stimuli, which initiates the response.

1.4 The hypothalamic-pituitary-adrenal (HPA) neuroendocrine axis

What remains a constant in most stress conditions, either real or perceived, is that the brains response involves the activation of the HPA axis. The HPA axis refers to an intricate set of direct influences and feedback interactions between: the hypothalamus, located below the thalamus; the pituitary gland, a pea-shaped structure located below the hypothalamus that sits in a small bony cavity (sella turcica) at the base of the brain; and the adrenal gland, a small, paired, pyramidal organ usually located on the top of each kidney. Species from humans to the most ancient organisms share several components of the HPA axis. Threats activate several central components of the HPA axis responsible for coordinating the behavioral, autonomic, and neuroendocrine response to stress. The HPA axis also regulates various body processes including digestion, the immune system, mood, sexuality, and energy mobilization.

The paraventricular nucleus (PVN) of the hypothalamus is the gatekeeper of the HPA axis. The neuroendocrine arm of the PVN that ultimately regulates the release of glucocorticoids
from the adrenal gland resides within cells occupying the mediodorsal parvocellular part of the nucleus (Sawchenko et al., 1996). These neurons project to the pituitary portal external zone of the median eminence. Upon activation, a cocktail of neuropeptides are released from their stores in the median eminence, and corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) are the most prominent regulators of the HPA axis. CRH and AVP are transported to the anterior pituitary corticotropes through the pituitary portal blood system. At the level of the anterior pituitary, CRH is required to stimulate both the synthesis and release of adrenocorticotropic hormone (ACTH), while AVP interacts with CRH to potentiate the ACTH secretory response (Antoni et al., 1993). ACTH is transported systemically to the adrenal cortex of the adrenal gland, where it stimulates the synthesis and release of glucocorticoids. Stress-induced elevations in plasma glucocorticoid concentrations serve to meet metabolic and physiological demands of homeostatic threat, as well as alert the organism to environmental or physiological challenges.

Glucocorticoids are recognized and bound by two subtypes of receptors that are expressed throughout the body and the brain. These are known as the mineralocorticoid receptors and glucocorticoid receptors, or glucocorticoid type I and type II receptors, respectively. Glucocorticoids and their receptors bind to form a complex that can act to modify transcription of key regulatory proteins or cell signaling processing. In the end, glucocorticoids act to mobilize energy (glycogenolysis) at the expense of immunity, bone and muscle growth, and the reproductive system. Under chronic stress conditions, these same systems are compromised, ultimately leading to several types of physiological, neurodegenerative, and psychological disorders, such as depression and anxiety (Dallman, 2006). Therefore, while acute glucocorticoid responses are initially adaptive, sustained glucocorticoid release is maladaptive. Thus, it is by no coincidence that the HPA axis is involved with so many different types of mood disorders and physiological disturbances, including anxiety disorder, bipolar disorder, post-traumatic stress disorder, clinical depression, burnout, chronic fatigue syndrome and irritable bowel syndrome (Barden, 2004; Johnson and RN, 2006; Shelton, 2007; Young, 2004).

Excitation of the HPA axis is driven by several central brain-circuits to the PVN motor neurons. Brainstem catecholamine producing pathways project directly to the CRH producing pathways of the PVN, where they are thought to activate via alpha-adrenoreceptors on the PVN. Physical challenges, be it hemorrhage, oxygen depletion, or compromised immunity, activate the
HPA axis via this pathway. For example, lesions of the ascending brainstem pathways inhibit Fos activation of hypophysiotropic neurons in the PVN in response to immune challenge (Li et al., 1998). HPA stimulatory input can also be communicated by way of the amygdala, and this pathway has been shown to initiate behavioral and cardiovascular responses to perceived stress (Davis, 1992). These effects appear to be mediated specifically by the centromedial and lateral nuclei of the amygdala as electrophysiological stimulation of these nuclei elicits corticosterone secretion (Dunn and Whitener, 1986). c-fos activation within these amygdala nuclei is also increased during restraint and swim stress (Cullinan et al., 1995). Lesions of these regions are capable of blocking the ACTH and corticosterone responses to acoustic, photic stimulation, restraint, and fear conditioning (Feldman et al., 1994; Van de Kar et al., 1991). The bed nucleus of the stria terminalis (BST) can also traffic stimulatory and inhibitory input to the PVN (Choi et al., 2007). This structure links structures such as the amygdala, hippocampus, and lateral septum, with regions responsible for controlling vital functions, including the PVN, various sub-regions of the hypothalamus, and the brainstem (reviewed in Herman et al., 2003; Herman and Cullinan, 1997). The BST is thus the link between the limbic structures and the PVN creating a limbic stress pathway. The anterior division of the BST provides a CRH-ergic link between the central nucleus of the amygdala and the PVN (Champagne et al., 1998), while posterior BST provides an AVP-ergic link between the medial amygdala and the PVN (Herman and Cullinan, 1997).

The limbic stress pathways, including these CRH- and AVP-ergic pathways to the PVN, are most sensitive to stressors involving higher order sensory processing or cognitive appraisal. HPA responses to restraint, fear conditioning, or exposure to a novel environment are all affected by lesions of the hippocampus or amygdala (Herman et al, 2003). These stressors all require the gathering, assembly and processing of signals from multiple sensory modalities prior to activation of stress response. None of the aforementioned stress paradigms involve immediate threat to survival, but rather represent stimuli that increase or decrease in impact only by comparison with previous experience. Once these threats are processed, information gathered is then diverted to multiple limbic structures capable of augmenting or diminishing the subsequent HPA response (Herman and Cullinan, 1997). In contrast, information regarding immediate threats to homeostasis and survival are directly and rapidly relayed to the PVN by way of the brain stem, bypassing the need for limbic and cognitive processing.
As mentioned earlier, because chronic elevations in circulating glucocorticoid levels can have detrimental effects, HPA axis activity must be maintained within tolerable limits. This is achieved by a process known as glucocorticoid mediated negative-feedback inhibition. While glucocorticoids can act via their receptors in the PVN to inhibit the HPA axis directly, glucocorticoid feedback regulation also occurs above the level of the PVN. Thus, deafferentation of the PVN results in increased expression of CRH and AVP under basal conditions, indicating that glucocorticoid sensitive inhibitory inputs to the nucleus are required for maintenance of basal HPA tone (Herman et al., 1990). Increases in glucocorticoids, as seen during chronic stress, are unable to prevent the increased synthesis of CRH and AVP within parvocellular neurons of the PVN despite the availability of glucocorticoid receptors. This suggests that there must be other forms of neuronally mediated negative-feedback inhibition (Sawchenko et al., 1996). The hippocampus is a specific site of interest, as it displays the highest level of both glucocorticoid and mineralocorticoid receptors of any brain structure (Herman, 1993). An inhibitory role for the hippocampus is supported by the finding that lesioning the hippocampus increases CRH and AVP expression in the PVN, while stimulation of the hippocampus results in decreased levels of corticosterone (Herman et al., 1995). Other limbic-related structures appear to participate in this negative feedback regulation, including the prefrontal cortex, lateral septum, amygdala, and the posterior division of the BST (Cullinan et al., 1995; Herman et al., 2003).

1.5 Neuropeptide regulation of neuroendocrine function and behavioral coping responses

Fear and anxiety circuits in the brain have evolved to protect the organism against potential threats. While some of these circuits are hard wired or reflexive, higher order animals such as mammals are capable of learning and anticipating problems rather than reacting to them. This is due to cognitive and emotional influence imparted by the limbic forebrain. Many signaling pathways are responsible for regulating anxiety, and neuropeptides likely play a role in this process as suggested by the prominence of their receptors throughout the limbic system and the diverse roles of neuropeptides in inter-neuronal communication. Neuropeptides are biologically active sequences of three or more amino acids that are produced in and released from several brain nuclei. Neuropeptides are released from axonal synapses and can thus communicate traditionally with their postsynaptic receptors. However, unlike classical
neurotransmitters, they are also released dendritically, and are thus capable of diffusing over great distances (up to 1mm), essentially freeing the brain from the constraints of wiring.

The regulation and influence of individual differences in coping styles on stress adaptation appear to be mediated by multiple neuropeptide systems and in several brain regions implicated in fear and anxiety in both experimental animals and in humans. Foremost among these include corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) emanating from the amygdala and bed nuclei of the stria terminalis and forebrain oxytocin (OT) projections originating in the paraventricular nucleus of the hypothalamus. In humans and rodents CRH and AVP may contribute to extreme trait anxiety and the co-morbid expression of depression-like symptoms, while OT appears to possess anxiolytic properties. All of these neuropeptides have been shown in a variety of animal models to coordinate, if not contribute to, individual differences in behavioral and adrenal responses to challenging situations.

**1.5a Arginine vasopressin**

The central arginine vasopressin (AVP) system is composed of three major components. First, the hypothalamic neurohypophyseal tract, originates from magnocellular neurons of the supraoptic nucleus and the paraventricular nucleus (PVN) of the hypothalamus. These nuclei project to the posterior pituitary, and upon stimulation AVP is secreted into the general circulation to facilitate water reabsorption and glycogenolysis. Second as discussed earlier, AVP is also involved with the HPA axis; it is produced by the medial parvocellular neurons of the PVN and directed at the median eminence and anterior pituitary. Third, AVP is expressed by a variety of brain nuclei that are responsible for mediating emotional appraisal, avoidance and anxiety behavior, and within brain regions capable of regulating the HPA axis indirectly (Landgraf and Neumann, 2004).

AVP is a nonapeptide that is produced in the PVN, the posterior part of the BST, the supraoptic nucleus (SON) of the hypothalamus, and within the medial nucleus of the amygdala (MeA). The MeA and BST project to multiple limbic regions, including the septum and ventral hippocampus (Caffe et al., 1987; De Vries and Buijs, 1983). AVP synthesis and transport to these limbic brain regions is very sensitive to the stimulatory effects of testosterone (De Vries et al., 1984). This could likely represent a mechanism for individual differences in behaviors associated with central AVP activity (Everts and Koolhaas, 1997).
The effects of AVP are mediated through two receptors; V1a receptors are most prominent throughout the limbic system and basal ganglia and are distributed within various hypothalamic nuclei, including the PVN. The V1b receptor subtype is distributed in the anterior pituitary and within the amygdala, hypothalamus, and hippocampus (reviewed in Tribollet et al., 1998). As both AVP receptor subtypes are plentiful within the limbic system, including those regions trafficking information to the HPA axis, this suggests an involvement of centrally released AVP in stress related behavior and neuroendocrine function (Tribollet et al., 1998). Of note, both receptor subtypes have been shown to regulate anxiety like behaviors (Landgraf et al., 1995; Griebel et al., 2002).

The AVP system was once thought to primarily be involved in cognition, mainly the consolidation of aversive memories and contextual learning (McEwen, 2004). However, central AVP pathways are now thought to play an essential role in several forms of limbic mediated emotional appraisal. Selective down regulation of V1a receptors in the septum via antisense targeting lead to decreased cognitive processing and reduced anxiety-like behaviors in the rodent, as measured on the elevated plus maze (Landgraf et al., 1995). Conversely, animals with AVP injections into the lateral septum show decreased open arm entries. Taken together, these findings suggest that endogenous AVP exerts anxiogenic effects at the level of the septum (Liebsch et al., 1996). It is quite possible that AVP acts at the level of the septum to coordinate contextual learning and memory as well as emotionality to determine an adequate behavioral response to environmental demands (Landgraf, 2005). Rodents selected for high anxiety behavior (HAB) show increased AVP mRNA expressions and release in the PVN than their low anxiety (LAB) counterparts (Murgatroyd et al., 2004; Wigger et al., 2004). This behavioral differentiation is attenuated by microdialysis injection of an AVP V1a/b antagonist into the septum of HAB rats (Wigger et al., 2004).

1.5b Corticotropin-releasing hormone

The corticotropin-releasing hormone (CRH) is responsible for mediating behavioral (Heinrichs and Koob, 2004), neuroendocrine (Smith and Vale, 2006), and autonomic (Dunn and Berridge, 1990) responses to stress. CRH is composed of 41 amino acids and is found in many regions of the brain (Vale et al., 1981). In addition to its role in regulating the HPA axis at the level of the PVN, central CRH also emanates from the anterior division of the BST and the
central nucleus of the amygdala (CeA). Thus, central CRH likely integrates autonomic and behavioral responses to stress within multiple brain regions (De Souza, 1995; Makino et al., 1999). There are at least two CRH receptor subtypes, including CRH R1 and CRH R2, and both of these have been shown to mediate anxiety related behaviors (Aguilera et al., 2004). CRH receptors are distributed throughout the brain, including within the hippocampus, cortex, olfactory pathways, amygdala, and brain stem regions processing stress information and mediating autonomic function (Koob and Heinrichs, 1999). Central administration of CRH can increase anxiety related behavior in rodents (Heinrichs and Joppa, 2001). CRH R1 appears to be involved with cognitive components of behavior, while CRH R2 is involved with more primal types of behavior, including reproduction and feeding. CRH mRNA levels in the PVN and amygdala have shown to increase in animals exposed to anxiety provoking stimuli (Makino et al., 2002). Animals bred for high and low anxiety traits; show no differences in CRH expression in the PVN and amygdala, arguing against a role for central CRH in mediating different anxiety traits. However, higher levels of CRH R2 have been detected in high anxiety bred rats (Wigger et al., 2004). Nevertheless, changes in CRH expression and responses to exogenous CRH have been observed in humans suffering from anxiety and depression (reviewed in Heinrichs and Koob, 2004).

1.5c Oxytocin

While CRH and AVP are the most prominent of the neuropeptides in the PVN regulating ACTH and glucocorticoid release, oxytocin (OT) cells in the PVN have been shown to influence the magnitude of the ACTH response, and under certain conditions this influence may be inhibitory. We now understand that OT is produced within different regions of the PVN and within the supraoptic nucleus (SON) of the hypothalamus. Moreover, the sole source of forebrain projecting OT neurons originates within specific subsets of neurons in the PVN. In addition, OT secreting neurons in the SON are also capable of reaching several targets in the brain by way of dendritic release and entry of the peptide into the ventricular system. Similarly, evidence has also shown that AVP may also gain access to far reaching areas of the brain, areas with no vasopressinergic projections, via this route (Barberis and Tribollet, 1996). OT is released both peripherally and centrally in response to physical and psychological stress (reviewed in Neumann, 2002). During forced swim exposure and social defeat in the rodent, OT
is released into the pituitary portal system and extracellular levels of the peptide are elevated in the septum and in the amygdala, as detected by microdialysis (Wotjak et al., 1996; Englemann et al., 1999). Injection of OT into the ventricular system, lateral septum, PVN, and amygdala cause a decrease in HPA activity and behavioral responses to stress (Neumann et al., 2000). These results suggest that endogenous OT is capable of integrating and inducing in the brain a general inhibitory effect on both the neuroendocrine and behavioral responses to stress. Depending upon the stimulus intensity and type, neurons within the PVN and SON are capable of regulating their peripheral (axon terminals) and central (dendritic) release of AVP and OT in a coordinated or independent manner (Landgraf and Neumann, 2004). Taken together with the widespread distribution of OT receptors throughout the limbic system, we now understand that central OT is capable of contributing to learning, memory, emotionality, and behavioral coping style (Engelmann et al., 1996; Ebner et al., 2005), in addition to modulating the HPA axis (Neumann et al., 2000; Neumann, 2002).

In view of the wide ranging effects of CRH, AVP, and OT on anxiety related behaviors, one can hypothesize that, similar to the manner by which the HPA axis is regulated, neuropeptides may shape to various degrees emotionality depending on the intensity and quality of the aversive stimulus that the organisms is exposed to (Landgraf, 2005). As there are many neuroanatomical parallels in rodents and humans, particularly those involving the neuropeptidergic circuits of the hypothalamus and limbic system mediating HPA function and anxiety, the rodent provides an excellent model for determining the paths of pathological anxiety in humans (Landgraf, 2005). As will be discussed below, determining an appropriate behavioral model for examining individual differences in coping styles is crucial to understanding the underpinnings of stress, anxiety, and emotional behavior.

1.6 Individual differences in coping style

Intrinsic to any discussion of behavior is the concept that individuals display remarkable differences in behavior in response to the same stimuli. In humans, behavioral differences have long been recognized as a tool for investigating the roots of neuropsychological disease. Rats appear to also display behavioral differences. This can be used to examine normal and extremes of anxiety related behavior. For example, Valenstein et al. (1972) showed that rats responding to electrical stimulation of the lateral hypothalamic area displayed consistent differences in their
behavioral responses in exploratory and freezing behaviors in an open field study. Dantzer et al., (1988) showed that animals selected for ingestive behaviors displayed differences in freezing behavior in the resident intruder paradigm. Similarly, individual differences in drug self-administration have been shown to correspond to novelty seeking behavior (Piazza et al., 1989). In fact, novelty-seeking behavior has also been correlated with different types of behavioral strategies in tests of anxiety (Kabbaj et al., 2000). These studies support the notion that individual differences in behavior can be used as a means for investigating the neurophysiological and neuropsychological underpinnings of behavior. It is of note that variability in testing is frequently observed in animals, but is typically disregarded as simply representing experimental error or representing the natural response range of a given species. While this could be true in some cases, individual differences in behavior could still nonetheless contribute to the within experiment variability encountered.

Modeling the basis for individual differences in stress-induced psychopathology and recovery in animal studies is difficult, on both practical and theoretical levels, if only because normal subjects are used in most cases. Thus, there exists a real need to tackle this problem in animal models of experimentally induced or innate differences in psychological state (as indicated by behavioral correlates). When confronted with a threatening stimulus, animals show a blend of behavioral responses that depend on the physical characteristics of the stressor and the degree to which it is appraised as aversive. The ability to cope or successfully meet the demands of unexpected threats rests, therefore, somewhere within the organism’s capacity for proper cognitive assessment and the ability to adapt to different environmental conditions.

1.7 The defensive burying paradigm

During traditional behavioral tests the rodent's behavior is generally limited to freezing, fleeing, or fighting. However, Hudson (1950) discovered that rats buried certain objects with bedding material when they encountered them for the first time. Defensive burying behavior has been observed under more natural settings in the wild (Calhoun, 1962) and is now the subject of several different behavioral paradigms in the laboratory (reviewed in De Boer and Koolhaas, 2003). These observations support the idea that burying behavior is naturally occurring and an emotionally motivated behavioral reaction of rats. It was not until 1978 that John Pinel brought the defensive burying paradigm to light, based on a seminal observation that rats previously
removed from the cage and injected with a toxin would block or defend the entrance to their cage with bedding. He then observed that rats were capable of burying a non-aversive prod if they were previously shocked by it during first encounter. Since then, the shock-induced defensive burying paradigm has been extensively used to determine the basis of normal anxiety in the rat.

Defensive burying refers to the rodent behavior of displacing bedding material with alternating forward pushing movements of the forepaws and shoveling movements with the head directed at localized sources of aversive stimulation or threat (Pinel et al., 1978; also reviewed in De Boer and Koolhaas, 2003). Rats appear to bury any stimulus source that is harmful, unfamiliar, or noxious. By burying sources of aversive stimuli animals can successfully avoid or remove the threatening stimulus from their environment. Defensive burying can thus be added to the list of flight, freezing, and fighting as unconditioned species-specific defensive reactions that are readily and innately available to the animal's repertoire.

In the laboratory, defensive burying can be observed in the animal's home cage or in a familiarized test chamber with ample bedding material on the floor. A previously neutral inanimate object is paired with noxious events to create an aversive stimulus. When the stimulus is delivered, the animal displays a behavioral repertoire that could be construed as an attempt to limit the stress felt by the individual. This behavioral repertoire in the rodent has been well documented and appears to be shaped to meet the demands of the threatening stimulus (De Boer et al., 1991). Studies of defensive burying focus, for the most part, on flexible purposeful behaviors used by the subject to investigate and cope with threatening test objects, rather than classical flight or fight behaviors. The defensive burying repertoire of the rodent has multiple components and can be subdivided into such indices as ambulation, rearing, immobility, burying, grooming, and object exploration. Ambulation consists of any horizontal locomotor activity, mostly accompanied by sniffing and exploratory activity directed at the floor and wall of the test chamber and at the air. Rearing behavior consists of raising the body vertically on the hind limbs usually accompanied by sniffing actions directed by upward movements of the head. Immobility consists of sitting and/or crouching, but with horizontal head movements. In contrast, freezing behavior is described as sitting motionless with a rigid body posture. Burying is defined as disrupting or pushing bedding material specifically towards the source of the aversive stimulus or object. Grooming consists of complex strings of movement to clean and maintain the skin and fur. Exploration of the stimulus includes object contact and stretch attend postures, in which the
body is horizontally stretched towards the stimulus, taken as indices of risk assessment (Pinel et al., 1989; Pinel and Mana, 1989; Blanchard and Blanchard, 1989; Dielenberg and McGregor, 2001).

During exposure to an aversive stimulus source, all of the behaviors listed above are altered, providing several indices of fear and anxiety. Ambulation is typically decreased, which reflects an inhibition of general exploration. In contrast, rearing behavior, taken as an index of explorative escape or as a flight response, is increased during exposure to a novel or threatening object. Immobility and/or freezing behaviors are elevated, which reflect passive avoidance and fear, respectively. Defensive burying behaviors, as reflected in the time spent burying and the amount of bedding material directed at the aversive stimulus source, increase in response to the intensity of the aversive stimulus, reflecting active avoidance. Lastly, exploration of the stimulus object decreases, taken as a sign of decreased exploration and avoidance (reviewed in De Boer and Koolhaas, 2003).

The behaviors demonstrated in the defensive burying paradigm have a cognitive component, implying that a rat actively gathers information about dangerous environments. This is reflected by the animal's ability to identify, gather information, and actively engage the source of the aversive stimulus specifically (reviewed in Pinel and Mana, 1989). Assessment of burying behavior is one standard measure of determining how the animal interacts with an object. Determining the number or frequency of stretched approaches provides an additional index of risk assessment. A stretched approach sequence begins with the rat advancing slowly towards the aversive object in a low stretched posture that ends when the rat typically reverses its movement away from the object. This movement often positions the rat in the immediate vicinity of the aversive stimulus where it can palpate with its vibrissae and contact the object directly. This allows the rat to gather information about the offending object as to its visual, olfactory, and tactile characteristics (Pinel et al., 1994). This form of risk assessment is perhaps the most common and prevalent behavior for any higher animal in situations involving unfamiliar or unpredictable hazards. In the shock prod paradigm, rats use information acquired during the post-shock period to direct their subsequent defensive activities. Rearing is thought to be another means of procuring information about the surrounding environment in addition to more common forms of exploration. While these behaviors are thought to represent active forms of coping responses to an aversive stimulus, it is known that some animals are capable of
choosing less active coping responses or simply avoid the stimulus altogether without showing burying behavior. Individual variation in coping forms and defensive burying behavior was first noted by Hudson (1950) and by Pinel and Treit (1978), who identified extreme differences in the propensity to bury between different strains and species of rodents.

1.8 Statement of the problem

Animals often show marked individual differences in their behavioral responses to threatening or novel stimuli, even under controlled or uniform testing conditions. Several emerging studies indicate that differences in coping style provide a strong basis for determining potential vulnerability to stress-related disorders as well as allowing us to determine the underpinnings of normal anxiety. As noted above, largely forgotten in the defensive burying literature, is the propensity for individual animals to show extreme differences in active coping responses. While the shock prod defensive burying paradigm provides an excellent model for determining the underpinnings of anxiety, the basis for individual differences in defensive burying behavior remains poorly understood. Before we can understand how variations in coping style differentially predispose certain individuals to the pathological effects of stress, developing a model that invites or exposes individual differences in defensive burying behavior would seem to be an important starting point.

1.9 The present study

In the traditional defensive burying paradigm, the severity of shock may afford little if any opportunity for rats to display individual differences in defensive behavior. Thus, distinct from the more traditional approach of inducing defensive burying with an electrified prod, we chose to remove the aspect of pain as a limiting factor towards inducing or inviting individual differences in defensive burying behavior and components of this response. By remotely triggering a mousetrap (without physically contacting the rat, modified from Terlecki et al., 1979) we hypothesized that this would invite extreme differences in several types of active and passive coping behaviors. In light of the endogenous roles for central CRH, AVP, and OT in mediating or contributing to anxiety as well as the central regulation of the HPA axis, we also hypothesized that differences in the expression of these factors would be represented to some degree in animals identified as showing differences in defensive burying behavior. This aspect
of the thesis involved anatomical assessments of CRH, AVP, and OT mRNA expression levels within brain regions previously identified as mediating avoidance behavior and/or regulating the HPA axis, including within different subsets of cell populations within the PVN. As the AVP transcript is highly sensitive to changes in gonadal status in males, we also hypothesized that individual differences in defensive burying would correlate with androgen receptor levels in the brain. Centrally acting neuropeptides appear to coordinate both normal HPA function and anxiety in the rat. Thus, we believe that characterizing how CRH, AVP, and OT vary as a function of defensive burying behavior provides an important starting point for unraveling the neural circuitries responsible for individual differences in emotionality and neuroendocrine function.
CHAPTER 2. Unmasking individual differences in conditioned and unconditioned defensive burying

A. Introduction

First noted by Hudson (1950) and extensively developed by Pinel et al. (1978), rodents show a remarkable propensity for displacing bedding material towards a variety of noxious stimuli. Since its introduction, the defensive burying test has proven incredibly reliable and informative with respect to determining the neurobiological properties of avoidance, fear, and anxiety-related behavior. What has emerged from some of these studies, however largely ignored, is that animals have the capacity to enter the experimental environment with different tendencies to bury as a conditioned response. Therefore, we used a mousetrap as an aversive stimulus source to induce defensive burying, yet triggered it remotely towards unmasking innate individual differences in defensive burying behavior. Beyond this aspect of the defensive response, we also assessed several types of behavior, including freezing, risk assessment, grooming, and exploratory behavior, to enhance our survey of individual differences in avoidance behavior.

B. Materials and methods

Animals. Thirty adult male Sprague Dawley rats (UBC Animal Care Centre, Vancouver, Canada) were used. Animals were pair-housed under controlled temperature and lighting conditions (12:12 hour light: dark cycle, lights on at 0600 hours), with food and water available ad libitum. The University of British Columbia Animal Care Committee approved all experimental protocols.

General treatment schedule. Two days after arrival, rats were weighed and handled on each of 7 consecutive days prior to behavioral testing, involving daily removal from the home cage room and transport to an adjacent behavioral suite. The rats were then habituated to the defensive burying chamber for 30 min on each of 4 consecutive days. During this phase, rats were exposed to a dummy (unloaded) single spring mousetrap (Victor Easy Set Mouse Trap Model M035, Woodstream Corporation, Lititz, PA), affixed 5 cm above the floor of the chamber (level with bedding). Rats were then exposed to a loaded mousetrap for 15 min on each of 3 consecutive
days. Under these test conditions, the trap was remotely triggered by an observer as the rat approached no closer than approximately 2 cm from the device. On day 8, the animals were then again exposed to the chamber-trap assembly, introducing an unloaded mousetrap as a test of conditioned aversion. Twenty four hours after the initiation of the conditioned aversion test, rats were anesthetized for perfusion and tissue collection.

**Behavioral testing.** The burying test apparatus consisted of an opaque plexiglass chamber measuring 30 x 44 x 44 cm (W x L x H) that was uniformly filled with wood chip bedding material to a depth of 5 cm. The chamber was cleaned and fresh bedding was applied between testing. As described above, the apparatus contained a mousetrap that was unloaded during habituation and a single test of conditioned aversion or remotely triggered during conditioned defensive burying. Habituation and test trial behaviors were recorded using a JVC video camera, initiated as soon as the rat was placed in the chamber away from the mousetrap, and analyzed using the event recorder Hindsight program (v1.5). Acclimatization, behavioral habituation, and testing were restricted to the light phase of the cycle (0900 to 1200 hours), and the order of presentation was counterbalanced on each consecutive day.

**Behavioral analysis.** Several behavioral dimensions were monitored to explore individual differences in spontaneous and conditioned defensive burying responses. These measures included temporal and qualitative assessments of defensive burying (latency, duration, amount of bedding pushed towards the trap), trap exploration (approach latency, contact time, stretch attend posture), field exploration (rearing, center chamber crossings, time spent in proximity or distal to the trap), passive avoidance was measured as freezing, while grooming was taken as the conflict between fear and normal behavior. Behaviors were analyzed as presented above, with field exploration containing components of rearing and ambulation. Burying was measured as any bedding directed towards the trap, whether measured in seconds or in peak height cm. Latency to bury was measured as the time in seconds for the rat to begin burying after trap snap. Approach latency was taken as the time to approach the trap upon being placed in the chamber. Contact time was the time in which the animal spent in contact with the trap. Stretch-attend is a distinct elongated posture, used for the rodent to gather information on the stimulus (more eloquently described by Pinel et al., 1989). Center crosses and time spent distal or proximal to the trap are
simply based on dividing the apparatus into two components separated by an imaginary centerline. With the trap in one end and nothing in the other line crosses and time-spent proximal/ distal are measured as the rat crosses the centerline in the chamber. To accurately gauge the intensity of each rat’s immediate reaction to the trap once remotely triggered, several additional behaviors were combined to provide individual trap-reaction scores. Based on the five-point scale employed by Gray et al. (1981), trap reactivity scores were: no discernible reaction (score = 0); startle, but no immediate withdrawal (score = 1); startle and withdrawal to the far end of the chamber (score = 2); jumping and or squealing followed by rapid withdrawal (score = 3); and finally, a reflexive jump to the far end of the chamber (score = 4).

**Statistical analysis.** To make certain that we were studying reliable defensive burying traits, we restricted our analysis only to those rats displaying stable defensive burying responses over 3 consecutive test trials. Thus, data derived from rats displaying rank-order shifts in the duration of burying over three consecutive triggered mousetrap exposures were removed from the study. Twenty rats were identified as showing stable patterns in bury duration profiles, and a median split of this data yielded two groups of rats (10 animals each). The inter-observer reliability of the duration measure was extremely high (r = 0.90). Behavioral data derived from Group x Trial designs were analyzed by two-way ANOVA (Bury, Trial) with one repeated measure (Trial), and significant main effects and interactions were analyzed using the Newman-Keuls post hoc test. Corelational analyses were performed using simple regression. Statistical comparisons were made observer-blind by assigning coded designations to the video recordings, data, and tissue sets in advance.
C. Results

Aversive Trap conditioning: Defensive burying behavior

Rats identified as showing stable patterns in defensive burying behavior in response to a remotely triggered mousetrap were ranked according to the mean of their bury duration levels determined over 3 consecutive test trials. Animals with bury durations greater than or less than the median duration value of 44.2 seconds were designated as either High- or Low-bury animals, respectively. This assignment yielded non-overlapping profiles in the time spent burying and the height of the highest pile of bedding material directed at the mousetrap (see figure 1).

Assessment of bury duration in response to mousetrap conditioning revealed significant effects of Bury \( [F(1, 18) = 30.7; P<0.001] \) and Trial \([F(4, 72) = 9.5; P<0.0001] \), and a significant Bury x Trial interaction \([F(4, 72) = 8.7; P<0.0001] \). Post hoc analyses of the interaction indicated a significant positive effect \((P<0.05)\) of trap exposure in High-, but not in Low-bury animals (see figure 2). With respect to bury latency, there were significant effects of Bury \([F(1, 18) = 19.9; P=0.0003] \) and Trial \([F(2, 36) = 27.9; P<0.0001] \), and a significant Bury x Trial interaction \([F(2, 36) = 15.6; P<0.0001] \). Post hoc analyses of the interaction indicated a significant negative effect \((P<0.05)\) of trap exposure in High-, but not in Low-bury animals (see figure 3).

Immediate reactions. Assessment of behavioral reactions during the first minute of trap exposure revealed no significant effect of Bury \([F(1, 18) = 1.0; P = 0.33] \) and no significant Bury x Trial interaction \([F(2, 36) = 1.1; P = 0.33] \), but a significant effect of Trial \([F(2, 36) = 21.7; P<0.0001] \) that was attributed to a progressive decrease in behavioral reactions scores by the third day of trap exposure \((P <0.05; \text{figure} \ 4)\). On the first day of exposure, reaction scores were mostly attributed to jumping and rapid withdrawal responses. By the third day of exposure the clear majority of animals displayed startle and slow withdrawal responses. A discernible response was observed in all cases, and reflexive squealing and jumping responses were extremely rare (4 out of 60 observations).

Stretch approaches. There were significant effects of Bury \([F(1, 18) = 22.1; P=0.0002] \) and Trial \([F(2, 36) = 19.6; P<0.0001] \), and a significant Bury x Trial interaction \([F(2, 36) = 14.8; P<0.0001] \) on the number of stretch approaches. Post hoc analyses of the interaction indicated a
significant positive effect (P<0.05) of trap exposure in High-, but not in Low-bury animals (see figure 5).

**Grooming and rearing.** There was a significant effect of Bury [F(1, 18) = 20.0; P=0.0003], no significant effect of Trial [F(2, 36) = 0.4; P=0.65], but a significant Bury x Trial interaction [(F(2, 36) = 5.8; P=0.007] on grooming frequency. Post hoc analyses of the interaction indicated a significant positive effect (P<0.05) of trap exposure in Low-, but not in High-bury animals (see figure 6). There was a significant effect of Bury [F(1, 18) = 6.1; P=0.0233], no significant effect of Trial [F(2, 36) = 3.0; P=0.06], and no significant Bury x Trial interaction [(F(2, 36) = 0.5; P=0.60] on rearing frequency. Post hoc analysis credited the main effect of Bury to a significantly higher incidence of rearing postures in Low-bury animals during trap habituation and after trap conditioning (P<0.05, see figure 7). Low-bury animals also tended to display higher levels of rearing during conditioning (P=0.0519).

**Freezing.** There were significant effects of Bury [(F(1, 18) = 6.4; P=0.0207 and Trial [(F(2, 36) = 4.8; P=0.0140], and no significant Bury x Trial interaction [(F(2, 36) = 2.0; P=0.1483] on freezing frequency. Post hoc analyses credited the main effects of Bury and Trial to a significantly higher display (P<0.05) of freezing postures in High-bury animals during repeated trap exposure (see figure 8).

**Field and trap exploration.** Multiple parameters were used to study exploratory behavior, including the number of chamber (center-line) crossings, the relative time spent occupying the proximal and distal halves of the chamber, the latency to approach and make first contact with the trap, and trap contact frequency once initiated. Assessment of the number of center chamber crossings revealed no significant effects of Bury [(F(1, 18) = 0.7; P=0.4045] and Trial [(F(2, 36) = 1.9; P=0.1552], and no significant Bury x Trial interaction [(F(2, 36) = 0.58; P=0.5667]. Determining the ratio of the time spent occupying the proximal and distal halves of the chamber revealed no significant effect of Bury [(F(1, 18) = 0.04; P=0.8381], a significant effect of Trial [F(2, 36) = 4.5; P=0.0176], and no significant Bury x Trial interaction [(F(2, 36) = 2.2; P=0.1295]. The effect of trial was credited to a significant increase (P<0.05) in the relative time spent within the proximal half of the chamber during repeated trap exposure.
With respect to trap contact latency, there were significant effects of Bury \([F(1, 18) = 19.9; P=0.0003]\) and Trial \([(F(2, 36) = 27.9; P<0.0001)\], and a significant Bury x Trial interaction \([F(2, 36) = 15.6; P<0.0001]\) on the latency to approach and contact the trap. Post hoc analyses of the interaction indicated a significant positive effect \((P<0.05)\) of trap exposure in Low-, but not in High-bury animals (see figure 9).

Assessment of trap contact frequency revealed significant effects of Bury \([F(1, 18) = 24.2; P<0.0001]\) and Trial \([(F(2, 36) = 114.2; P<0.0001)\], and a significant Bury x Trial interaction \([F(2, 36) = 4.9; P=0.0136]\). Post hoc analyses of the interaction indicated a significant negative effect \((P<0.05)\) of trap exposure on trap contact frequency. This inhibitory effect was significantly greater \((P<0.05)\) in High-bury animals both during and after trap conditioning.
Figure 1. Defensive burying profiles in High- and Low-bury rats. Rats identified as showing stable patterns in defensive burying behavior in response to a remotely triggered mousetrap were ranked according to the mean of their bury duration levels determined over 3 consecutive test trials. Animals with bury durations greater than or less than the median duration value of 44.2 seconds were designated as either High- or Low-bury animals, respectively. This assignment yielded non-overlapping profiles in the time spent burying and the height of the highest pile of bedding material directed at the mousetrap (n = 10 animals per group).
Figure 2. Mean ± SEM defensive burying durations (seconds) in High- and Low-bury rats during different phases of the paradigm. Relative to the last day of habituation (Hab.4), High-bury animals, but not Low-bury rats, showed significant levels in bury duration (* P<0.05 vs Low-bury) over three consecutive aversive tests (Trap.1 to Trap.3) and during a single (Test) exposure to a neutral mousetrap (n = 10 per group).
Figure 3. Mean ± SEM latency to bury (minutes) following trap snap exposure in High- and Low-bury animals. There were significant effects of Bury [F(1, 18) = 19.9; P=0.0003] and Trial [(F(2, 36) = 27.9; P<0.0001], and a significant Bury x Trial interaction [F(2, 36) = 15.6; P<0.0001]. Post hoc analyses of the interaction indicated a significant negative effect (*P<0.05, vs. Low-bury rats) on the latency to bury in High-bury rats (n = 10 per group).
Figure 4. Mean ± SEM trap reactivity scores measured across aversive test days in High- and Low-bury animals. To accurately gauge the intensity of each rat’s immediate reaction to the trap once remotely triggered, several additional behaviors were combined to provide individual trap-reaction scores. *P<0.05 vs. Trap.1 and Trap.2 (n = 10 per group). Data based on the five-point scale employed by Gray et al. (1981) as described in Methods.
Figure 5. Mean ± SEM number of stretch approaches directed at the mousetrap during habituation (Hab), aversive testing (Trap), and during a single test of retention (Test) in High- and Low-bury rats. *P<0.05 vs. Low-bury animals (n = 10 per group).
Figure 6. Mean ± SEM grooming duration (seconds) during habituation (Hab), aversive testing (Trap), and during a single test of retention (Test) in High- and Low-bury rats. *P<0.05 vs. Low-bury animals (n = 10 per group).
Figure 7. Mean ± SEM rearing duration (seconds) behavior during habituation (Hab), aversive testing (Trap), and during a single test of retention (Test) in High- and Low-bury rats. *P<0.05 vs. Low-bury animals (n = 10 per group).
Figure 8. Mean ± SEM freezing duration (seconds) during habituation (Hab), aversive testing (Trap), and during a single test of retention (Test) in High- and Low-bury rats. ANOVA indicated significant Bury-group differences [(F(1, 18) = 6.4; P=0.0207] and trial effects [(F(2, 36) = 4.8; P=0.0140]. Post hoc analyses credited the main effects of Bury and Trial to a significantly higher display of freezing postures in High-bury animals during aversive trap exposure (*P<0.05 vs. Low-bury animals; n = 10 per group).
Figure 9. Mean ± SEM mousetrap approach latency (seconds) during habituation (Hab), aversive testing (Trap), and during a single test of retention (Test) in High- and Low-bury rats. *P<0.05 vs. Low-bury animals (n = 10 per group).
Non-aversive trap habituation
To explore differences in unconditioned behavior, behavioral responses over the course of repeated exposure to a neutral mousetrap during habituation were retrospectively analyzed in rats designated as High- and Low-bury animals during the conditioning phase of the paradigm. No significant effects of Bury and Trial, and no significant Bury x Trial interactions were detected with respect to field and trap exploration parameters ($P > 0.1141$ in all cases), although animals tended to display an increase in trap contact frequency ($P=0.0588$) over the course of habituation. Significant effects were revealed, however, with respect to bury duration and the frequency of stretch attends, grooming, rearing, and freezing postures.

**Bury duration.** There was a significant effect of Bury ($F(1, 18) = 4.9; P=0.0396$), no significant effect of Trial ($F(1, 18) = 2.9; P=0.1069$), and no significant Bury x Trial interaction ($F(1, 18) = 2.4; P=0.1381$). The main effect of Bury was credited to significantly higher bury duration levels in High-bury animals during initial neutral trap exposure (figure 10). By the fourth day of trap habituation this response was comparable to Low-bury animals. Inspection of individual response profiles indicated that the number of animals displaying burying activity over the first two days of habituation was never less than 7 in the High-bury group, and never more than 2-3 animals in the Low-bury group.

**Stretch approaches.** There was a significant effect of Bury ($F(1, 18) = 7.08; P=0.0159$), no significant effect of Trial ($F(1, 18) = 2.5; P=0.1329$), and no significant Bury x Trial interaction ($F(1, 18) = 1.3; P=0.2757$). The Bury effect was credited to a significantly higher incidence of stretch approaches displayed by High-bury animals during initial trap exposure (figure 11). This differential response was rapidly extinguished by the second trap exposure (data not shown).

**Freezing.** There were significant effects of Bury ($F(1, 18) = 6.9; P=0.0171$) and Trial ($F(1, 18) = 7.8; P=0.0121$), and a significant Bury x Trial interaction ($F(1, 18) = 6.5; P=0.0202$). The interaction was attributed to an overwhelming greater display of freezing postures in High-bury animals on the first day that was nearly abolished by the fourth day of neutral trap exposure (see figure 12).
Grooming and rearing. There was no significant effect of Bury \( (F(1, 18) = 0.8; P=0.3836) \), a significant effect of Trial \( (F(1, 18) = 12.6; P=0.0023) \), and no significant Bury x Trial interaction \( (F(1, 18) = 0.8; P=0.3918) \) on grooming. Post hoc analysis attributed the Trial effect to an overall positive effect \( (P<0.05) \) of trap habituation on grooming frequency. With respect to rearing, there were significant effects of Bury \( (F(1, 18) = 5.6; P=0.0294) \) and Trial \( (F(1, 18) = 7.3; P=0.0144) \), and no significant Bury x Trial interaction \( (F(1, 18) = 0.2; P=0.6884) \). Post hoc analyses attributed the Bury and Trial effects to a significantly higher display of rearing in Low-bury animals \( (P<0.05) \) and an overall negative effect of trap habituation on rearing frequency \( (P<0.05) \).

To determine the extent to which differences in conditioned defensive burying could be accounted for by differences in unconditioned behavior, the duration of burying during aversive trap conditions was analyzed as a function of stretch attend postures and the time spent freezing and burying during the habituation phase of the paradigm. Reliable relationships were found with respect to stretch attend \( (P=0.0276) \) and freezing postures \( (P=0.0087) \) but not with bury duration \( (P=0.2852) \). Taken together, these findings suggest that the difference in defensive burying behavior under aversive trap conditions could be predicted by variations in stretch attend and freezing postures during habituation.
Figure 10. Mean ± SEM defensive burying duration (seconds) during the first (Hab.1) and last day (Hab.4) of neutral mousetrap exposure in High- and Low-bury rats. *P<0.05 vs. Low-bury animals (n = 10 per group).
Figure 11. Mean ± SEM number of stretch attend postures during the first (Hab.1) and last day (Hab.4) of neutral mousetrap exposure in High- and Low-bury rats. *P<0.05 vs. Low-bury animals (n = 10 per group).
Figure 12. Mean ± SEM freezing behavior duration (seconds) during the first (Hab.1) and last day (Hab.4) of neutral mousetrap exposure in High- and Low-bury rats. *P<0.05 vs. Low-bury animals (n = 10 per group).
D. Discussion

Aversive trap conditioning. Employing a repeated measures and test design we ranked and explored animals showing reliable and sustainable differences in conditioned defensive burying. We extended our behavioral survey beyond burying, to explore indices of fear, exploration, and avoidance behavior. This allowed us to further examine how high and low bury animals differentially cope over the course of repeated (aversive) trap exposure. Rats appeared to respond to the aversive trap in either one of two burying patterns, showing robust signs of defensive burying (High-bury rat), or little to no responses at all (Low-bury rat) (See fig. 1). In High-bury rats, the response on the final day was still far greater than that seen during habituation, indicating that the aversive nature of the trap was continuing to influence behavior. Despite selecting for rats on the basis of burying behavior we found that rats responded in altogether different behavioral repertoires; displaying different profiles in fear, exploration, and avoidance behavior.

The latency to bury was also significantly different between High- and Low-bury rats. High-bury rats were far more inclined to initiate burying behavior once the trap was remotely triggered. Thus the high-bury animals appeared to be more proactive. High-bury rats showed substantially higher levels of stretch attend postures compared to Low-bury rats during repeated aversive trap exposure and during a single test of retention. This indicates that risk assessment of the stimulus source differed between groups. Freezing behavior was also relatively greater in the group of High-bury rats, however, closer inspection of this response indicated that this was true only during the first trial of aversive trap testing (data not shown). Freezing response likely diminished upon learning the regularity of the stimulus. Grooming behavior was relatively lower in the High-bury group, indicating a reluctance to engage in normal behavior. This is likely due to fear as normal behaviors are replaced with behaviors tailored to meet the demands of the aversive stimulus. While increased freezing and decreased grooming responses have been taken to represent aspects of fear, the group differences in burying behavior do not appear to be explained by differences in fearfulness. Consistent with this argument, high-bury animals continued to show lower latencies to approach the trap before it was remotely triggered during the repeated aversive phases of the paradigm. Thus, as discussed in greater detail below, at this point we are compelled to conclude that our results reflect very different coping strategies between the High- and Low-bury groups of animals.
Burying has long been considered an index of anxiety. While this may be true, we prefer to think of burying as just another tool in the rats’ behavioral repertoire to assess aversive stimuli. By displacing bedding material onto the trap the animal can gain additional information regarding the aversive quality of the stimulus at hand. Burying, taken together with an increase in stretch attends (highly validated as a source of risk assessment, Pinel et al., 1989; Pinel and Mana, 1989; Blanchard and Blanchard, 1989; Dielenberg and McGregor, 2001), indicates that the High-bury rats focused their attention on the source of aversive stimulus and continued to engage and assess the mousetrap more actively than their Low-bury counterparts. In addition, freezing behavior and a near zero contact time shown by the High-buriers in respect to the Low-buriers showed that the stimulus was indeed registered as aversive. Freezing behavior has long been taken as an index of fear, as most predators of small rodents see based on movement the behavior is rooted in evolution (reviewed in Blanchard and Blanchard, 2005). Freezing behavior amongst High-buriers also decreased with repeated exposure indicating an increased familiarity or habituation to the paradigm. Previous studies have found freezing behavior to be the inverse of burying, at least in response to an electric shock. However, this does not seem to be the case in our study, as freezing behavior did not appear to compete with the level of burying in the High-bury group of animals. The lack of time spent in contact with the trap, taken together with the increase in stretch attends displayed by the High-bury group of animals, provides some indication that these animals remain interested in actively engaging the trap directly.

The Low-bury group showed higher levels of rearing throughout all of the test phases of the paradigm, indicating a higher propensity for explorative escape in these animals (De Boer and Koolhaas, 2003). This behavior is thought to be indicative of fear and anxiety, although it may reflect a general response to the testing chamber rather than a specific response to the mousetrap. In agreement with this argument, is the fact that the contact time with the trap was greater in low-bury animals, indicating a lack of fear associated with the trap. One issue that remains, however, is whether the low-bury animals failed to register the mousetrap as the source of the aversive stimulus or whether these animals simply had other means by which to master the situation at hand. Because the reactivity scores were remarkably similar between the groups as well as the first minute behavioral scores of each successive aversive mousetrap exposure, we are inclined to conclude that both groups of animals were equally capable of reacting to and identifying the mousetrap as an aversive stimulus source. Furthermore, the High- and Low-bury
animals displayed similar declines in reactivity by the third aversive test trial, providing some indication that the information gained between the groups was equivalent, at least in terms of the threatening nature, location, and regularity by which the mousetrap dispensed an aversive stimulus. Taken together, at this point it would seem reasonable to conclude that our paradigm unmasked two very different coping profiles. High-bury rats "avoid" the trap by actively burying it, while Low-bury rats appear to actively avoid the trap as indicated by higher approach latency scores.

**Non-aversive trap habituation.** The results of the first aversive test trial suggested that the groups entered the aversive phase of the experiment with different and already established tendencies to bury the mousetrap. To explore this differentiation further, behaviors over the habituation phase were retrospectively analyzed as a function of defensive burying during the aversive phase of the paradigm. In a novel environment the animal is driven to explore the unknown. At the same time the animal is determined to avoid potential danger. This can lead to risk assessment behaviors and/or decreased exploration. Rats designated as High-bury animals showed substantially higher levels of burying, stretched approaches, and freezing behavior than did Low-bury animals on the first day of neutral mousetrap exposure. These behavioral differences were extinguished by the fourth day. These animals also showed differences in the latency to approach the trap upon being placed in the test chamber. Latency to approach was lower in High-bury rats. Previous reports have shown that there are considerable individual differences in the tendencies of rats to explore new environments and novel objects that are generally related to coping style (Steimer and Driscoll, 2003). Thus, it appears that rats designated as High-bury animals during the aversive phase of the paradigm were more willing to investigate the trap under novel environmental conditions. Defensive burying is greater in High-bury animals under these non-aversive conditions. As in the arguments raised above, it would seem reasonable to assume that these group differences may have occurred as an unconditioned component of the rat’s neophobic response. When re-introduced to the mousetrap as an aversive stimulus source for the first time, the approach latency and behavioral reactivity scores were no different between High- and Low-bury animals. This suggests that the neophobic response or experience in High-bury rats during the habituation phase may not have a negative impact on the propensity to respond and approach the trap in this group of animals.
We found a strong correlation between the propensity for individual animals to bury under aversive conditions and their capacity to engage the trap during the habituation phase of the paradigm. This is supported by Robert Bolles (1970) who stated that rats and other animals’ performance in aversive conditioning experiments are determined to a large degree by the defensive predispositions that they bring into the experiment with them. It seems the animals enter the paradigm with a predisposition to investigate and bury the object of interest this behavior is then shaped to suit the degree of adversity of the stimulus resulting in burying of an aversive object and investigation of a novel object. Thus, individual differences in defensive burying under aversive test conditions reflect appear to be rooted in the animals natural tendency to explore, risk-assess, and respond to challenging situations. As Weiss (1972) has argued, it is not the physical nature of the aversive stimulus, but rather its predictability and controllability that drives the behavioral response. Because the High-bury animals showed a greater tendency to engage and familiarize themselves with the trap under non-aversive conditions, this could have guided their behavioral response to the trap when triggered.
Chapter 3. Rats showing high and low tendencies in defensive burying display differences in AVP and OT mRNA expression levels within hypothalamic nuclei

A. Introduction

It has long been known that exposure to a variety of stimuli promote the synthesis and release of a cocktail of neuropeptide hormones from the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) of the hypothalamus. Foremost among these include corticotropin-releasing hormone (CRH), arginine vasopressin (AVP), and oxytocin (OT). These peptides are released from both the parvocellular and magnocellular cell populations of the PVN, acting both centrally and peripherally to coordinate the organism’s response to homeostatic threat or stress. AVP and OT release from the SON are predominantly released into the general circulation via the posterior pituitary; these peptides can also act centrally via their presynaptic release into the ventricular system (Ludwig and Leng, 2006).

Differences in coping styles are not only defined by a range of behavioral differences (as described in Chapter 1), but by differences in the expression and release of CRH, AVP, and OT in the hypothalamus, including the PVN and SON (reviewed in Koolhaas et al., 1999; Wigger et al., 2004; Landgraf and Neumann, 2004; Landgraf, 2006). Furthermore, individual differences in coping styles are also associated with differences in hypothalamic-pituitary-adrenal (HPA) function (reviewed in Koolhaas et al., 1999).

In this study we tested the hypothesis that individual differences in defensive burying behavior are associated with differences in neuropeptide expression levels in the hypothalamus. To implicate a potential role for CRH, AVP, and OT in mediating the differential behavioral responses observed we examined the transcript levels of these peptides in the PVN and SON. Brain tissues were obtained from the same animals studied in Chapter 2 to enhance our ability to detect differences in peptide expression as a function of individual differences in defensive burying activity.
B. Materials and methods

**Animals and treatment schedule.** As noted, the in situ hybridization analyses were performed on tissues obtained from the same animals used in Chapter 2.

**In situ hybridization.** To correlate steady-state differences in brain CRH, AVP, and OT mRNA expression levels as a function of burying behavior, animals were deeply anesthetized for perfusion twenty-four hours after the last exposure to the defensive burying chamber. The rats were anesthetized with a lethal dose of chloroform (35 % w/v, 350 mg/kg, intraperitoneal). Deep anesthesia occurred within approximately 2 min of chloroform administration, as verified by corneal, pedal, and tail-pinch reflexes. Saline (125 ml) and 4 % paraformaldehyde fixative (500 ml) were sequentially perfused via the ascending aorta at a flow rate of 20-25 ml/min. Brains were post-fixed for 4 h in fixative and then cryoprotected overnight in 15% sucrose, 0.1M potassium phosphate-buffered saline (KPBS). Five 1/5 series of frozen coronal sections (30 µm) were collected and stored in antifreeze (30% ethylene glycol and 20% glycerol in 0.05 M sterile KPBS) at –20 C until processing.

An in situ hybridization approach was used to measure the relative expression levels of neuropeptide transcripts using [³³P]UTP-labeled (Amersham, Arlington Heights, IL) antisense cRNA probes encoding for CRH, AVP, and OT. The CRH probe was transcribed from a full-length (1.2-kb) cDNA encoding CRH mRNA (Dr K. Mayo, North-western University, Evanston, IL, USA); the AVP probe from a 230-bp cDNA fragment encoding the vasopressin-specific 3’ end of AVP (Dr. D. Richter, University of Hamburg, Germany); and the OT probe from a 190-bp cDNA fragment containing exon C and part of the 2nd intron of the rat OT gene (Dr. D. Richter). Briefly, free-floating sections were first rinsed in KPBS to remove cryoprotectant and then mounted and vacuum dried on glass slides overnight. After postfixation with 10% formaldehyde for 30 min at room temperature, sections were digested in proteinase K (10 mg/ml, 37 C) for 30 min, acetylated for 10 min (2.5 mM acetic anhydride, 0.1 M triethanolamine, pH 8.0), rapidly dehydrated in ascending ethanol concentrations (50–100%), and then vacuum dried. Radionucleotide antisense cRNA probes were used at concentrations approximating 3 x 10⁷ cpm/ml in a solution of 50% formamide, 0.3 M NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA, 0.05% tRNA, and 10 mM dithiothreitol, 1x Denhardt’s solution, and 10% dextran sulfate and applied to individual slides. Slides were coverslipped and then incubated overnight at 57.5 C,
after which the coverslips were removed and the sections washed three times in 4x standard saline citrate (SSC; 0.15 M NaCl, 15 mM citric acid, pH 7.0) at room temperature, treated with ribonuclease A (20 µg/ml) for 30 min at 37 C, desalted in descending SSC concentrations (2–0.1x SSC), washed in 0.1x SSC for 30 min at 60 C, and dehydrated in ascending ethanol concentrations. Hybridized sections were then exposed to x-ray film (β-max, Amersham), defatted in xylene, subsequently coated with Kodak NTB2 liquid autoradiographic emulsion, and exposed at 4 C in the dark with desiccant, the duration determined by the strength of signal on x-ray film (12 and 9 d for AVP mRNA and CRH mRNA in the PVN, respectively). Slides were developed at 14 C with Kodak D-19 for 3.5 min, briefly rinsed in distilled water for 15 sec, fixed in Kodak fixer for 6.5 min, and then washed in running water for 45 min at room temperature.

Based on the strength of the autoradiographic signal, exposure time to emulsion was optimized to ensure that CRH mRNA and AVP mRNA were within the linear range of the assay and could be quantified by making relative comparisons in OD levels. Analysis of the relative levels of CRH, AVP, and OT mRNA levels in the PVN and SON was performed on silver grain developed emulsion-coated slides using Macintosh-driven Open Lab Image Improvistion software version 3.0.9 (Quorom Technologies). OD readings, corrected by background subtraction, were taken at regularly spaced (150-µm) intervals through each region of interest. Average OD values were determined bilaterally from three or five sections taken through the rostrocaudal extent of the medial parvocellular portion of the PVN and most of the extent of the SON, respectively. The hybridization patterns of the antisense CRH, AVP, and OT mRNA probes are in exact agreement with previous hybridization localization studies. Adjoining series of sections were stained with thionine for anatomical reference purposes. Labeled sense probes did not yield any positive hybridization signal (data not shown). Relative intensities in hybridization are expressed as mean (± SEM) OD values.

Light- and dark-level images were captured using a Retiga 1300 CCD digital camera (Qimaging, Burnaby, BC), cataloged using a Macintosh OS X-driven Open Lab Image Improvistion software v 3.0.9 (Quorum Technologies, Guelph, ON), quantified using Image J (v. 1.3.5j) for densitometric analysis, and exported to Adobe Photoshop (v. 7.0) for final assembly at a resolution of 300 dpi.
**Statistical analysis.** Neuropeptide data in the PVN and SON were analyzed as a function of cell type, within parvicellular and magnocellular neurons, by two-way ANOVA (Group x Region) and Newman-Keuls post hoc tests, where appropriate. Correlational analyses were performed using simple regression. Statistical comparisons were made observer-blind by assigning coded designations to the video recordings, data, and tissues sets in advance.

C. Results

**Paraventricular and Supraoptic Nuclei.** In figure 13, parceling and terminology used for describing CRH, AVP, and OT expression within the paraventricular nucleus (PVN) of the hypothalamus followed Swanson and Kuypers (1980) and Swanson and Simmons (1989). There was no significant difference in CRH mRNA in the medial parvocellular dorsal part of the PVN (data not shown). Relative to Low-bury animals, High-bury rats displayed significantly lower levels of the AVP transcript within the posterior magnocellular part of the PVN and the supraoptic nucleus (see figure 14). High-bury animals also tended to show lower levels of AVP mRNA within the medial parvocellular dorsal (P=0.0778) and ventral (P=0.0690) parts of the PVN. OT mRNA levels were significantly lower in High-bury animals in the dorsal parvocellular and posterior magnocellular parts of the PVN, as well as in the supraoptic nucleus. High-bury animals tended to show lower levels of the OT transcript in the lateral parvocellular part of the PVN (P=0.0549). There were no differences in OT mRNA levels in the medial parvocellular ventral or medial magnocellular parts of the PVN (see figure 15).
Figure 13. AVP and OT mRNA expression and distribution in the paraventricular nucleus (PVN) of the hypothalamus. Representative darkfield photomicrographs at 150 µm intervals through the caudal half of the paraventricular nucleus (PVN) showing the relative strength and distribution of AVP (top) and OT (middle) mRNA within different PVN compartments (bottom panels). As described in the schematic (bottom panels), the AVP transcript is concentrated within the posterior magnocellular (pm) part of the PVN, with substantially lower densities in the dorsal body of the medial parvocellular (mpd) part and in the medial parvocellular ventral (mpv) region. The OT transcript shows a relatively wider and unique distribution, including within the periventricular (pv) part, the dorsal parvocellular (dp) and lateral parvocellular (lp) parts, and a noticeable distribution within the lateral and anteroventral aspects of the posterior magnocellular division adjoining the mpv region. Scale bar = 100 µm (applies to all).
Figure 14. Mean ± SEM of the relative optical density (OD) of AVP mRNA expression within the supraoptic nucleus (SON) and different compartments of the PVN (mpd, mpv, MM, and PM regions; defined in figure 13) in High- and Low-bury rats. *P<0.05 vs. Low-bury (n = 10 per group).
Figure 15. Mean ± SEM of the relative optical density (OD) of OT mRNA expression within the supraoptic nucleus (SON) and different compartments of the PVN (mpd, dp, lp, MM, and PM regions; defined in figure 13) in High- and Low-bury animals. *P<0.05 vs. Low-bury (n = 10 per group).
D. Discussion

The PVN has been shown to be responsive to a number of stressors, and neuropeptide expression in the PVN is responsible for mediating behavioral, neuroendocrine, and autonomic responses to stress. Therefore it was interesting, but not surprising, to see that rats showing differences in behavior in the defensive burying paradigm also showed differences in AVP and OT expression in the PVN. We observed no group difference in CRH expression within a region of the PVN known to project to the amygdala and to the locus coeruleus, a brainstem region responsible for driving anxiety related behavior (Charney et al., 1998). Previous studies, however, have shown that rats selected and bred for extreme differences in anxiety-like behavior show differences in AVP expression, but no differences in CRH expression in the PVN (Aubry et al., 1995; Landgraf and Wigger, 2002, 2003). Therefore, CRH levels in the PVN may not contribute to the group differences in defensive burying and avoidance behavior.

The Bury-group differences in AVP expression identified amongst the magnocellular neurons of the PVN and SON predict different neuroendocrine capacities in High- and Low-bury animals. AVP derived from these hypothalamic nuclei may also gain access to the ventricular system. Thus, the group differences in AVP expression within these nuclei could also play a pivotal role in determining the differential expression of avoidance and/or anxiety behavior in High- and Low-bury rats. In agreement with this possibility, rats bred for high anxiety show increased levels of AVP in the PVN, and this behavioral trait is abolished following inverse microdialysis of a mixed (V1a/b) vasopressin receptor antagonist into the PVN (Murgatroyd et al., 2004; Wigger et al., 2004). Altemus et al. (1994) showed that increased freezing behavior in fawn hooded rats is associated with a reduced expression of AVP mRNA in the parvocellular part of the PVN. In our study, High-bury animals tended to show a decrease in AVP mRNA and significantly higher levels of freezing behavior, at least during the initial phases of testing. While several previous studies have shown that AVP pathways emanating from extra-hypothalamic sources provide the bulk of AVP's central effects on avoidance behavior and anxiety (discussed in Chapter 4), our current findings, in the very least, suggest that changes in AVP levels in the PVN could contribute to the group differences in freezing behavior.

To date, the vast majority of studies exploring the behavioral and neuroendocrine effects of OT have been performed in the female rodent. However, recent studies have shown that OT is released differentially, both peripherally and centrally, upon both physical and psychological
types of challenges (reviewed in Neumann, 2002). Furthermore, several emerging studies indicate that in contrast to AVP, central OT exerts anxiolytic-like behavioral effects and/or generates passive coping strategies (Neumann et al., 2000; Landgraf and Neumann, 2004). Therefore we were delighted to see a difference in OT expression levels within the PVN between High- and Low-bury animals.

As illustrated in Figure 16, the PVN (and the SON) represents a critical if not the sole source for OT's forebrain influences on avoidance and anxiety-like behavior (Sofroniew, 1983; Devries and Bujis, 1983, Pittman et al., 1981). We observed significantly higher levels of OT mRNA levels in Low-bury animals than in High-bury animals in the SON and within the dorsal parvocellular (dp) and posterior magnocellular (PM) parts of the PVN. The dp portion of the PVN projects to various brainstem centers controlling autonomic and behavioral responses to stress. Furthermore, several reports suggest that the magnocellular neurons of the PVN and SON are capable of releasing AVP/OT from their dendrites into the ventricular system, independent of their terminal release into the median eminence and posterior pituitary (reviewed in Landgraf and Neumann, 2004; Engelmann et al., 2004; Ludwig and Leng, 2006). As the High- and Low-bury animals showed remarkable differences in AVP/OT expression within magnocellular neurons, this could provide a source of individual differences in AVP/OT concentrations in the cerebral spinal fluid. The implications of this are vast and exciting, since neuropeptides in the ventricles are capable of acting at multiple brain sites and evoking long-lasting changes in behavior. It is important to note that various subregions of the PVN, including those represented by OT-expressing neurons, contain a mixture of forebrain- and brainstem-projecting neurons as well as those directed at the pituitary. Thus, there may be other sub-regions of the PVN showing bury-group differences in OT expression that could only be identified using a connective (tract-tracing) approach. For example, one recent report employing a retrograde tracer injection approach in the central nucleus of the amygdala, has shown that differences in anxiety behavior are associated with differences in OT expression within amygdala-projecting neurons seated within the anterior aspect of the PVN (Marroni et al., 2007), a region of the PVN we have yet to examine.
**Figure 16.** Candidate targets and pathways in the brain mediating the central effects of OT on behavior. The pathways shown are by no means complete, and several of these may be indirect. In the literature, several mismatches have been noted between the distribution of OT receptors and OT immunoreactivity in the brain. This mismatch is perhaps salvaged by the fact that magnocellular neurons of the PVN and SON are capable of secreting OT directly into the ventricular system. As such, OT could gain access to several brain regions, especially those situated most proximal to the ventricles, including the septum, hippocampus, and periaqueductal grey, for example.

Abbreviations: Amy, amygdala; BNST, bed nucleus of the stria terminalis; CPu, caudate putamen; DMX, dorsal motor nucleus of the vagus nerve; Hpc, hippocampus; LS, lateral septum; MD, medial dorsal nucleus of the thalamus; MPOA, medial preoptic area; Nacc, nucleus accumbens; NTS, nucleus tractus solitaries; PAG, periaqueductal grey; PVH, paraventricular nucleus of the hypothalamus; SC, spinal cord; SN, substantia nigra; VT, ventral tegmentum.
Chapter 4. Rats showing high and low tendencies in defensive burying display differences in AVP mRNA levels within limbic-related nuclei

A. Introduction

The regulation and influence of individual differences in coping styles on stress adaptation appear to be mediated by multiple neuropeptide systems in brain regions implicated in fear and anxiety in both experimental animals and in humans. Foremost among these include corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) emanating from the amygdala (AMY) and bed nuclei of the stria terminalis (BST). The AMY and BST project to multiple regions implicated in the behavioral and neuroendocrine response to stress, including the lateral septum and hippocampus.

In humans and rodents CRH and AVP may contribute to extreme trait anxiety and the comorbid expression of depression-like symptoms, while OT appears to possess anxiolytic properties as discussed above. CRH and AVP have been shown in a variety of animal models to contribute to individual differences in behavioral and adrenal responses to challenging situations. In the current study we extended our in situ hybridization approach to include the BST and AMY, to test the hypothesis that High- and Low-bury animals show differences in CRH and AVP expression within these brain regions.

B. Materials and methods

Animals and treatment schedule. The in situ hybridization analyses were performed on tissues obtained from the same animals used in Chapters 2, using a protocol identical to that described in Chapter 3.

Statistical analysis. Neuropeptide data in the AMY and BST were analyzed by two-way ANOVA (Group x Region), with Newman-Keuls post-hoc tests. Statistical comparisons were made observer-blind by assigning coded designations to the video recordings, data, and tissues sets in advance.
C. Results

Amygdala. Densitometric analysis of CRH mRNA levels through the extent of the central nucleus of the amygdala revealed no significant differences (Mean ± SEM relative OD values in High- and Low-bury animals = 13.5 ± 0.5 and 12.4 ± 0.5, respectively). Through the rostrocaudal extent of the posterodorsal part of the medial amygdala, AVP mRNA levels were significantly greater in High- than in Low-bury animals (see figures 17-18).

Bed nucleus of the stria terminalis. Parceling and terminology used for describing CRH and AVP expression followed Dong and Swanson (2004, 2006 a,b,c). Within the anterior division of the bed nuclei of the stria terminalis (BST), there were no differences in CRH mRNA levels in the anteromedial group, encompassing the anterodorsal and anteroventral nuclei (Mean ± SEM relative OD values in High- and Low-bury animals = 14.3 ± 0.7 and 14.6 ± 0.6, respectively). There were no differences in CRH mRNA levels within the lateral part of the mid extent of the anterior BST, including the oval and fusiform nuclei (Mean ± SEM relative OD values in High- and Low-bury animals = 18.4 ± 0.9 and 19.8 ± 0.9, respectively). Within the posterior division of the BST, encompassing the principle, transverse, and intrafascicular nuclei, AVP mRNA levels were significantly higher in High-bury animals (see figures 17-18).
**Figure 17.** Mean ± SEM of the relative optical density (OD) of AVP mRNA expression in the medial nucleus of the amygdala (MeA) and within the posterior division of the bed nucleus of the stria terminalis (BST) in High- and Low-bury rats. *P<0.05 vs. Low-bury (n = 10 per group).
Figure 18. Representative darkfield photomicrographs of AVP mRNA hybridization through comparable levels of the medial nucleus of the amygdala and the posterior division of the bed nucleus of the stria terminalis (BST) from Low- and High-bury rats (left and middle panels, respectively). The localization of AVP mRNA to the anterodorsal (MEAad) and posterior division of the BST are outlined (right panels). Structures labeled for reference: fx, fornix; int, internal capsule; ot, optic tract; sm, stria medullaris; st, stria terminalis; if, interfascicular nucleus; pr, principle nucleus; tr, transverse nucleus of the posterior BST. The relative rostrocaudal coordinates (mm) through each region of interest are based on the atlas of Swanson (1998). Scale bar = 100 µm (applies to all).
Vasopressinergic pathways

**Figure 19.** Candidate targets and pathways in the brain mediating the central effects of AVP on behavior. Central AVP pathways are more hardwired than central OT pathways in the brain. However, AVP can gain also access to various targets via the intraventricular route.

Abbreviations: Amy, amygdala; BNST, bed nucleus of the stria terminalis; CPu, caudate putamen; DMX, dorsal motor nucleus of the vagus nerve; DR, dorsal raphe nucleus; Hpc, hippocampus; LC, locus coeruleus; LS, lateral septum; MD, medial dorsal nucleus of the thalamus; MPOA, medial preoptic area; Nacc, nucleus accumbens; NTS, nucleus tractus solitarius; PAG, periaqueductal gray; PVH, paraventricular nucleus of the hypothalamus; SC, spinal cord; SN) substantia nigra; VT, ventral tegmentum.
D. Discussion

The amygdala is viewed as an interface between brain structures conveying sensory information and those mediating avoidance and defensive behaviors in response to stress, fear, and anxiety (Davis, 2006; LeDoux, 2000; 2003). The BST relays information from the amygdala, cortex, and additional limbic structures that, in turn, project to various hypothalamic and brainstem regions driving autonomic and behavioral responses to aversive or threatening stimuli (Dong and Swanson, 2004). Depending on the type and aversive quality of the stimulus, lesion studies have implicated the amygdala, BST, lateral septum, and the dorsal hippocampus in avoidance behavior and defensive burying (Lehmann et al., 2003; Pesold and Treit, 1992; Treit et al., 1993; Treit and Menard, 1997). CRH and AVP have been shown to exert anxiogenic responses in the rat, and their receptors are distributed within putative circuits mediating avoidance behavior (reviewed in Landgraf, 2005). For these reasons, we hypothesized that High-bury animals display higher levels of CRH in the central nucleus of the amygdala and within its forebrain relays in the anterior division of the BST. Likewise, as central AVP appears to evoke anxiogenic responses as well as induce active forms of avoidance behavior, we likewise expected to see higher levels of AVP expression within the medial nucleus of the amygdala and within the posterior division of the BST in High-bury compared to Low-bury animals.

We found no group differences in CRH expression within the central amygdala, nor within the anterior division of the BST. Previous reports have shown that exogenous administration of CRH into the central amygdala increases defensive burying behavior in response to shock (Diamant et al., 1991; De Boer and Koolhaas, 2003). Furthermore, rats bred and selected for high trait anxiety show lower levels of CRH mRNA in the anterior BST and central amygdala compared to their low anxiety bred counterpart (Wigger et al., 2004). Finally, CRH mRNA levels have been shown to increase in the central amygdala following repeated exposure to anxiogenic or stressful stimuli (Makino et al., 2002). Based on our findings we are compelled to conclude the following: 1) Repeated mousetrap exposure may have been equally threatening to both groups of animals; or 2) Central CRH pathways may not be associated with the differential display of burying and avoidance behavior between the two groups of animals. Admittedly, changes or lack thereof in central CRH mRNA levels may not be entirely reflective of changes in synthesis, output, and release. Thus, the extent to which High- and Low-bury animals show differences in CRH protein synthesis, as well as changes in receptor levels remains to be seen.
The central release of AVP is known to contribute to learning and memory, emotionality, stress coping, and control of the HPA axis (Engelmann et al., 1994, 1996; Ebner et al., 1999, Landgraf and Wigger, 2003; Wotjak, 2005). These effects are thought to be coordinated by central AVP at the lateral septum and other key nuclei containing AVP receptors (Figure 19) (Koolhaas et al., 1998; Tribolet et al., 1998). The sole sources of AVP to these nuclei are the posterior bed nuclei of the stria terminalis and the medial nucleus of the amygdala. These nuclei in turn represent key sites mediating the central effects of AVP on behavior and emotionality (Dong and Swanson, 2006; Debiec, 2005). Although several other AVP-expressing regions are probably capable of influencing behavior, including the descending population of pre-autonomic AVP neurons in the PVN.

However, unlike a clear majority of AVP-producing neurons in the brain, AVP neurons in the medial amygdala and within the posterior division of the BST are particularly sensitive to stress exposure, gender, and individual difference in expression levels (DeVries and Miller 1998). Most intriguingly, animals bred and selected for high levels of aggression display higher levels of AVP in the BST and MeA, and projection densities of AVP within the lateral septum compared to animals bred for low aggression (Compaan et al., 1992, 1993; Everts et al., 1997; Koolhaas et al., 1998). Further, animals bred for high anxiety behavior also show higher expression and release of AVP, compared to their low anxiety bred counterparts (Murgatroyd et al., 2004; Wigger et al., 2004). Although previous studies had examined AVP expression in the PVN and peptide release in the lateral septum of animals selected and bred for anxiety and avoidance behavior, these studies had failed to examine the limbic source of these peptides, the BST and amygdala. Finally, AVP receptor antagonism or knockdown in the lateral septum has been shown to decrease active avoidance behavior and/or behavioral indices of anxiety during aversive tests of social interactions and open field exposure (Engelmann and Landgraf, 1994; Landgraf et al., 1995; Landgraf et al., 2003; Liebsch et al., 1996; Engelmann et al., 2000; Greible et al., 2002).

Comparing these findings with our own data, our findings implicate AVP as an causal factor driving differences in burying behavior between High- and Low-bury animals. Conversely, differences in AVP expression may be driven by behavior. Several future experiments are required to test directly whether AVP drives or changes in response to higher burying responses in some animals. However, our findings provide a strong indication that differences in defensive
burying levels can be predicted based on the tendency for animals to show different levels of defensive burying and stretch attend postures during the habituation phase of the paradigm. Based on this data, we predict that AVP receptor blockade could affect aversive mousetrap induced burying only in those animals identified as showing High-bury tendencies during trap habituation. Conversely, animals showing Low-bury tendencies should be uniquely sensitive to OT receptor blockade, if these factors do in fact drive the behavioral response. Finally, if the behavior does indeed shape neuropeptide expression, then receptor blockade should have no affect.

As discussed above for extra-hypothalamic CRH mRNA levels, the group differences in AVP mRNA in the BST and MeA remain uncertain with respect to peptide synthesis, turnover, and transport. However, unlike CRH expression in the BST and central amygdala, several studies have shown that stress-induced changes and steady-state differences in AVP mRNA expression in the BST and MeA parallels the number of AVP immunoreactive neurons detected in these nuclei, as well as the density of AVP immunoreactive terminal fibers in the lateral septum (discussed in Landgarf and Neumann, 2004; Ludwig and Leng, 2006; Leng and Ludwig, 2006). Thus, we remain confident that the group differences that we observed in AVP within the BST and MeA are indicative of differences in synthesis, turnover, transport and function at sites of release including the lateral septum. Nonetheless, future studies directed at the lateral septum in High- and Low-bury animals remain worthy of pursuit.
Chapter 5. Rats showing high and low tendencies in defensive burying display differences in androgen receptor staining within limbic-related nuclei

A. Introduction

The medial amygdala and posterior division of the BST are amongst the most sexually dimorphic regions of the brain, and show the highest densities of sex steroid hormone receptors in the brain, including androgen and estrogen receptors (Reviewed in De Vries and Panzica, 2006). Furthermore, unlike AVP expressing neurons in the PVN, SON, and SCN, AVP producing neurons in the amygdala and posterior BST are uniquely and extremely sensitive to changes in plasma testosterone (Zhou et al., 1994). Several previous reports have shown that AVP expression levels within both of these nuclei are decreased by gonadectomy, reversible with testosterone replacement, and are reduced in animals receiving the androgen receptor antagonist, flutamide ((Devries et al., 1983, 1984, 1986; Axelson et al., 1999). As our findings in Chapter 4 showed higher levels of AVP mRNA levels in the medial amygdala and posterior bed nuclei in High- compared to Low-bury animals we hypothesized that High-bury animals show higher levels of androgen receptors within these same nuclei. In the current study we compared androgen receptor cell numbers using an immunocytochemical detection method for androgen receptor immunoreactivity, in an adjacent series of brain tissue taken from the same animals studied in Chapters 2 - 4.

B. Materials and methods

Animals and treatment schedule. As noted above, the immunocytochemical detection of androgen receptors were performed on tissues obtained from the same animals used in Chapter 2.

Immunohistochemistry. To correlate steady-state differences in brain androgen receptor-immunoreactivity (ir) as a function of burying, animals were deeply anesthetized for perfusion twenty-four hours after the last exposure to the defensive burying chamber.

Androgen receptor-immunoreactivity (AR-ir) was localized in a series of sections through the posterior division of the bed nuclei of the stria terminalis (BST) and the posterodorsal part of the medial nucleus of the amygdala (MeApd) using a polyclonal antibody (PG-21; 0.67 µg/ml) raised in rabbit against amino acids 1-21 of the rat androgen receptor (Upstate, Charlottesville,
Sections were stained by using a conventional nickel-intensified, avidin-biotin-immunoperoxidase (Vectastain Elite ABC kit; Vector Laboratories, Burlington, CA) procedure (Sawchenko et al., 1990). Briefly, free-floating sections were first rinsed in KPBS buffer to remove cryoprotectant, then pretreated with 0.3% hydrogen peroxide for 10 minutes to quench endogenous peroxidase activity. This was followed by four rinses in KPBS, and then in sodium borohydride (1% w/v in KPBS) for 5 minutes to reduce free aldehydes. Sections were then incubated for 48 hours at 4°C in a KPBS-Triton (0.3% Triton-X; Sigma-Aldrich, Oakville, ON) solution containing 2% normal goat serum and the primary AR detecting antiserum (1:8000 dilution). A biotinylated anti-rabbit IgG secondary (vector laboratories, 1:4444 in KPBS-Triton) was used and then amplified using the previously mentioned ABC kit. Adjoining series of sections were stained with thionine for reference purposes. Tests for immunolabeling specificity involving the preadsorption of the primary antiserum with a synthetic peptide corresponding to the N-terminal region of the androgen receptor, or substituting the primary antibody with nonimmune serum yielded no specific labeling (Williamson and Viau, 2007). AR positive cells were identified as those showing a black reaction product in the nucleus, evaluated under bright field illumination and assisted by redirected sampling of adjacent thionine stained tissue series for location. The number of AR positive cells were derived from cell counts taken in complete, regularly spaced (150 μm) intervals through the posterior BST and MeApd, and corrected for double-counting error using Abercrombie’s formula (Guillery, 2002). Quantitative data are extrapolated estimates derived from the total corrected number of profiles encountered in each series of sections multiplied by the sectioning interval of 5.

Light- and dark-level images were captured using a Retiga 1300 CCD digital camera (Qimaging, Burnaby, BC), cataloged using a Macintosh OS X-driven Open Lab Image Improvision software v 3.0.9 (Quorum Technologies, Guelph, ON), and exported to Adobe Photoshop (v. 7.0) for final assembly at a resolution of 300 dpi. The Improvision quantitative software was also used to estimate the total number of neurons displaying AR-ir through the extent of the BST and MeA regions.

**Statistical analysis.** Androgen receptor data in the amygdala and bed nuclei of the stria terminalis were analyzed by two-way ANOVA (Group x Region) with Newman-Keuls post-hoc tests. Correlation analyses were performed using simple regression. Statistical comparisons
were made observer-blind by assigning coded designations to the video recordings, data, and tissues sets in advance.

C. Results

Amygdala. Total cell count estimates derived from androgen receptor staining through the extent of the posterodorsal aspect of the medial amygdala revealed significantly higher numbers of androgen positive cells in High-bury animals (figure 20, and see 21).

Bed Nucleus of the Stria terminalis. There was no significant difference in the number of androgen receptor positive cells within the anterior division of the BST, including the anterodorsal, anteroventral, oval, and fusiform nuclei (data not shown). Within the posterior division of the BST, androgen receptors were most conspicuously concentrated within the principle nucleus, and relatively moderate to lower numbers of androgen receptor positive cells were detected within the transverse and interfascicular nuclei (figure 21). High-bury animals displayed significantly higher numbers of androgen receptor positive cells throughout the posterior division of the BST (figure 20), and this difference was most apparent within the principle nucleus (figure 21). Redirected sampling of adjacent series of thionine-stained tissue revealed no significant bury effect on the volumes of the anterior and posterior bed nuclei of the BST or in the central and medial nuclei of the amygdala.

Correlational analysis

To further explore relationships between neuropeptide mRNA and androgen receptor levels as a function of conditioned burying, simple regression analyses of these parameters were performed using the time spent burying as the independent variable, as summarized in Table 1. When Low-bury animals were removed from the analysis, a single reliable relationship remained with respect to bury duration and androgen receptor number in the medial nucleus of the amygdala. This suggests that the difference in androgen receptor levels in the medial amygdala could be accounted for by variations in defensive burying, both within High-bury and between High- and Low-bury groups of animals. Similar results were found in all cases using bedding pile height as the independent variable, in line with the high correlation between bury duration and pile height (r = 0.96) across all animals.
Table 1. Regression analysis of neuropeptide mRNA and androgen receptor levels as a function of defensive burying activity.

<table>
<thead>
<tr>
<th>Variable/Region</th>
<th>Across High- and Low-bury</th>
<th>Within High-bury</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$P$ value</td>
<td>$r$ value</td>
</tr>
<tr>
<td>AVP mRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medial amygdala</td>
<td>0.0245</td>
<td>+0.50</td>
</tr>
<tr>
<td>Bed n. Stria Terminalis</td>
<td>0.0034</td>
<td>+0.62</td>
</tr>
<tr>
<td>Posterior magno. (PVN)</td>
<td>0.0273</td>
<td>-0.49</td>
</tr>
<tr>
<td>Supraoptic n.</td>
<td>0.0027</td>
<td>-0.64</td>
</tr>
<tr>
<td>OT mRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dorsal parvo. (PVN)</td>
<td>0.0024</td>
<td>-0.64</td>
</tr>
<tr>
<td>Posterior magno. (PVN)</td>
<td>0.0235</td>
<td>-0.50</td>
</tr>
<tr>
<td>Supraoptic n.</td>
<td>0.0074</td>
<td>-0.58</td>
</tr>
<tr>
<td>AR cell No.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medial amygdala</td>
<td>0.0009</td>
<td>+0.68</td>
</tr>
<tr>
<td>Bed n. Stria Terminalis</td>
<td>0.0792</td>
<td>+0.42</td>
</tr>
</tbody>
</table>
Figure 20. Mean ± SEM of the relative numbers of androgen receptor positive cells in the medial nucleus of the amygdala (MeA) and within the posterior division of the bed nucleus of the stria terminalis (BST) in High- and Low-bury rats. *P<0.05 vs Low-bury (n = 10 per group).
Figure 21. Androgen receptor staining in the medial amygdala and posterior division of the bed nucleus of the stria terminalis (BST) in High- and Low-bury rats. Representative brightfield photomicrographs of androgen receptor immunoreactive (AR-ir) staining through comparable levels of the medial nucleus of the amygdala and the posterior division of the BST from Low- and High-bury rats (left and middle panels, respectively). The localization of AR-ir to the posterodorsal (MEApd) and posterior division of the BST are outlined (right panels). Structures labeled for reference: cpd, cerebral peduncle; fx, fornix; int, internal capsule; ot, optic tract; sm, stria medullaris; st, stria terminalis; if, interfascicular nucleus; pr, principle nucleus; tr, transverse nucleus of the posterior BST. The relative rostrocaudal coordinates (mm) through each region of interest are based on the atlas of Swanson (1998). Scale bar = 100 $\mu$m (applies to all).
D. Discussion

Normal AVP expression in the medial amygdala and BST require testosterone and depend on androgen receptors (Devries et al., 1984,1985; Miller et al., 1992). Thus, we hypothesized that the increased display of AVP mRNA in these brain regions by the High-bury group of animals would be likewise associated with an increase in androgen receptor staining. Indeed, High-bury animals showed substantially higher levels of androgen receptor positive cells through the rostrocaudal extent of the posterior division of the BST and through the longitudinal extent of the medial amygdala, most apparent within the posterodorsal part of the nucleus.

While both of these nuclei have not been studied with respect to defensive burying behavior, several previous studies have shown that central AVP exerts stimulatory effects on active avoidance and anxiety-like behavior in the male rodent (reviewed in Landgraf and Neumann, 2004; Landgraf, 2005, 2006). Anatomically, both the medial nucleus of the amygdala and certain sub-nuclei of the posterior BST division (most likely intrafascicular and transverse nuclei) send robust projections to the lateral septum, a key nucleus mediating defensive burying behavior (Caffe et al., 1987; De Vries and Buijs, 1983). Since previously labeling studies have shown that virtually all of the AVP expressing cells in the BST and medial amygdala stain for the androgen receptor (Zhou et al., 1994), these projections to the septum are likely sensitive to testosterone.

Our pilot studies indicated no differences in plasma testosterone levels following the final day of testing (data not shown). However, this may not be an absolute requirement, given the group differences in androgen receptors. At this point, one obvious question is whether testosterone is capable of exerting effects on avoidance behavior and/or anxiety behavior. Adult rodents show major sex differences in anxiety behavior on the elevated plus maze (Frick et al., 2000), but do not show sex differences in defensive burying in response to shock prod (Treit et al., 1980, but see Arakawa, 2007). However, clear age dependent differences in defensive burying levels emerge with age amongst males, which increase during adolescence, peak at the onset of adulthood, and decline with age (Treit et al., 1980). As plasma testosterone levels and AVP expression in the MeA and BST show similar age related profiles, variations in plasma testosterone and androgen receptors levels could contribute to individual differences in defensive burying behavior. A recent study by Frye and Seliga (2001) showed that gonadal-intact rats displayed decreased shock prod induced freezing behavior compared to gonadectomized male
rats. As our study indicates, freezing behavior does not fully explain or account for an individual animals' propensity to bury, as the group difference in this response was only apparent on the first day of aversive trap testing. More recently, Fernández-Guastia and Martínez-Mota (2005) showed that testosterone can effectively reverse the stimulatory effects of gonadectomy on the time spent burying in response to shock prod. Moreover, the androgen receptor antagonist blocked the effects of testosterone on burying behavior. These findings stand in stark contrast to our findings, in which defensive burying correlated strongly and positively with the number of androgen receptor positive cells in the medial amygdala and posterior BST. While the findings, described by Fernández-Guastia and Martínez-Mota (2005), suggest that androgen receptors are capable of mediating the anxiolytic-like actions of testosterone, the opposite may be true under less aversive conditions, as our data would suggest.
Chapter 6. General Discussion

Animals live in a dynamic and complex natural environment full of challenges, they must deal with predators as well as conspecifics and react to change in environment. Fear and anxiety are normal emotions that animals have derived to help avoid these threats and dangers. Fear and anxiety bring about behavioral and physiological responses tailored to meet these challenges. These emotions are represented and altered in the brain via activation of specific regions, unique firing patterns, and neuropeptide expression. The end result of these emotions is altered state of mind and behavior. However, this altered state of mind and behavior is not the same for all animals, as considerable differences exist with respect to how animals respond to different types of challenges.

One area in which individuals can differ considerably is in their vulnerability to stress. Individuals seem to differ in their capacity to cope with environmental demands due to genotype, ontogeny, experience age and social support. These individual differences manifest themselves in behavior and physiological differences. Several attempts have been made to attempt to classify personalities or coping styles that will predict responses to environmental stressors. Individual differences in neurobiological makeup may account for these differences in coping strategies and subsequent predisposition to distress and disease.

Studies employing shock-induced defensive burying and less intense or aversive behavioral paradigms have made several important inroads on the neurobiological correlates of anxiety. However, these studies have focused primarily on animals showing burying responses only. Our findings underscore the utility of using a less aversive variant of the defensive burying paradigm, because it allowed us to identify rats that chose to bury and those that did not. Although we are not the first to notice this phenomenon, the central basis for this differential behavior has not been systematically approached. Our data leads us to hypothesize a role for centrally acting AVP, OT, and androgens in driving or responding to naturally occurring variations in defensive behavior, and to predict different neuroendocrine capacities in rats identified as High- and Low-bury animals.

Beyond using a pain-free, but no less aversive test of defensive behavior, our model is unique on several fronts. First, we employed a repeated measures and test design to rank and explore animals showing reliable and sustainable differences in conditioned defensive burying. Second, retrospective analysis of behavioral recordings taken during the habituation phase of the
paradigm allowed us to track the potential for spontaneous differences in unconditioned defensive behavior in individual rats that were designated as High- and Low-bury animals during the aversive phase. Third, we extended our behavioral survey to include indices of fear, exploration, avoidance, and trap reactivity scores to further differentiate how High- and Low-bury animals cope in response to repeated mousetrap exposure. Finally, subsequent hybridization and histochemical analyses of brain tissue taken from the same animals allowed us to determine how naturally occurring variations in defensive behavior are associated with differences in androgen receptor and neuropeptide mRNA levels within brain regions driving emotional and neuroendocrine motor responses.

Rats seem to have entered the experimental paradigm with already established tendencies to display defensive burying or not. This was observed when the mousetrap represented an aversive stimulus source (remotely triggered) and during habituation (unconditioned defensive burying). We selected and focused our attention on rats showing stable individual differences in burying display. It is important to note that the burying of bedding material was always directed towards the trap and that this occurred even in rats showing major rank order shifts in burying activity (high burying displayed during one trial and little to no burying on the next, for example), indicating that the mousetrap was readily identified as the specific source of aversive stimulation. Further, the magnitude of the behavioral responses observed, including burying duration and stretch attend, freezing, and grooming postures, fell well within the range previously observed in studies using a shock prod as an aversive stimulus. Taken together, the present findings reflect genuine individual tendencies in defensive behavior that were unmasked, rather than shaped by any underlying limitations of using a pain-free test of defensive burying.
6.1 Contributions to original knowledge

A list of our most salient findings is presented here towards discussing their individual implications, limitations, and future considerations (provided in section 6.2).

1. Removing the aspect of pain from the defensive burying paradigm invited extreme individual differences in defensive burying activity.

2. Individual animals appeared to have entered the paradigm with different tendencies to show defensive burying behavior both under aversive and non-aversive (habituation) conditions.

3. Designated and grouped as High- and Low-bury rats, animals also showed marked departures in the level of risk assessment, fear- and anxiety-like behavior.

4. As a group, High-bury animals showed higher levels of AVP mRNA within limbic regions of the brain that form part of several circuits mediating avoidance and/or anxiety-like behavior.

5. In contrast, Low-bury animals showed higher levels of OT mRNA within regions of the PVN that may be responsible for driving inhibitory effects on anxiety.

6. Bury-group differences in AVP and OT mRNA were also evident within other sub-regions of the PVN that are related to neuroendocrine and pre-autonomic function.

7. Finally, consistent with the known stimulatory effects of androgens on central AVP expression unique to regions of the medial amygdala and posterior BST, High-bury animals also showed higher levels of androgen receptor cell numbers within these same regions.
6.2 Limitations and future considerations
At this point, our findings with respect to central AVP and OT pathways related to anxiety and avoidance behavior are correlational. However, these peptides have been shown to evoke stimulatory and inhibitory effects, respectively, in several other tests of anxiety, including open-field and social recognition. We predict that future studies examining the effects of central administration of AVP and OT receptor antagonists will show a bias towards AVP and OT utilization in High- and Low-bury animals, respectively.

These considerations are now possible, because our paradigm shows that we can reliably predict which animals will show high or low tendencies to bury based on their performance under neutral trap conditions. As well, animals showing stable individual differences in defensive burying over the course of aversive trap testing, retained this differential response during a single test of retention.

Unlike most studies examining the effects of anxiolytics on defensive behavior, we have examined several behavioral dimensions, including risk assessment, exploratory, freezing, and rearing behavior. Taking the all of these behaviors into consideration, our findings would suggest that High- and Low-bury animals possess different motivations for actively engaging or avoiding the mousetrap altogether. How each of these behaviors change, individually or in concert, in response to anxiolytic treatment will permit us to study whether these behaviors occur as a function of defensive behavior or represent components of the animal's defensive behavior repertoire.

Differences in AVP and OT expression within sub-regions of the PVN predict that High- and Low-burying animals show different neuroendocrine capacities in response to psychological or physiological challenges. As discussed earlier, there is a fair bit of heterogeneity in the PVN. Thus, further functional and connectional studies are required to determine whether High- and Low-bury animals show differences in neurons peptide expression in the PVN that occur within neurons directed at the brainstem, pituitary, or to the forebrain. Likewise, the extent to which the group differences in AVP expression in the medial amygdala and posterior BST occur within neurons projecting to the septum, as well as the PVN, remains to be seen.

Taken together, our findings have provided a reliable and testable framework for examining the central nervous system bases for individual differences in defensive behavior.
References


Appendix 1. Animal care certificate

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A05-0323

Investigator or Course Director: Victor Vianu

Department: Cellular & Physiological Sc.

Animals:

Rats Sprague Dawley, Long-Evans 150
Sprague Dawley 100

Start Date: April 1, 2004

Approval Date: May 30, 2007

Funding Sources:

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Androgen-sensitive pathways to the paraventricular nucleus of the hypothalamus

Funding Agency: Natural Sciences and Engineering Research Council of Canada (NSERC)
Funding Title: Critical influence of neonatal testosterone on stress pathways in the adult brain

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.