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Adaptations to chronic unpredictable threat stress: Effects on defensive behaviors and working memory

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Abstract of the Dissertation

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In humans, stressors are often psychological and anticipatory in nature rather than directly harmful. In order to understand the consequences of chronic psychological stress and behavioral adaptations to repeated unpredictable threats, an ethologically relevant rodent model was developed that included the anticipation of a predator attack but without direct harm. In the unpredictable threat stress condition, rats faced risk while seeking resources through a tunnel over a three week period. When rats cross the tunnel, they were presented with random (p=0.25), simultaneous presentations of predator odor, flashing LEDs, and an abrupt auditory stimulus. The control group was housed in identical tunnels but never experienced threat stimuli.

The aims of the study were the following: (1) to quantitatively test the effectiveness of the stimuli and the duration with which they remain effective over repeated presentations, (2) to further test whether the stimuli were perceived as threats of harm by measuring the frequency of behaviors known to be elicited in the face of predators, (3) to test for adaptations in direct defensive responses as well as behaviors with predictive validity for symptoms of anxiety and

depression, (4) to test for changes in cognitive behavior with a standard rodent test of spatial working memory, and (5) to identify candidate sites of plasticity by metabolic factors that indirectly reflect rates of neural activity.

While in the manipulation, foraging was reduced but there were no differences in food/water consumption and weight gain indicating no significant challenges to homeostasis. The stress group produced significantly more risk assessment behaviors than the control group confirming the aversive nature of the stimuli. After removal from the stress manipulation, the stress group exhibited greater defensive responding consistent with threat-related vigilance and hyper-reactivity, both of which would be expected to increase an organism's survival in high threat environments. There were no changes in passive avoidance or behavioral symptoms of depression. Metabolic changes were observed in brain regions associated with threat, which included the dorsal premammillary nucleus of the hypothalamus, a critical region in response to predator. Repeated exposure to threat over three weeks enhanced defense behaviors tested in high arousal conditions, but at the expense of spatial working memory tested in neutral test conditions. These shifts in behavior may be adaptive in unpredictable, high threat environments but may be maladaptive in safe environments.

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Chapter 1

General Introduction

Stress is an everyday occurrence when an organism aims to uphold a steady equilibrium in response to a constantly changing environment. It is a fundamental component of life as it provides balance between activity and rest (R. M. Nesse, Bhatnagar, & Young, 2007). Energy is needed to maintain life and activity is needed in order to consume energy. All living organisms face demands such as seeking food and shelter (Kavaliers & Choleris, 2001; R. M. Nesse et al., 2007). These inevitable trade-offs and decisions are daily occurrences that require comparisons between the benefits and costs of resource attainment (Kavaliers & Choleris, 2001). After all, homeostasis requires avoidance of harm and yet acquisition of resources is crucial for survival. There is a push and pull between approach and avoidance that is analogous to foraging behavior when organisms must leave safe locations and expend energy in order to obtain resources. When the costs and risks outweigh resources, the organism is at a risk of enduring a chronic stress response. A stress stimulus or stressor, which may be either external and internal demands, induces an integrated stress response, which includes physiological (autonomic, neuroendocrine and endocrine responses) and behavioral responses (Herman & Cullinan, 1997; Jankord & Herman, 2008a, 2008b; McEwen, 2007; Ulrich-Lai & Herman, 2009b; Ursin & Eriksen, 2004).

Coping can be described as the behavioral and physiological efforts to overcome the stressors and is fundamentally necessary to adapt (Folkman, Lazarus, Dunkelschetter, Delongis, & Gruen, 1986; Koolhaas et al., 1999). After repeated exposure to stressors, both physiological and behavioral responses adjust to match demands (R. M. Nesse et al., 2007); adjustments may

improve efficiency or lower the threshold for the detection of stressors (Masten, 2001; Ulrich-Lai & Herman, 2009b). Effective generalization of stress adaptations to novel stressors is referred to as resilience (Feder, Nestler, & Charney, 2009). However, when environments change and adaptations are ineffective, the organism may be more vulnerable. When stress is chronic, the accumulating damage from stress may precipitate disorders such as anxiety and depression (Kessler, 1997). The accumulation of damage and the types of adjustments are likely related to unique characteristics of stressors. Adaptations to stressors, in the form of adaptations in coping, are influenced by the frequency and duration of stressors as well as key environmental features such as controllability, predictability, and novelty (Amat, Paul, Zarza, Watkins, & Maier, 2006). Although these factors have long been discussed in relationship to stressors (Mason, 1968b), systematic manipulation of these factors to differentiate outcomes has been carried out only after acute stress (Amat et al., 2006). Environmental factors shape coping and to better understand the behavioral adaptation to chronic stress will require a better understanding of the role environmental factors play in shaping adaptations.

In a dynamic environment, both human and non-human animals share the need to leave the safety of home, and risk harm to gather resources (Aupperle, Sullivan, Melrose, Paulus, & Stein, 2011; Goldstone & Ashpole, 2004; Kavaliers & Choleris, 2001). To better understand the relationship between environmental variables and behavior, a platform that essentially strips environments down to the basic approach-avoidance conflict within a simplified foraging environment is necessary. Furthermore, an approach/avoidance conflict during foraging is ethologically relevant and highly instrumental in the understanding of our relationship with environmental parameters that contribute to behavioral shifts and associated neural plasticity.

I. 2. Parameters of stimuli that elicit the stress response

Generally, the activation of the stress response is not detrimental when stressors are infrequent and low in intense (McEwen, 2000a). In shorter periods of time, acute stress responses may be beneficial as they restore homeostasis or prepare the body for an impending homeostatic challenge. Chronic stress responses involve the continuous exposure to a stressor or anticipated stressors (Kovacs, Miklos, & Bali, 2004). A stressor may not have harmful effects itself, but when a homeostatic challenge detrimentally affects the normal homeostatic state, the allostatic load is increased (McEwen, 1998b). Allostatic load occurs when the stress response surpasses the normal homeostatic range of the organism (Day, 2005; McEwen, 2008). The intensity, frequency, and duration of the stressor may influence the stress-related neuronal response (Kovacs et al., 2004). In regards to intensity, when a relatively mild stressor ends, the stress response is terminated shortly after the stressor termination, but after a severe stressor, the response may exceed exposure to the stressor or signals of threat (Garcia, Marti, Valles, Dal-Zotto, & Armario, 2000; McEwen, 1998b). The high frequency or occurrence of stressors may have debilitating effects, because of a failure to respond to the repeated stressors. Prolonged or severe stress may lead to a high allostatic load (McEwen, 1998b). This may lead to imbalanced and inadequate responses to the stressors (Herman & Cullinan, 1997; Krishnan & Nestler, 2008; McEwen, 2008).

The key features of stress stimuli that initiate a stress response are controllability, predictability, and type of stressor (physical or psychological) (Anisman & Merali, 1999b; Herman & Cullinan, 1997; McEwen, 1998b). The controllability of the stress stimuli refers to the actual or perceived control over the occurrence or outcomes (Mineka & Hendersen, 1985; Weiss, 1972a). It also includes the mastery of positive outcomes through prior exposure to the

stimuli (Mineka & Hendersen, 1985). Uncontrollability refers to the lack of control or perceived control over the occurrence, such as unavoidability, and consequences (e.g. termination) (Maier, Ryan, Barksdale, & Kalin, 1986; Mineka & Hendersen, 1985). For example, if there is an impaired ability to detect response-outcome contingencies, or if there is a disassociation between the organism's potential ability and its effectiveness, the organism develops learned helplessness (Mineka & Hendersen, 1985; Overmier & Seligman, 1967; Weiss, 1972b). Additionally, having previous behavioral control (escapable shock stress) reduces the effects of inescapable shock stress at a later time point. It may not be the physical shock of the aversive stimuli, but the degree to which the stimulus cannot be controlled once it is presented that may produce impairments (Amat et al., 2006; Weiss, 1972a). Also, experiencing inescapable shock stress decreases later social exploration behaviors, a measure of anxiety while escapable shock stress actually increases later social exploration as compared to controls (Kubala, Christianson, Kaufman, Watkins, & Maier, 2012). Not having a means of escape or control over the outcome may produce both symptoms associated with anxiety and depression. The experiences with either controllable or uncontrollable stressors shape behavioral, physiological, and response-outcome contingency learning that affects future coping mechanisms.

The predictability of the stress stimuli depends upon the availability of a cue that precedes a stressor (Grillon, Baas, Lissek, Smith, & Milstein, 2004; Mineka & Hendersen, 1985). Some studies suggest that the unpredictable delivery of the presentation of a stressor, such as shock, may increase the stress response (Tsuda, Ida, Satoh, Tsujimaru, & Tanaka, 1989), but other studies have shown that predictability may be more stressful (Pitman et al., 1995). Having predictability may lead to habituation (Grissom & Bhatnagar, 2009; Thompson & Spencer, 1966). The unpredictable nature of a stimulus can produce a sustained generalized

stress response (Masini, Sauer, & Campeau, 2005; Selye & Fortier, 1949) followed by risk assessment (D. C. Blanchard, Blanchard, Griebel, & Nutt, 2008). In animals, there are unconditioned responses to predator threat, which likely reflect the potential fatal outcome of ignoring predator cues (Kavaliers & Choleris, 2001). Although many studies have tested the outcomes of uncontrollable stressors, few animal studies have tested behavioral adaptations to repeated unpredictable threats that are never followed by pain or harm. In chronic unpredictable stress (CUS) models, there are a variety of unpredictable stress presentations such as intermittent footshock and food/water deprivation, but nevertheless the anticipation of the stress presentation always leads to the actual stimuli presentation (Bondi, Rodriguez, Gould, Frazer, & Morilak, 2008; Papp, Willner, & Muscat, 1991). The expectancy of the anticipated stimuli led to the actual occurrence. Since humans detect threat often, despite few actual damaging events, it is important to identify the behavioral adaptations to unpredictable threat with high control. Studies should seek to observe adaptations to stressors (threat and abrupt stimuli) that cannot be predicted in time, which are also not paired with direct physical harm.

I.3. Brain structures involved in psychological stress

The type of stressor, physical or psychological, is detected and initiated at different key regions of the brain that ultimately elicit the stress response. The initiation of a stress response is dependent upon the detection of challenges to homeostasis, which include extreme temperatures, hypoxia, hypoglycemia, dehydration, and injury (Ulrich-Lai & Herman, 2009b). Direct, physical signals reflecting homeostatic imbalance, pain, and inflammation activate the brainstem, hypothalamus, and circumventricular organs. Stressors that originate in the periphery such as fluid and electrolyte imbalance and blood pressure signal the lamina terminalis system in the forebrain which includes the median preoptic nucleus, the subfornical organ, and organum

vasculosum (Ulrich-Lai & Herman, 2009b). Physical stress responses are dependent upon the detection at the level of the brainstem, midbrain and hypothalamus, and response initiation at these levels (Anisman & Merali, 1999a; Chrousos & Gold, 1992; Herman & Cullinan, 1997; Ulrich-Lai & Herman, 2009b). External cues may cause anticipation of internal challenges and in turn elicit a preemptive stress response. Preemptive responses rely upon the cognitive interpretation of external stimuli carried out by higher order brain areas (Ulrich-Lai & Herman, 2009b) that act on the physical stress systems to generate a stress response.

Indirect threats such as stress related to work (i.e. job insecurity and role overload) (Coverman, 1989) or threats of traumatic events (i.e. terrorism, violence, and natural disasters) (Cancro, 2004; Cohen & Eid, 2007) or social conflict (Rayner & Hoel, 1997) require anticipation of challenges to homeostasis, but are also capable of inducing a stress response. Psychological stress responses are typically dependent upon previous experience (Herman & Cullinan, 1997; Jankord & Herman, 2008b). Three higher order brain areas detect threat and initiate the psychological stress response: mesocortical/mesolimbic systems respond to the anticipation of threat, the limbic areas of the amygdala, hippocampus, and prefrontal cortex initiate and terminate the stress response, and the arcuate nucleus set the pain sensation (Herman et al., 2003; Tsigos & Chrousos, 2002). Detecting environmental predictors of homeostatic challenge allows organisms to engage in preparatory physiological and behavioral responses. Multiple sensory modalities can detect cues predicting direct stressors. The limbic regions, including the hippocampus, amygdala, and the prefrontal cortex, process these sensory modalities.

Although the psychological stress response is initiated by higher order structures, those structures act on and modulate the physical stress system (Herman & Cullinan, 1997). The mPFC is the limbic association area which receives input from multiple sensory modalities and

has descending projections to the hypothalamus (Radley, Arias, & Sawchenko, 2006), which is the involuntary motor control center. In the rat, the infralimbic area of the prefrontal cortex projects extensively to the medial and central nucleus of the amygdala, which can promote and regulate the stress response. It also projects to the anterior bed nucleus of the stria terminalis, a major integrator of stressor information, and the nucleus of the solitary tract, which is a major efferent of the SNS (Radley et al., 2006; Sesack, Deutch, Roth, & Bunney, 1989). The prelimbic area of the prefrontal cortex projects to the ventrolateral preoptic area, dorsomedial hypothalamus, and peri-PVN regions which inhibits the stress response (Sesack et al., 1989). The prelimbic mPFC processes information about psychological stressors and is implicated in regulating the duration of the stress response (Radley et al., 2006). The orbitofrontal area of the prefrontal cortex and anterior cingulate cortex (ACC) are involved in the appraisal process, which in turn may affect stress regulation (Dedovic, Duchesne, Andrews, Engert, & Pruessner, 2009). The medial prefrontal cortex also has connections with the amygdala, which processes threatening stimuli and relays the information for an appropriate behavioral output (M. Davis & Whalen, 2001).

The hippocampus and amygdala are two other limbic regions associated with the psychological stress response. The amygdala regulates the stress response through feed-forward mechanisms as opposed to the mainly inhibitory roles of the hippocampus and mPFC (Herman, Ostrander, Mueller, & Figueiredo, 2005). The hippocampus projects to the BNST, which in turn projects to the PVN of the hypothalamus, which is the region that secretes hormones needed to produce a stress response. The hippocampus is part of a negative feedback loop to terminate the stress response. This region has relatively high density of glucocorticoid receptors (GC). The GC receives input from glucocorticoid hormones (or cortisol in humans) that dictates the

cessation of the stress response (Sapolsky, Krey, & McEwen, 1984a). The amygdala plays an excitatory role in the stress response and is comprised of different subnuclei that are differentially activated by either physical or psychological stressors. The central amygdala (CeA) is generally activated by physical stressors that directly disrupt homeostasis, but has a role in integrating the autonomic elements of psychological stressors (Herman & Cullinan, 1997). The central nuclei project to the BNST which integrates the information and projects onto the PVN (Ulrich-Lai & Herman, 2009b). Psychological stressors preferentially activate the medial (MeA) (Canteras, Kroon, Do-Monte, Pavesi, & Carobrez, 2008; Canteras, Simerly, & Swanson, 1995) and basolateral (BLA) amygdala (Cullinan, Herman, Battaglia, Akil, & Watson, 1995). Output from the excitatory regions such as CeA, MeA, and infralimbic mPFC regions are integrated with the inhibitory outputs from the ventral subiculum and prelimbic cortex to control neurons of the PVN, which play a critical role in activating the stress response (Herman et al., 2003).

These limbic regions may also be modulated by the locus coeruleus (LC) (Ziegler, Cass, & Herman, 1999), a structure activated by both intrinsic (i.e. hypoglycemia and decreased blood volume and pressure) and extrinsic (i.e. environmental stress) stressors (Charney, 2004). It has been referred to as the "cognitive arm" of the stress response. Through activation of the LC, stressors generate heightened awareness and sensitivity to the environment by modulating forebrain neuronal activity and, in turn cognitive functions including attention, memory, and sensory information processing (Espana, Reis, Valentino, & Berridge, 2005). Selective states of LC activity promote selective attention to stimuli (Aston-Jones, Rajkowski, & Cohen, 1999). The LC plays an important role in the discrimination of relevant versus irrelevant environmental threat cues. These regions are necessary for the detection of psychological stressors and the

onset of the appropriate stress response activation and termination. Sustained stress reactivity may contribute to both functional and structural damage to the key regions associated with the stress response (McEwen, 1998a).

I. 4. Neural systems that detect stress stimuli and produce the stress response

The stress response involves three physiological pathways including the Autonomic Nervous System (ANS), Sympathetic Nervous System (SNS), Hypothalamic-pituitary-adrenal (HPA) axis and a behavioral response. The Autonomic Nervous System (ANS) is the system with the most immediate response to stressor exposure, whereas the endocrine response through the HPA axis is much slower. The ANS has two components; the slightly delayed response through neural innervation of the adrenal medulla, which provides the circulating epinephrine and norepinephrine (Tsigos & Chrousos, 2002) that are broadly distributed through the vascular system. The fastest response is through the sympathetic nervous system (SNS) innervation of the peripheral organs. The SNS innervation is derived from efferent preganglionic fiberswhich are mostly cholinergic, and are located closely to the intermediolateral column of the spinal cord. The nerves synapse onto the postganglionic sympathetic neurons and are mostly noradrenergic and innervate many organs such as the heart, gut, and smooth muscle of the vasculature, and skeletal muscles (Tsigos & Chrousos, 2002). In contrast to the direct projections of the SNS, the sympatho-adreno-medullary system is slower but has a longer duration. The slowest and longest lasting response (Ulrich-Lai & Herman, 2009b) is the endocrine response, which is carried out by the hypothalamic-pituitary-adrenal (HPA) axis. This response is initiated by the parvocellular corticotropin-releasing hormone (CRH) and arginine-vasopression (AVP) neurons in the paraventricular nuclei (PVN) (Chrousos & Gold, 1992; Herman & Cullinan, 1997). CRH and AVP, in turn, promote the secretion of adrenocorticotropic hormone (ACTH) from the

anterior pituitary gland. ACTH is released into the vascular system, where it travels to the adrenal cortex. There, it initiates the synthesis and release of glucocorticoid hormones (Ulrich-Lai & Herman, 2009b). In humans, the primary glucorticoid is cortisol, whereas in rats, it is corticosterone. Glucocorticoids (GC) are necessary hormones to mobilize energy resources for adaptive responses to stressors (Sapolsky, Romero, & Munck, 2000), and inhibit the immune system (Keller, Weiss, Schleifer, Miller, & Stein, 1983). In the CNS, GCs also increase arousal and attention (Charney, 2004; Lupien, McEwen, Gunnar, & Heim, 2009; Tsigos & Chrousos, 2002), which serve to enhance awareness of external threat.

The ANS and HPA-axis collaborate to cover short and long-term physiological responses that effectively manage the need for both immediate and long lasting behavioral responses. The vital targets of physical (systemic) stress responses are the HPA axis and ANS (Herman et al., 2003), which are controlled by the hypothalamus and brainstem nuclei such as the NTS. These structures receive descending input from higher order brain areas. The ANS response can be driven by brain areas as high as the medial prefrontal cortex (mPFC) and central amygdala (CeA), which projects to the ventrolateral bed nucleus of the striatum (vlBST), the paraventricular nucleus (PVN) of the hypothalamus and, in turn, to the nucleus solitary tract (NST). This provides descending projections outside the brain to the ANS (Ulrich-Lai & Herman, 2009b). The hypothalamic PVN is the primary integrator of stress signals. In addition to its projections to the NTS, the PVN projects directly to the median eminence, brainstem, intermediolateral cell colum (IML), the parabrachial nucleus (PBN), the dorsal motor nucleus of the vagus nerve (DMX)(Ulrich-Lai & Herman, 2009b). Just as the ANS can be driven directly by ascending physiological input; it can also be initiated indirectly by cognitive interpretation of impending challenges to homeostasis.

As previously discussed, higher order brain regions have been implicated in the initiation of the HPA axis to psychological stress response, a process that requires processing of external stimuli to detect signals of impending threat. Acute stress responses involve a relatively short-lasting and rapid activation and termination of the HPA axis with enhanced secretion of the ACTH and GC (McEwen, 1998a). Adaptation to the stress state may produce functional alterations in the limbic regions that may influence the HPA axis and GC hyper- or hyposecretion (Herman et al., 2005). The frequency and duration of psychological stressors may be more chronic and repetitive than physical stress, in turn, producing a sustained or frequent stress response. Whereas physical stressors have immediate reflexive responses, the anticipatory nature of psychological stressors increases the likelihood of longer duration, and the possibility of a false negative increases the frequency of psychological stress. Accordingly, psychological stressors may produce a more sustained state of physiological responses, making them more damaging. In psychological stress response, there is a top down control of the stress response system where higher order regions can both influence in the initiation and termination.

The HPA axis stress response is controlled by a negative feedback mechanism from the hippocampus and mPFC. Both glucocorticoids receptors (GR) and mineralcorticoid receptors (MR) are located in these regions (De Kloet, Vreugdenhil, Oitzl, & Joels, 1998; Reul & de Kloet, 1986). The GRs are involved in regulating both the basal levels and termination of the stress response (Furay, Bruestle, & Herman, 2008). The MRs have high affinity for glucocorticoids while the glucocorticoid receptors have a lower affinity. The low affinity receptors dramatically increases the range for detection of high levels of GC (De Kloet et al., 1998). Elevated plasma ACTH acts on receptors of the adrenal cortex, which lead to an upsurge of plasma glucocorticoids (GC) (Lopez, Akil, & Watson, 1999). ACTH controls cortisol

release. CRH expression and ACTH secretion are inhibited when circulating GC binds to cytoplasmic receptors in the hypothalamus (Lopez et al., 1999). The increase in glucocorticoids inhibits further activation and advancement of the HPA axis. As the concentration of glucocorticoids increase the binding to MR reaches maximum capacity, leaving GR critical for controlling homeostatic balance when circulating glucocorticoids are high (Lopez et al., 1999). The initiation and termination of the HPA axis is crucial for adaptive homeostatic regulation.

I. 5. Risk Detection and Assessment

Coping behaviors are a consistent feature of the stress response (Lazarus, 1984). Coping can be described as both behavioral and physiological responses to stress stimuli (Koolhaas et al., 1999). The characteristics of the stressors shape emotional coping behaviors. It may be the cognitive appraisal of the stressor, such as perceived controllability and predictability that determines the style of emotional coping (Christianson, Thompson, Watkins, & Maier, 2009; Koolhaas et al., 1999). There are two general types of emotional behavioral responses, active and passive. The controllability of the stressor may initiate a set of emotional responses. If the stress is escapable, there is control, (Floyd, Price, Ferry, Keay, & Bandler, 2000; Keay & Bandler, 2001; Weiss, 1971) and active coping such as flight is employed. In contrast, inescapable stressors produce passive (reactive) coping, which are characterized by immobility and decreased responsiveness (Keay & Bandler, 2001; Koolhaas et al., 1999). The two styles of coping are accompanied by physiological responses that vary in the balance between the ANS and endocrine responses. Proactive coping results in low HPA-axis reactivity with high sympathetic reactivity, and reactive coping involves higher HPA axis reactivity and high parasympathetic reactivity (Koolhaas et al., 1999). The ANS is also activated when active coping is anticipated but not yet produced. The sympathetic system provides the energy in

preparation for fight or flight (Misslin, 2003). The heart rate and cardiac activity are increased, and blood vessels are dilated, particularly in the periphery, as the animal is preparing for a defensive movement (Misslin, 2003). Both neural and behavioral responses are complementary in the initiation and reaction to the stress stimuli.

In animals, the type of coping behavior varies with distance from a predator or threat. These include: circa-strike, pre-encounter, and post-encounter. Circa-strike defense occurs when the predator threat is near or physical contact is made. This type of defense is associated with phasic fear, which is defined as a reaction to immediate and proximal threat. It occurs and dissipates as soon as the threatening stimulus is removed (Jovanovic et al., 2010) . The smallest defensive distances result in attack whereas intermediate distances elicit flight or freezing (McNaughton & Corr, 2004). Pre-encounter defense occurs when a predator is not present but has been previously encountered in the present location, and post-encounter defense occurs when a predator is present but at a distance (Jovanovic et al., 2010). Therefore, detection of risk and distance to risk,determine both neural and behavioral responses to the threat (D. C. Blanchard, Griebel, Pobbe, & Blanchard, 2011).

I. 6. Threat and defensive behavior

During foraging, there is a conflict between energy consumption and activity such as feeding and reproduction and energy expenditure activity and risks such as predation and the inability to obtain resources (Kavaliers & Choleris, 2001). There is a motivational conflict between energy intake activity and the potential harm that may arise from leaving a safe area to obtain sources of energy. The decision-making depends on the benefits of gaining resources such as food consumption and mating and the susceptibility to predation (Bednekoff & Lima, 1998). When threat is perceived close in distance, an animal may give complete attention to the threat

and terminate all other activities such as feeding, and is in a state of vigilance (D. C. Blanchard et al., 2011; Kavaliers & Choleris, 2001). In order to gain information of an impending threat, the animal portrays risk assessment behaviors to confront potential risk (R. J. Blanchard & D. C. Blanchard, 1989a).

Risk assessment includes behaviors associated with allocating attention for detection and response to potential threat (D. C. Blanchard et al., 2011; Kavaliers & Choleris, 2001). In animals, there is an apprehension gradient ranging from no attention to a predator to complete preoccupation with a predator; this would lead to a range from foraging with no analysis of potential risk to complete cessation of ongoing activities to attend to the potential threat (Kavaliers & Choleris, 2001; S. L. Lima & Bednekoff, 1999). As more information is gathered from the environment, both behavioral and stress responses are fined-tuned to match the impending threat. Risk assessment behaviors are utilized to investigate if the stimulus is an actual threat. Stretched attend behaviors consist of hind limb immobility while the front limbs are approaching the stimulus. Also head-scanning behavior is used to scan the environment for potential threat (Hubbard et al., 2004). These risk assessment behaviors are utilized when the threat stimulus is ambiguous (D. C. Blanchard et al., 2011).

As risk assessment behaviors range from assessment to heightened vigilance (complete cessation of all ongoing activity and complete attention to the impending threat) (Kavaliers & Choleris, 2001), a variety of defensive behaviors are utilized with information from the environment. Animals will escape if a route is available (Pinel, Treit, Ladak, & MacLennan, 1980), hide and freeze in safe areas (Dielenberg & McGregor, 1999, 2001) and defensively bury a potential threat (Pinel et al., 1980). In animals, one misjudgment of potential threat may be detrimental (Kavaliers & Choleris, 2001). Environmental parameters such as predictability are

highly useful in order to gain information about the impending harm and to optimize behaviors to counteract the possibility of harm. If an environment is unpredictable, risk assessment behaviors are necessary in order to evaluate the environment properly. Depending on the distance and the actual potential for harm, both risk assessment and defensive behaviors are utilized in response to threat distance. There is a continuum between risk assessment and defensive behaviors, and both can be displayed in sequence.

I. 7. Fear and Anxiety

The defense behavior system is involved in response to fear (Misslin, 2003) and is crucial to the survival of the animal (R. J. Blanchard & D. C. Blanchard, 1989a; R. M. Nesse, 2001). Fear is an emotion accompanied by adaptive physiological and behavioral responses to immediate or anticipated threat (Bishop, 2007). Fear is distinct from anxiety (Lang, Davis, & Ohman, 2000). It is associated with short, discrete cues that are strongly predictive of aversive stimuli, whereas anxiety is associated with diffuse cues and the timing of the aversive event, and therefore is related to lack of predictability in a threatening environment (M. Davis, 2006). Since fear is immediate, the animal may be more likely to produce behaviors such as fight, or flight, or freezing. Generally, fear is associated with the animal potentially moving away from the threat. Pre-encounter (potential threat) and post-encounter (distal threat) are involved in sustained fear, or anxiety, where immediate defensive behavior shifts to sustained risk assessment (Jovanovic et al., 2010) a behavior associated with fear to potential, non-immediate threats (Misslin, 2003). Risk assessment behaviors may be sustained as the animal is moving towards the threat and assessing the potential threat. Vigilance and attention are heightened to diffuse threats that are unpredictable (R. J. Blanchard, Yudko, Rodgers, & Blanchard, 1993; M. Davis, 2006). States of

anxiety last longer and therefore termed sustained fear (M. Davis, 2006; Walker, Toufexis, & Davis, 2003).

The defensive and coping behaviors can be initiated by the same brain structures implicated in the physiological stress responses. Thus, the physiological responses are coordinated with the behavioral responses. These immediate reactions include freezing, increased alertness, and escape (Vianna, Graeff, Brandao, & Landeira-Fernandez, 2001). Both involve the limbic areas and forebrain circuitry. The fear circuitry involves the CeA, anterior cingulate, medial hypothalamus and PAG (M. Davis, 2006; McNaughton & Corr, 2004). As the organism assesses the potential threat, the amygdala and PAG are involved in the process of triggering appropriate fear defense responses to the assessment (Misslin, 2003). The amygdala is part of the sensory region that detects and integrates the cues in the environment for potential threat and the PAG is a motor output region influencing defensive behaviors (Cezario, Ribeiro-Barbosa, Baldo, & Canteras, 2008). Defensive avoidance involves the prefrontal (ventral stream), anterior cingulate, amygdala, medial hypothalamus, and PAG (McNaughton & Corr, 2004). Defensive approach behavior involves neural areas that overlap with those that control defensive avoidance behavior: prefrontal (dorsal stream), posterior cingulated, septo-hippocampal-system, amygdala, medial hypothalamus, and PAG (McNaughton & Corr, 2004). The PAG includes subregions that, when stimulated, produce either active (dlPAG) or passive responses (vlPAG). The active and passive responses include escape and freezing behaviors, respectively (Vianna, Graeff, et al., 2001; Vianna, Landeira-Fernandez, & Brandao, 2001). These responses may be modulated by descending projections from the forebrain, which processes aversive stimuli (Keay & Bandler, 2001; Vianna & Brandao, 2003). Thus, within the

brain regions generally tied to stress, fear and anxiety, there are subregions that control the specific nature or strategy of the coping response.

The limbic system plays an essential role in the neurocircuitry of fear and anxiety, just as it plays a role in stress. The basolateral amygdala (BLA) may play a key role in the acquisition of fear and fear-learning (Walker et al., 2003). The BLA projects to the central nucleus (CeA) and the bed nucleus of the stria terminalis (BNST); both are involved in the integration of fear responses, for example, respiratory distress and corticosteroid release (Jovanovic et al., 2010; Walker et al., 2003). The CeA have direct projections to the hypothalamus and brainstem which may be involved in the activation of the autonomic nervous system and neuroendocrine responses (M. Davis, 1992). The CeA is the key region for the fear response. The BNST is required for the sustained state of fear and apprehension (Walker & Davis, 1997). The BNST integrates the information from the limbic regions and is central to the regulation of the HPA-axis (Walker et al., 2003). As the organism navigates through an environment with potential threat, the key regions associated with the stress and fear responses are interlinked and physiological responses and defensive behaviors.

The development and progression of fear is likely dependent upon selective characteristics of aversive stimuli, including whether they are predictable and controllable. For example, the novelty of a stimulus may provoke fear responses in animal models (R.J. Blanchard, M.J. Kelley, & D.C. Blanchard, 1974). The fear response may be driven by the uncertainty of the novel stimuli, which have no predictive value. Novel situations activate the CeA, hypothalamus, and brainstem (M. Davis, 1992) which are involved in behaviors characterized by fear including a complex pattern of defensive reactions including both flight and freezing.(R.J. Blanchard et al., 1974; Kabbaj, Devine, Savage, & Akil, 2000). With anxiety,

the prefrontal cortex, BLA, and BNST (M. Davis, 1992) are involved in the top-down control mechanisms involved with association, attention, and interpretive processes (Bishop, 2007). The hippocampus plays the central role in the acquisition of learned fear responses; it processes the contextual information of the stressful stimuli and projects to the amygdala for the production of behavioral and endocrine response (Bishop, 2007). Organisms often face conditions in which they attempt control (+C) over stressors or attempt to predict (+P) the timing and associations of stressors and cues. When threat is highly probable but uncontrollable (-C) or unpredictable (-P), then the organism will be constantly vigilant and prepared to defend itself, features of anxiety. Thus lack of control or prediction (-C/-P) may be key environmental factors that induce anxious states. In animal models, inescapable stressors can produce a state of anxiety (Amat, Matus-Amat, Watkins, & Maier, 1998). Similarly, repeated unpredictable threat may be a significant factor in the development of sustained apprehension, a key characteristic in anxiety (Grillon et al., 2004). Alternatively, lack of prediction *with control*, may be sufficient to induce risk assessment and defense behaviors, but not anxiety. Different parameters of the environments produce the development of behaviors optimized to those parameters. Currently, there are models without control or escapable parameters but few with low predictability but full control and escape (Amat et al., 2006). If there is no prediction, behaviors will not be finely tuned and appropriately responsive.

I. 8. Stress effects on working memory

The hippocampus is highly involved in the regulation of the stress response (de Kloet, Reul, & Sutanto, 1990; Sapolsky, Krey, & McEwen, 1984b), including the termination of the stress response (Furay et al., 2008). Hippocampal sensitivity to stress is through its high concentration of GC receptors ((Woolley, Gould, & McEwen, 1990)). As the HPA axis is

sustained due to chronic stress, there is constant release of glucocorticoids that bind to the GC receptors (de Kloet et al., 1990). This may lead to changes in the structure and function of the hippocampus (Woolley et al., 1990). With the changes, it may lead to either enhancement or impairment of behavior associated with hippocampal involvement.

The hippocampus is recognized as a region associated with learning and memory (Kim & Diamond, 2002) and is highly implicated in working memory (Barnes, 1988). Administration of corticosterone impairs working memory and performance on memory tasks (Conrad, Galea, Kuroda, & McEwen, 1996; McLay, Freeman, & Zadina, 1998). To study the consequences of stress manipulation on working memory, a number of stress models have been used, including foot shock (Mizoguchi et al., 2000; Yoshioka, Matsumoto, Togashi, & Saito, 1996), restraint (Conrad, LeDoux, Magarinos, & McEwen, 1999; Magarinos & McEwen, 1995), and social defeat (Buwalda et al., 2005; Calvo, Cecchi, Kabbaj, Watson, & Akil, 2011). Chronic mild stress and chronic variable stress models consist of random presentations with a variety of intermittent stressors such as restraint, forced swim, footshock, and food/water deprivation (Katz, Roth, & Carroll, 1981; Ostrander, Ulrich-Lai, Choi, Richtand, & Herman, 2006; Willner, 1997). These models utilize the randomness of exposure to the aversive conditions to induce anticipation of threat between events. The onset of the aversive stimuli may be unpredictable yet the actual stimuli may still be physical in nature. In other words, the anticipation always led to a physical stress presentation. Memory and learning deficits may be tied to the direct physical challenges. Since the anticipation may be completely isolated from an actual physical experience, anticipatory forms of stress may be prolonged and therefore more damaging (McEwen, 1998a). Therefore, a full understanding of the cognitive effects of anticipatory stress without accompanying physical stress is important.

Chapter 2

Brief Discussion and Rationale for Current Studies

Homeostasis includes maintaining stability in response to changes to both inner and outer environments. Organisms seek to achieve stability despite being confronted with stressors continuously (Day, 2005; McEwen, 1998b) in order to sustain life (Day, 2005; McEwen, 1998b). This stability is achieved by resource acquisition and avoidance of harm. The conflicts and decisions faced between the benefits and costs of resource attainment are analogous to foraging where energy is expended in order to obtain more energy resources. The ability to calculate the tradeoff between risks and resources is essential for the most effective homeostatic maintenance.

To calculate the risk when an animal seeks resources requires understanding of the relationships between cues in the environment and risk and reward. Some questions remain to be understood: how the key environmental parameters, and the interaction between the key parameters influence the body's response to the stressors physiologically, and also how the environmental parameters shapes and develops different coping styles. The parameters of controllability and predictability may shape an organism's behavior and stress response at time point A which in turn, affects the organisms' response at a later time point B (Maier & Watkins, 2010). Exposure to stressors that are tolerable and controllable may promote resilience, defined as successful adaptation to stressors (Feder et al., 2009; Maier & Watkins, 2010; McEwen & Gianaros, 2010). It will be beneficial to understand how key parameters of the environment shape and strengthen patterns of behaviors and whether or not certain behaviors are malleable and can be easily shifted to new environmental parameters.

The types of stress most humans often encounter are psychological in nature and may not always consist of direct harm. The chronic anticipatory nature of psychological stressors may promote vulnerabilities to behavioral and physiological consequences and may play a role in precipitating clinical disorders. There are models of chronic unpredictable threats that utilize a variety of unpredictable, physical stressors (e.g. swim stress followed by restraint stress) (Conrad et al., 1996; Roth & Katz, 1981), but few that utilize only anticipatory threat. Previous studies have used physical stressors such as a water bath or foot shock (Mizoguchi et al., 2000; Yoshioka et al., 1996) (Conrad et al., 1999; Magarinos & McEwen, 1995) and social defeat (Buwalda et al., 2005; Calvo et al., 2011) to induce psychological stress. In the chronic unpredictable stress model, random exposure to adverse conditions induces the anticipation of threat between events (Roth & Katz, 1981) yet the model still possesses a physical characteristic. The expectancy of the anticipated stimuli was psychological but also led to the presentation of a physical stressor. Since humans may detect threats or anticipate threats that do not lead to actual damaging events, studies should seek to observe adaptations to stressors that cannot be predicted in time, but also are not paired with direct physical harm.

An ethologically valid model should be utilized in order to investigate the natural pattern of behavioral responses. Manipulating specific environmental factors will allow us to investigate the behavioral patterns associated with the manipulation. We are specifically interested in testing whether chronic unpredictable threat without direct harm is sufficient to reliably elicit risk assessment and vigilance throughout a three week manipulation. Psychological stressors may occur completely segregated from physical harm, and a full understanding of the behavioral effects due to the manipulated environmental factor would be beneficial. If the environment remains unpredictably threatening, the behaviors are adjusted to efficiently counteract the

unpredictable threat. Later potential threats may promote increased behaviors associated with unpredictable threat.

Physiological and behavioral adaptations serve to match environmental demands. If the threat in the environment is ambiguous or highly unpredictable, it is difficult to refine strategic physiological and behavioral responses; instead, hard-wired or reflexive responses are dominantly displayed. The organism resorts to behaviors associated with risk assessment such as scanning the environment and approach toward potential threat to further assess risk. When harm is unpredictable, the organism is dependent upon feedback responses. In predictable environments, there is time and distance to strategize, prepare, and display both neural and behavioral responses that could counteract the predicted threat, allowing avoidance. These feed-forward responses require higher order systems such as the frontal cortex for decision making and planning. In order to observe the dynamic relationship between the environment and the behavioral responses of the organism, we need to monitor and code the behavioral changes and adaptations over time.

Challenges to the organism can produce the stress response which consists of physiological and behavioral changes. Stress can precipitate anxiety and depression (Kendler, Karkowski, & Prescott, 1999; Kessler, 1997). As mentioned before, stress that is uncontrollable may lead to deficits in response contingency learning that may lead to learned helplessness, a feature of depression. Both uncontrollable and unpredictable stress may also affect the onset of anxiety symptoms (Kubala et al., 2012). Previous studies have utilized unpredictable and uncontrollable models such as chronic unpredictable stress (CUS) and chronic variable stress (CVS) respectively to produce measures of anxiety and depression. The models use a variety of stressors such as intermittent foot shock and food/water deprivation. The presentations were

always physical in nature. Yet most human potential threat experiences do not lead to actual physical harm. The current dissertation addresses studying coping and adaptations to stressors (threat and abrupt stimuli) that cannot be predicted in time, but also that does not lead to actual physical harm. More specifically, the studies observed the effects of unpredictable threat presentation with successful control (avoidance or escape) from the potential challenges. We measured the adaptations and coping involved with the unpredictable threat. Previous studies have shown that repeated unpredictable threat associated with harm can produce anxiety (D'Aquila, Brain, & Willner, 1994; van Dijken, Mos, van der Heyden, & Tilders, 1992; Willner, 1997), and others have shown that uncontrollable stress can lead to symptoms of depression (Maier & Watkins, 2010). The current study addressed whether there were similar outcomes when organisms successfully avoid the potential challenges to homeostasis consistently. Experiments 1 through 3 tested the effects of three week exposure to unpredictable threats with no harm. Experiment 1 reports behavioral responses during and after the condition, including risk assessment and defense behaviors.

Stress can lead to structural and functional changes in the brain, and it may also affect learning and memory that rely on those regions, more specifically the hippocampus and mPFC. In particular, the hippocampus has a high density of gluccocorticoid receptors (Woolley et al., 1990). Corticosterone, stress, and disorders with elevated plasma glucocorticoids alter hippocampal pyramidal cell morphology (Woolley et al., 1990) and neurogenesis (Joels, Karst, Krugers, & Lucassen, 2007). Similarly, the prefrontal cortex pyramidal cell morphology is affected by corticosterone and restraint stress (Cook & Wellman, 2004). Previous research has shown that chronic psychological stress may impair working memory (Conrad et al., 1996; Luine, Villegas, Martinez, & McEwen, 1994; McLay et al., 1998). Many models have utilized

chronic variable stress or restraint to induce psychological stress, however, these models may include physical stressors. Experiment 2 tests for the effects of chronic unpredictable threat without direct harm on spatial working memory.

Behavioral adjustments are interlinked with neural plasticity. As behaviors are repeated in response to unpredictable threat, the neuronal activity of neurons is expected to increase. Neuronal activity depolarizes the resting membrane potential energy in the form of ATP is consumed in order to restore the resting membrane potential. Since neural activity requires energy expenditure, energy needs to be replenished. Cytochrome c oxidase (COX), complex IV of the electron transport chain, is an enzyme located in the inner mitochondrial membrane. It catalyzes the oxidation of cytochrome c to oxygen to form water and translocate protons across the membrane, which is used to produce the proton electrochemical potential in the membrane that is critical for driving ATP synthesis. This process yields ATP needed energy for neuronal activity (Hatefi, 1985). There is a tight coupling between neural activity, cytochrome c activity and ATP synthesis (M. T. Wong-Riley, 1989).

COX activity can provide indirect measure of neural activity in different neuronal regions. COX activity is down regulated after complete sensory deprivation within 3 days (M. Wong-Riley & Carroll, 1984). Less severe yet chronic manipulations such as access to exercise wheels for six months change COX activity (McCloskey, Adamo, & Anderson, 2001). With evidence that changes in both sensory input and behavior can influence COX activity, I hypothesized that sensory, behavioral, or physiological changes that are sustained in our chronic unpredictable threat manipulation will be reflected by regulation of the cytochrome oxidase enzyme levels. Testing for COX up or down-regulation allows for localization of the brain regions activated by our chronic unpredictable threat stress manipulation.

There were three aims of the dissertation:

1. Test whether the chronic unpredictable threat manipulation can persistently elicit risk assessment behaviors. After exposure to chronic unpredictable threat, we tested for active and passive defensive behaviors.
2. Test the effects of unpredictable threat without harm on spatial working memory. The chronic unpredictable threat environment will produce working memory impairments tested after the cessation of the stress manipulation.
3. Identify candidate locations for plasticity underlying the behavioral adaptations to chronic unpredictable threats by testing for sites activated by unpredictable threats (i.e. medial hypothalamic zone). The chronic unpredictable threat exposure will increase cytochrome oxidase activity in regions that process predator odor.

Chapter 3

Experiment 1: Chronic Unpredictable Threat Increases Risk Assessment and Defensive Behaviors

III. 1. Introduction:

In humans, stress is recognized as a precipitator of anxiety and depression (Kendler et al., 1999; Kessler, 1997). Over the course of life, stress is often experienced as we take risks to gain rewards, an integral feature of life. Humans subject themselves to social assessment (e.g. to gain a job), whereas rats risk attack by a predator while searching for mates, food or water. These challenges are characterized as approach-avoidance conflicts that are analogous to a foraging task in which the individual (human or non-human animal) must leave safe locations to search for resources (Aupperle et al., 2011; Kavaliers & Choleris, 2001). Although repeated unpredictable threat associated with harm can produce generalized fear/anxiety (D'Aquila et al., 1994; van Dijken et al., 1992; Willner, 1997), it is not clear that the same outcome will occur when individuals successfully avoid or escape challenges to homeostasis on a consistent basis (i.e., have high control). In a number of human environments, awareness of risk is high, but the frequency of harmful events is relatively low. Although there are epidemiological indicators that repeated threat is damaging, few animal models are available to test the effects of threat without harm. To further understand the relationships between types of stressors and responses, we developed a chronic living environment that includes a foraging feature that allows the manipulation of factors that induce stress.

The generalized stress response has long been recognized as resulting specifically from unpredictable threats, lack of control and novelty (Mason, 1968a; Seligman & Maier, 1967). Resources and risk are predicted by external cues (A. A. Lima et al., 2010), some learned within and others across generations. Some predictive cues guide navigation, but others are abrupt, unexpected and ambiguous. Abrupt unexpected cues, innately threatening cues and conditioned cues can produce a generalized stress response (Masini et al., 2005; Selye & Fortier, 1949), as well as eliciting behavioral responses like startle, risk assessment (D. C. Blanchard, Blanchard, Griebel, et al., 2008) and coping behaviors (Coppens, de Boer, & Koolhaas, 2010). Repeated activation of the risk assessment and coping behaviors, and the success or failure of those responses during exposure to chronic and repeated stressors will produce adaptations that may later generalize to novel situations (Maier & Watkins, 2010). Adaptations and deficits following uncontrollable stressors are well described, but few animal studies have tested behavioral adaptations to repeated unpredictable threats that are never followed by pain or harm. Rather than the low control of common stress manipulations, we seek to study adaptations to stressors (threat and abrupt stimuli) that cannot be predicted in time, but also are not paired with harm. Thus, we seek to study the anticipation of harm (psychological stress) without direct challenges to physiology (physical stress).

To develop a platform for the study of the effects of unpredictable threat stress, we first set out to capitalize on stimuli that produce anticipation of a predator attack in rodents. Inspired by the elegant work demonstrating behavioral responses to predators and predator odors (D. C. Blanchard, Griebel, & Blanchard, 2003), and the demonstration of behavioral and c-Fos responses to predator odors (Cezario et al., 2008; Dielenberg, Hunt, & McGregor, 2001), including ferret odor (Masini et al., 2005), we capitalized on the rodent's innate aversion to ferret

dander odor. In our model, odor was presented when rats traversed a tunnel separating two tub cages, all comprising their home territory. To increase and sustain the novelty of the stimuli, and therefore reduce the likelihood of habituation, we presented ferret dander odor simultaneously with a flash of light and sound, both abrupt and therefore with features of surprise. The stimuli were presented randomly with a probability of 0.25, and therefore were relatively unpredictable. Consistent with a foraging task, animals were motivated to pass through the threatening location by placing food and water on opposite ends of the tunnel, much like the spatial segregation of resources in the natural environments. Therefore, resource location was predictable but threat timing was not. Using this platform, we investigated the behavioral adaptations to repeated threat that is unpredictable, but always avoidable.

Threat that is non-specific will elicit vigilance (D. C. Blanchard et al., 2003), and risk assessment behaviors that serve to detect predators and harm. It is not clear whether threat alone, without direct harm will continually elicit risk assessment. Chronic vigilance and risk assessment are features of hyper-arousal, a core symptom of PTSD (Solomon, Horesh, & Ein-Dor, 2009). Hyper-arousal would be expected to be adaptive in high threat environments, but maladaptive in low threat environments. High threat environments should produce risk assessment and vigilance, regardless of the presentation of harm, as long as the stimuli remain threatening. Therefore, we predict that unpredictable threat without harm in a chronic living environment will increase risk assessment and defense behaviors, much like the hyper-arousal and impulsive aggression reported in PTSD (Pavic et al., 2003). In contrast, we do not expect to see helplessness, which can be produced by uncontrollable pain, and is associated with depression (W. R. Miller & Seligman, 1975; Pryce et al., 2011). Furthermore, although the stimuli may remain threatening, they are escapable by both avoidance and escape from the center

stimuli location. It is highly unlikely for learned helplessness due to motivational deficits often attributed to uncontrollable stimuli (Maier & Watkins, 2005; Overmier, 2002).

There were three aims of the study. Rats in the threat stress group were presented with repeated unpredictable presentations of aversive stimuli with 100% successful avoidance and 100% successful resource acquisition as a model of unpredictable threat stress in contrast to rats free to traverse their tunnel without presentation of stressors. Our first aim was to test the effectiveness of the stimuli on risk assessment and defensive behaviors, and their effectiveness over repeated presentations. We tested whether rats consistently avoided the threat stimuli, or whether they habituated over the three weeks as this is the standard amount of time for chronic stress. With the expectation that rats in the stress condition would avoid the threat stimuli, we were concerned that they would have less access to food and water so we monitored food and water consumption and body weight to ensure our ability to produce threat without the direct physiological challenges of deprivation. Second, to further test whether the stimuli were perceived as threats of harm, we measured the frequency of behaviors known to be elicited in the face of predators (D. C. Blanchard et al., 2011). These behaviors included retreat/withdrawal, head scanning, and stretch attend behaviors. After the three week manipulation, we tested for adaptations in direct defense response (Anderson, Nash, Weaver, & Davis, 1983; Dallas Treit & Pinel, 2005), as well as behaviors with predictive validity for anxiety (elevated plus maze) (Pellow & File, 1986; Rodgers & Dalvi, 1997), and depression (Porsolt forced swim test) (Willner, 2005).

III. 2. Methods:

2.1.1 Subjects. Male Sprague-Dawley rats were born to dams shipped to our colony (Taconic Farms, Inc.). Animals were housed in a reversed light-dark cycle (10PM/10AM). Each litter was weaned on postnatal day 21. All rats were handled for one week and were then randomly assigned to one of two conditions (ensuring that initial body weights were evenly distributed across groups): unpredictable threat/stress tunnel (ST) and control tunnel (CT). Twenty cohorts, ranging from 2-4 subjects per group per cohort were run. Food and water consumption were measured for all cohorts except 4 (first 4 cohorts; n=23, CT=11, ST=12)

2.1.2 Apparatus and experimental conditions. Tunnels (91.44cm) consisted of an aluminum lane (15.88cm wide) covered with hardware cloth separating two standard tub cages (38.1 X 20.32 X 17.78cm), one containing ab libitum food and one with free access to water. The tub cages were attached to the tunnel by square aluminum tubes (7.62" X 7.62") equipped with light-emitting diode (LED) sensors designed to detect head/body entry into the tunnel (see Fig. 1). The tunnels were interfaced with a computer that recorded behavioral activity and controlled stimulus presentations. A break between an infrared LED and a detector LED was used to record when the rat poked its head into, or made a full entry into the tunnel. When the two beams on each end of the tunnel were broken in succession, a traversal was recorded. In the center of the tunnel was a detector that allowed timing of stimuli when a rat was present. Presentations of the stimuli occurred with a probability of 0.25 in a random sequence. Stimuli included the opening of a valve attached to a pressurized (Whisper 40 Air Pump 120 Volts AC 60 Hz 2.9 Watts, Tetra, Melle, Germany) bottle (500 mL) containing a 15.25 X 20.32cm cloth or hammock that had previously been in a cage with ferrets for at least 2 weeks. By failing to pair the stimuli with pain or the removal of resources, habituation seemed likely. To reduce that

possibility, stimulus complexity was increased by pairing the puff of air with ferret dander odor with two additional mildly aversive stimuli. The first was the sound of a solenoid that hit the side of the tunnel in the center position and the second was a flash of 10 ultra-white LEDs, 5 on each side (3500 millicandles of light of 600 nanometer wavelength) for 1 second duration. The tunnels used for the control condition were an exact replica (without the accompanying stimuli) of the stress tunnels, but were housed in an identical, but separate room to avoid exposure to any of the aversive stimuli.

At the beginning of each experiment, all rats were habituated to the tunnels for 3 days. After habituation the treatment condition commenced and lasted three weeks. Food and water measurements were taken at the end of the habituation period, and after each week of the experimental conditions. Body weight was also measured before and after tunnel housing and analyzed with a one-way ANOVA.

2.1.3 Behavior in the Tunnels. Sequential LED detection across opposite ends of the tunnel was recorded as a full tunnel traversal. Daily values for total breaks and traversals were analyzed with a repeated measures ANOVA with group as the between subjects factor and day as the within subjects factor. The habituation period was analyzed separately as a repeated measures ANOVA with group as the between subjects factor and day as the within subjects factor. Traversals on the last day were analyzed with a one way ANOVA to test for group differences at the end of the manipulation.

Rats (CT: n=13 ST: n=12) were video recorded for one hour in their tunnels during the habituation period, and again at the beginning of the treatment condition and end of every week thereafter. The timing of recordings occurred between the second and seventh hours of the dark cycle under red light. Two independent observers coded behaviors previously demonstrated to

be associated with risk assessment (Robert J. Blanchard & D. Caroline Blanchard, 1989; Dielenberg, Carrive, & McGregor, 2001) vigilance (Jovanovic et al., 2010) and glucocorticoid elevations (Lupien, Maheu, Tu, Fiocco, & Schramek, 2007). These behaviors include a) stretching out to look from the tub cage, b) stretch attend/retract within the tunnels, c) head-scanning, d) withdrawal/retreating back into the tub cage rather than advance to the other side, and e) freezing. Stretching out to look from the tub cage was defined as half or more of the body enters the tunnel while hind legs remain in the tub cage, a movement that generally occurs when the animal observes a potential threat from a distance, but is near the safety of the tub cage. When the animal leaves the tub cage, stretch attending/retracting occurred when the animal elongated its body to observe the center stimuli and then retracts to a normal posture. Unlike stretching out to look, the whole body is within the tunnel for stretch/attends. Head-scanning was coded when a rat produced slow horizontal movements of the head and neck to scan the environment while the limbs were immobile. Head-scanning permits the animal to assess the environment (R.J. Blanchard et al., 1974). Withdrawal was coded when the animal completely entered the tunnel but retreated back into the closest tub cage, avoiding contact with the central location and foregoing access to resources on the other side. Freezing was defined as complete cessation of all movements in limbs, head, and neck. Freezing may occur after the animal stretch attends and retracts its body from the central location and remains motionless, a behavior utilized to prevent detection (Kavaliers & Choleris, 2001). Risk assessment behaviors during the habituation period were analyzed with a one-way ANOVA, but during the treatment periods were analyzed with a within subjects ANOVA with group as the between subjects factor and week (1-3) as the within subjects factor.

2.2 Testing after the treatment conditions

2.2.1 Adrenal gland weight. In a separate study, we measured the weights of the adrenal glands immediately after the cessation of the threat stress manipulation. Rats (CT: 4; ST: 6) were injected with sodium pentobarbital (100 mg/kg) and decapitated. The adrenal glands were collected and the average weight was taken from both adrenal glands for each rat. The weights were analyzed with an independent measures t-test (one-tailed, alpha .05).

Not all cohorts were tested on all of the following tests. When cohorts were tested on more than one test, the tests were ordered from least to most aversive to avoid carry over effects.

2.2.2 Defensive Burying. To test for habituation to the ferret odor, we measured defensive burying two days after treatment cessation during the dark cycle. Animals were placed in clear, plastic tub (polycarbonate, 38.1 X 20.32 X 17.78cm) with the same dimensions as their home cage with ¼ inch (3.8 cm) deep corncob bedding for 15 minutes. One group of rats (CT: n=11; ST: n=12) were tested with a 2 X 3 cm ferret scented rag sandwiched between hardware cloth taped to the one wall of the cage 4 cm above the top of the bedding. On the opposite wall is a 2 X 3 cm unscented rag between hardware cloth. Rags were replaced for every fourth rat. Equal numbers of rats from each group were tested with each rag to control for any potential differences in the intensity of odor across rags. Two investigators coded the latency to begin burying both rags and the amount of time spent burying both rags (Anderson et al., 1983).

2.2.3. Defensive Burying of Highly Novel Stimulus. A second set of rats (CT: n=36 ST: n=39) was tested for burying in response to a highly novel, noisy stimulus. The auditory stimulus was from a small 6V motor encapsulated in a plastic Eppendorf tube so that it could not harm the animal. Each animal was placed in the cage for 15 minutes. When animals were within 1 cm of the tube, the motor was turned on three times in rapid succession over a period of three

seconds. An experimenter blind to the groups coded behavior. The behaviors included latency to begin burying the highly novel stimulus and the amount of time spent burying. After each rat, the bedding was removed and the tub was cleaned with 30% alcohol solution and new bedding was added. Since there was a tendency for a higher proportion of rats in the threat stress condition to bury the ferret rag, we increased the sample size in order to test for both a difference in the proportion of animals that buried, as well as differences in the time spent burying. Proportion of animals that buried was analyzed with Chi-Square analysis, whereas the time spent burying was analyzed with a one-way ANOVA.

2.2.4 Elevated Plus Maze. The Elevated Plus Maze (EPM) is a validated model for measuring anxiety and risk taking behavior in rats (Rodgers, Davies, & Shore, 2002; Walf & Frye, 2007). The apparatus is a plus-shaped maze with 2 closed arms (walls on the maze) and 2 open arms (no walls with direct view of the environment) that were elevated 65 cm from the floor placed in a dimly lit room. Each arm was 67cm X 15.5 cm separated by a 15.5cm square area with two arms that had 19.5 cm high walls. The rats (CT: n=10 ST: n=12) were habituated in the testing room for 30 minutes. Each rat was placed in the center of the apparatus at the junction of the open and closed arms, facing the open arm opposite of the experimenter. After 7 minutes, the rat was returned to the tub cage and the maze was cleaned and dried with 30% alcohol solution. The numbers of open, closed and total arm entries as well as open and closed arm time were each measured with a one-way ANOVA. Arm entries were coded when all four paws entered the arm.

2.2.5 Open Field. To determine general activity levels and exploration to a novel environment, we measured mobility in an open field (90 cm x 90 cm with 28.5 cm high walls around the perimeter) divided into 16 equal sized sectors (22.5 cm x 22.5 cm) for 6 minutes in a

dimly lit room. All animals were habituated to the room for 30 minutes prior to testing. We measured the number of outer and inner crossings, latency to enter the middle grids, and frequency of rearing (standing on hind limbs not touching the wall), and wall climbing; the data were analyzed with a one-way ANOVA.

2.2.6 Novelty Suppressed Feeding: To test for neophagia (novelty induced feeding suppression), a set of rats never previously tested in the open field (CT n=10; ST n=11) were food restricted for twenty-four hours. At the beginning of the test, a single pellet of food chow was placed in the center of the open field (90 cm^2) in a brightly lit room. Each animal was placed in a corner of the open field with a single pellet of standard chow placed in the center. For ten minutes, the following measures were recorded: latency to approach the food (sniffing or grabbing the pellet), latency to eat (sitting down and biting the pellet), and locomotor activity as measured by total number of squares entered with all 4 paws. Data was analyzed with a one-way ANOVA.

2.2.7 Porsalt Forced Swim Test (PFST). The PFST is a measure for depression and helplessness (Sun & Alkon, 2003). Rats (CT n=29; ST n=23) were immersed in a tub filled with water (26-28° C) with no means of escape for 7 minutes. Immobility, climbing out, and swimming were coded in blocks of five-seconds each. Immobility (a measure of helplessness and defeat consists of no movement of the 2 front paws. Climbing out attempts (a measure of active coping and total activity (climbing out attempts and swimming) were also calculated. Data were analyzed with a mixed factor ANOVA with group as the between subjects variable and minute as the within subjects variable.

2.2.8 Sucrose Preference Test: To introduce sucrose, the day before placement in the tunnel conditions, twenty-three subjects, singly housed, had two bottles, one with water and one

with 2% sucrose in water, placed on their cage for 24 hours. The bottle positions in the cage were counterbalanced. On the following day subjects were placed in tunnel conditions and again had access to two bottles for 48 hours of the tunnel habituation period. During the experimental manipulation sucrose bottles were not available. When animals were returned to individual tub cages following the experimental phase, they were water deprived for 18 hours (18:00-12:00(+1)) while food was available ad libitum. Then two bottles for each rat, one with water and one with sucrose solution, were weighed and then placed in the cages for one hour, again counterbalancing the position across rats. The weight of liquid consumed in each bottle was measured. We analyzed consumption of sucrose solution and water, to calculate preference (sucrose consumption/total consumption). Group differences were tested with a one-way ANOVA.

III. 3. Results:

3.1 Behavioral differences *during* the treatment condition:

The fundamental goal of the present experiment was to develop and test a platform that allows us to induce chronic psychological stress through the presentation of unpredictable threats. Therefore we first set out to test whether the intensity and duration of threats was effective and sustained by testing for a reduction in entrances and crossings in the tunnel, and further to confirm that any reductions did not lead to challenges to homeostasis measured as deprivation (decreased weight or food/water consumption).

3.1.1 Traversals. The intermittent presentations delivered over 21 days produced persistent avoidance of the location of stimulus presentations. Tunnel data for two rats in the ST group were excluded because of equipment difficulties. Data from individual cohorts were consistent, therefore the traversal data from all 20 cohorts (CT n= 50, ST n= 50) were collapsed.

During the habituation trials, the threat stress and control condition produced similar numbers of traversals ($F(1,98)=.060$, $p=0.807$). During the three week treatment condition, however, the threat stress group made significantly fewer traversals than the control group ($F(1,98)=109.591$, $p<.0001$) (see Fig. 2A), a pattern consistent with previous observations that sensory signals representing nearby predators are associated with avoidance. The traversals changed significantly over the treatment condition ($F(20,1960) = 6.274$, $p < .0001$). There was a significant interaction between the group (treatment condition) and treatment period (21 days) ($F(20,1960) = 4.693$, $p < .0001$) with the control group maintaining habituation levels over the 21 days, whereas the threat stress group dramatically decreased traversals after experimental manipulation commenced. After day 5, the threat stress group slowly increased traversals, but they remained consistently lower than control levels even on the last day ($p<.05$).

3.1.2 Entries without traversals: Entries was calculated at the total number of LED detections. There were no differences in the number of tunnel entries between the two groups ($F(1,98)=2.365$, $p=0.127$) (see Fig. 2B) and no interaction between group and treatment period (21 days) ($F(20,1960) = 1.281$, $p=0.180$). However, the ST group made an average of 45 entries per full traversal in contrast to 7 entries per traversal by the CT group ($F(1,98)=71.486$, $p<.0001$) (see Fig. 2C), suggesting the threat stress group was monitoring the tunnel, despite making fewer crossings. This pattern is consistent with vigilance in response to the potential threat of a predator. The data violated the assumption of sphericity so the Greenhouse-Geisser correction was used to analyze the effect of day and the day by group interaction. There was a day effect ($F(6.283,615.704) = 3.497$, $p<.05$), and an interaction between the group and treatment period ($F(6.283,615.704) = 3.044$, $p<.05$). The control group maintained a steady ratio of entries to traversals over the treatment period. In contrast, the threat stress group rapidly increased the

ratio of entries to full crossings, reaching a peak on day 5. This change over time appears to be the inverse of the change in traversal number over time.

3.1.3 Risk assessment behaviors. We also tested whether the threats used in our manipulation elicited risk assessment behaviors, positive indicators of threat. During the habituation period, there were no group differences for the 4 different risk assessment behaviors. After the onset of the stress stimuli, the groups differed on 4 variables. The threat stress group had a higher frequency of stretching out to look from tub cage ($F(1,23)=10.20$, $p=.0040$) (see Fig. 3A), stretching and attending to the central position ($F(1,23)=11.18$, $p=.0028$) (see Fig. 3B), head-scanning in the tunnel ($F(1,23)=10.7$, $p=.0033$) (see Fig. 3C), and withdrawing from the center of the tunnel back to closest tub cage ($F(1,23)=34.02$, $p<.0001$) (see Fig. 3D). There were no differences in time spent freezing ($F(1,23)=0.138$, $p=.714$; CT M =.433± .165; ST M = .521 ± .171). The significantly higher frequency of risk assessment behaviors supports the effectiveness of the threat stress manipulation.

3.1.4 Body Weight and Intake of Food and Water. Physical stress could include inadequate energy supply and dehydration. Since our model was designed to induce psychological stress without physical stress, it is imperative that food and water consumption were adequate. We measured both groups (CT=41 and ST=40) at four time points during the experimental conditions and found no group differences (Food, $F(1,80)=2.73$, $p=.1027$; Water, $F(1,80)=.80$, $p=.3732$) or interactions with time in food and water consumption (Food, $F(4,316)=.90$, $p=.4626$; Water, $F(4,316)=.59$, $p=.6673$) (see Table 1) As the experimental condition progressed, both groups increased consumption of both food ($F(4,316)=17.69$, $p<.0001$) and water ($F(4,316)=14.90$, $p<.0001$). Body weight data was not measured for 8

cohorts. In the remaining cohorts (CT n=30 ST n=34) body weight gain did not differ significantly ($F(1,63)=2.07$, $p=.1548$) (see Table 1).

3.2 Behavioral differences *after* the treatment condition:

3.2.1 Adrenal gland weight. Average adrenal gland weight was significantly higher for the ST group ($t(8)=2.210$, $p=.0353$) (see Fig. 4).

Behavioral tests after the treatment condition:

3.2.2 Defensive Burying. To test whether rats in the threat stress condition habituated to the ferret dander odor after repeated exposure without harm, we measured the time spent burying a rag with ferret dander odor and the latency to bury. Proportionately more threat stress rats buried, but the difference was not significant ($X^2(2, N=23) = 2.01$, $p=0.157$)(see Fig. 5A). The ST group and CT group had similar latencies to bury the rag with ferret dander ($F(1,16)=.52$, $p=.4809$) (see Fig. 5B), but the ST group spent more time burying the ferret dander rag ($F(1,22)=5.13$, $p=.0341$) (see Fig. 5C). Repeated exposure to ferret odor had not led to habituation. Instead, the data suggest that the ST group was either selectively sensitized to the ferret dander odor, had greater sensitivity to aversive stimuli, or had generalized fear. No rat in either group buried the control rag.

3.2.3 Defensive Burying of Highly Novel Stimulus. To determine if heightened defensive burying of the odor rag in the ST group was due selectively to the sensitization to the ferret dander, we tested burying of a highly novel stimulus in separate cohorts. There were no significant differences in the average number of times the rats in each group approached the stimulus (CT: $M=10.86$ +/- 1.42, ST: $M=13.72$ +/- 1.82, $p>.05$). There was a tendency for a higher proportion of ST rats to bury than the CT rats ($X^2(2, N=61) = 3.79$, $p=0.052$) (see Fig. 6A). To avoid confounding the duration of burying by the difference in the proportion of rats that

buried, subsequent analyses were limited to only those animals that buried. Again, there were no group difference in the average number of times that rats in each group approached the stimulus (CT: *M*=12.31+/- 1.85, ST: *M*=14.63 +/- 1.89), and there were no group differences in the latency to bury the novel stimulus ($F(1,60)=0.03$, $p=.8701$) (see Fig. 6B). However, the ST group spent more time burying the novel stimulus ($F(1,60)=4.98$, $p=.0294$) (see Fig. 6C), suggesting the enhanced burying to the ferret odor was more general than sensitization to an odor previously encountered.

3.2.4 Elevated Plus Maze. Since burying could reflect anxiety, we tested rats in the EPM, as entry into open arms is sensitive to anxiolytics. There were no group differences in open arm time (OAT), ($F(1,21) = .007$, $p=0.935$, open arm entries (OAE), ($F(1,21)=.02$, $p=.9025$), total arm entries, ($F(1,21)=0.00$, $p=.9706$, (See Table 2), or proportion of time spent in the open arm ($F(1,21)=.109$, $p=0.744$) between groups. A preliminary study with 13 animals (unpublished observations) that was run with slightly different methods also failed to yield group differences.

3.2.5 Open Field. There were no differences between groups in mobility as measured by the number of outer crossings ($F(1,23)=.195$, $p=0.663$), inner crossings ($F(1,23)=.511$, $p=0.482$), and total crossings ($F(1,23)=.294$, $p=0.593$ (See Table 2)) in the open field. There were no group differences in general activity measured as rearing ($F(1,23)=2.555$, $p = 0.126$) and wall climbing ($F(1,23)=0.554$, $p = 0.464$). There was a tendency for the threat stress group to take longer to enter the middle ($F(1,23)=3.767$, $p = 0.065$), indicating a tendency toward behavioral avoidance, but there was no group difference in the time spent in the inner/central part of the box ($F(1,23)= 0.966$, $p = 0.336$), used as a measure of anxiety.

3.2.6 Novelty Suppressed Feeding. To test for neophagia, we measured the latency to approach the food pellet and latency to eat. There were no group differences in latency to

approach ($F(1,20)=1.862$, $p=0.188$) (See Table 2) and latency to eat ($F(1,20)=.005$, $p=0.943$). However, there was a tendency for a group difference in locomotor activity ($F(1,20)=4.18$, $p=0.055$) indicative of behavioral differences when food provides a motivating factor for mobility and exploration.

3.2.7 Forced Swim Test. Since stress can precipitate depression (Kendler et al., 1999)both groups were tested for depression symptoms with a test that measures active and passive coping behaviors. There were no differences between the groups in passive coping, immobility, ($F(1,51)=0.00$, $p=0.95$) in active coping, climbing out ($F(1,51)=1.04$, $p=0.31$), or total activity ($F(1,51)=1.34$, $p=0.27$) (See Table 3). For both groups immobility increased for the first 4 minutes and rates were then maintained for the remaining 3 minutes, with a significant effect of time for both immobility ($F(1,51)=42.59$, $p<.0001$) and climbing out ($F(1,51)=45.29$, $p<.0001$).

3.2.8 Sucrose Preference Test. Post-treatment, there were no group differences in sucrose consumption (CT 10.5 ± 1.40; ST 11.45 ± 1.07; $F(1,22)=0.286$, $p=0.598$), water consumption (CT 3.33 ± 0.53; ST 2.18 ± 0.26; $F(1,22)=3.607$ $p=0.071$), or sucrose preference (sucrose consumed divided by total liquid consumption) ($F(1,22)=2.826$, $p=0.108$) (see Table 3).

III. 4. Discussion:

To begin to understand how environmental factors such as predictability and control shape behavior, we developed a novel housing platform that will allow manipulation of these variables in a living environment that includes a foraging feature. In this first use of the platform, we tested the behavioral effects of unpredictable threat given intermittently over 21 days. This manipulation is novel for the field of stress, because we induced unpredictable threat,

without the reliance on exposure to direct threats to homeostasis. Thus, we attempted to induce anticipation of harm without introducing harm. To study the effects of threat exposure over weeks rather than a single episode, we reduced the possibility of habituation to the threatening stimuli by increasing stimulus complexity; predator odor was paired with abrupt flashes of light and abrupt sound. This strategy was successful. Upon commencement of the threat stress condition, full tunnel crossings were reduced in the threat stress group by 60% (range over weeks: 55 – 68%) relative to the habituation period, and remained low throughout the treatment condition. In contrast, the control group exhibited no reductions. The stress group entered the tunnels at frequencies similar to the control group, suggesting they were monitoring events in the tunnel despite lower levels of crossing to the opposite side. The initial reductions demonstrate the effectiveness of the stimuli, whereas the persistence of the reductions demonstrates the ability to avoid habituation to the stimuli.

In our model, the timing of the threat stimuli was unpredictable, but the spatial location of threat provided some control over their fear of predation. The threat stress group slowed and terminated their approach to the central location of threat presentation, behaviors strongly associated with the allocation of attention to predator cues (Kavaliers & Choleris, 2001). Arousal and threat detection were further confirmed by the dramatically higher frequency of the risk assessment behaviors, including complete termination of approach and scanning, indicating full search for potential prey. These behaviors emerged quickly and persisted throughout the threat treatment phase, again supporting maintained effectiveness of our experimental manipulation. The threat group exhibited more withdrawals into the closest tub cage, which is consistent with fleeing, an active form of coping. Although they could have avoided the central position for a significant period of time, they were exhibiting an average of 11 behaviors

categorized as risk assessment per hour during the active period of the day. In previous studies, these behaviors were associated with elevations in plasma corticosterone. The quality of behaviors illustrated by the threat stress group supports the detection of threat, and the persistent demonstration of these behaviors over the full treatment phase confirms the persistence of threat detection.

To develop a model that induces psychological stress, which is the anticipation of harm with no direct challenge to homeostasis, requires exclusion of the possibility that subjects in the threat stress group are responding to harm or deprivation. In pilot studies, random presentation of stressful stimuli with a probability of 0.33 reduced crossings enough to slow growth rates (unpublished observations). When presentation probability was reduced to 0.25, the threat stress group continued to avoid the stimuli, but crossed enough to obtain adequate food and water, and maintain body weights equivalent to the control group. The ability to maintain body weight with far fewer crossings suggests that the control group crosses more than necessary to obtain resources. This method of stress induction is novel; the experimental conditions manipulated parameters of a living environment to present threat and surprise in an ethologically relevant context while excluding direct challenges to homeostasis such as harm or deprivation.

It has long been recognized that the unpredictability of stressors exacerbates psychological stress or increases the duration of stress (Dickerson & Kemeny, 2004; Mason, 1968a), since resolution of the stress response is often delayed. In our condition, the threat stress group could have chosen to avoid the central position altogether since they could not predict the presentations of threat, but instead they repeatedly assessed risk. When the threat stress rats crossed, it is possible that they calculated a low probability of threat given the repeated assessment in the center position. Alternatively, hunger or thirst became prioritized over risk as

time passed, leading to crossing (Kavaliers & Choleris, 2001; S. L. Lima & Bednekoff, 1999; Olff, Langeland, & Gersons, 2005). In either case, the condition was designed so that the threat group eventually needed the resources beyond the central location with no ability to continually avoid the threat. However, harm was never presented so they were able to cross safely every time, and always obtained resources on the other side. Despite the repeated safe crossing, they more often chose to retreat into the closest tub cage, each time successfully avoiding harm. The ability to alter exposure to direct harm, or in this case entirely avoid harm is referred to as control (Maier, Amat, Baratta, Paul, & Watkins, 2006). Since the animals in our stress manipulation were able to avoid harm and deprivation altogether, they had full control despite exposure to threat and the inability to predict threat. The ability to induce consistent risk assessment responses while avoiding the induction of deprivation or presentation of harm demonstrates the ability to directly test for adaptations to threat and psychological stress without the confounds of accumulating physical damage from direct challenges to homeostasis (e.g., circulatory system challenges leading to energy deficits, or deprivation leading to energy depletion).

Despite repeated successful avoidance, risk assessment behaviors remained high and tunnel traversals remained lower over the full three week manipulation, raising a relatively old question; what reinforcer maintains the frequent avoidance and risk assessment behaviors during the treatment phase? Any autonomic arousal resulting from the threat stimuli would be expected to produce an emotional response, for example, acute fear. Retreat into the nearest tunnel, or crossing without harm would both be expected to reduce fear, which has been proposed to serve as a reinforcer (Thorndike, 1911). Thus, reduction of fear may have served to repeatedly reinforce risk assessment and avoidance behaviors.

The persistent reduction in traversals and repeated risk assessment may reflect the ability of predator dander odor to repeatedly signal the threat of predators, a pattern inconsistent with previous reports that rats habituate to repeated thirty minute exposure to ferret dander odor (Campeau, Nyhuis, Sasse, Day, & Masini, 2008; Weinberg et al., 2009), and to cat odor (Dielenberg & McGregor, 1999). The finding that the threat stress group spent more time burying a rag with ferret dander odor than the control group further supported the lack of habituation to the ferret dander odor. It is possible that the combination of ferret odor and abrupt auditory and visual stimuli prevented habituation, or that the acute nature of presentations, in contrast to longer duration exposures used by others, was critical for the avoidance of habituation. In this study, repeated presentations may have produced sensitization to ferret dander odor.

Just as rats in the threat stress group spent more time burying a rag with ferret dander odor, they also spent more time burying a highly novel stimulus. And a higher proportion of the threat stress group buried the novel stimulus than the control group. Similar to our findings, ten minutes of emotional stress from hearing and smelling another rat being shocked, but not shocked themselves, caused rats to bury for a longer time (Gutierrez-Garcia et al., 2006). In contrast, when stress manipulations are uncontrollable, defensive burying decreases (Bhatnagar, Huber, Lazar, Pych, & Vining, 2003; Overmier & Seligman, 1967; Simpson, Menard, Reynolds, & Beninger, 2010) or is not affected (Pijlman, Wolterink, & Van Ree, 2003). Therefore, control during stress may be a critical factor that affects adaptations of the burying response: without control, burying decreases, but with control, burying increases. The unpredictable threat stress condition increased active coping behavior, which is inconsistent with any expectation of stress-induced depression, a condition associated with a bias toward passive coping.

Approach to threat has been posited as a symptom of anxiety, whereas withdrawal is associated with depression (McNaughton & Corr, 2004) although this distinction fails to represent the analytical feature of approach (D. C. Blanchard et al., 2011). If this is true, then the frequent tunnel entries, despite lower crossing, and elevated risk assessment behaviors may represent the development of anxiety in the threat stress condition. Since the burying task is sensitive to anxiolytics (Sietse F. De Boer & Jaap M. Koolhaas, 2003; Njung'e & Handley, 1991), and the threat stress group spent more time burying, there was additional support for the possibility of anxiety. Alternatively, potentiated burying could simply represent an enhanced response during acute fear since burying is elicited only when stimuli are distinct and unavoidable (S. F. De Boer & J. M. Koolhaas, 2003; Dallas Treit & Pinel, 2005), conditions of acute fear. Therefore, to distinguish between fear and anxiety it is helpful to test responses to more ambiguous threats as well as in conditions that allow avoidance of threat. To do so, we measured avoidance of open arms in the elevated plus maze, which involves caution to more ambiguous threat, and a behavior sensitive to anxiolytics (Pellow & File, 1986; Rogoz & Skuza, 2011). In contrast to burying, an active form of coping, avoidance of the open arms is a passive form of coping. In this test, we found no group differences in contrast to previous studies of uncontrollable or inescapable stressors, which led to avoidance of open arms (Korte, De Boer, & Bohus, 1999). A critical distinction between our manipulation and those of the previous studies is *control* since our animals experienced no pain or harm. Therefore it is possible that control over the distance to threat in our stress manipulation, despite the unpredictable timing, may have been critical in leading to selectively enhanced responding to focused novelty and threat (e.g., highly novel stimulus) without affecting responses to ambiguous stimuli (e.g., open arms), a feature of anxiety. Since anxiety and depression are often co-morbid (Kalueff & Nutt, 2007) and

stress can precipitate both (Anisman & Zacharko, 1982; Hammen, 2006; Shih, Eberhart, Hammen, & Brennan, 2006), we also tested for helplessness, a symptom of depression. Studies have shown that models of Chronic Mild Stress (CMS) and Chronic Variable Stress (CVS) as well as chronic social stress, which all involve a direct challenge to homeostasis and therefore lack of control, increase immobility on the forced swim test (Garcia-Marquez & Armario, 1987; Prince & Anisman, 1984; Rygula et al., 2005; Willner, 2005) (Rygula et al., 2005). In contrast, we found no group differences in immobility, which is consistent with reports that *control* protects animals from depression (Amat et al., 2005; Anisman & Matheson, 2005).

Our findings suggest that unpredictability is not a key factor for stress induction of anxiety and depression, whereas other studies support the important role of control over harm for the induction of anxiety and depression. Although our manipulation separates the two factors, they are likely to be related in natural environments where predictability should moderate the probability of control. If prediction increases likelihood of control, and conversely lack of prediction decreases the probability of control, then unpredictable threats will indirectly lead to low control, anxiety and depression. Nevertheless, high resource environments with low harm, not uncommon for humans in industrialized nations, include unpredictable threats without harm, which may not be sufficient to produce anxiety and depression.

Taken together, the data suggest that the unpredictable threat condition in the present study required risk assessment that later generalized to new and novel environments to support an increase in threat detection and responding. The highly novel stimulus used in the defensive burying task involved both a dynamic response, turning a motor on in response to the rat approach, and a focal stimulus. In contrast, the elevated plus measure was a response to a static and ambiguous condition, a better test of anxiety. Defensive burying is not elicited by the first

approach to either the source of ferret dander odor, or the highly novel stimulus. The threat stress group spent more time burying, but did not bury sooner, or approach more times. The enhanced time burying may represent either a) a lower threshold for risk, b) longer time in individual approaches to assess the stimuli, or c) a more robust defense response. Since open arm avoidance did not differ, it is unlikely that the enhanced burying represents a lower threshold for risk. In contrast, if rats in the ST group spent more time risk assessing on single approaches, the probability of detecting risk increases. The mechanism would be easily explained by the generalization of the use of risk assessment behaviors in the tunnel. It seems less likely that the tunnel condition, where the rats had no bedding material in close proximity to the aversive stimuli, would have potentiated the motor response of burying. Therefore, we suspect it is unlikely that the plasticity underlying these behavioral adaptations is associated with the specific motor pattern generation or initiation of the burying response, which is initiated in the PAG. Instead it is likely that there is enhancement of risk assessment behaviors, which may also be initiated within the PAG. It is unclear, however, whether the enhancement arises from plasticity at the level of sensory processing or motor generation.

Regardless of the mechanism of plasticity, the results raise the possibility that repeated exposures to unpredictable threats are sufficient to potentiate aggressive behaviors. Aggression can be divided into two forms, impulsive aggression and motivated aggression (Nelson & Trainor, 2007). In contrast, the successful acquisition of resources after exposure to threat may increase the likelihood of motivated aggression. Future studies will need to directly compare our manipulation with and without control to determine if control is critical in determining the direction of coping behaviors from active forms like aggression to passive forms of coping such

as helplessness. Future studies with the current manipulation will test whether threat is sufficient to potentiate both forms of aggression.

With little support for the ability of unpredictable threats to produce anxiety or depression, the findings of facilitated active coping to specific stimuli, may be more consistent with resilience, which is defined as the generalization of adaptive coping responses (Feder et al., 2009). In humans, active coping is characterized by facing fears, fewer avoidance behaviors, and active problem solving. In contrast, passive coping is characterized by immobility, submission, and withdrawal, which may be more consistent with depression, behaviors not observed after unpredictable threats alone. The greater time assessing and responding to aversive or highly novel stimuli, without effects on withdrawal, is consistent with reports of the greater sense of self-efficacy reported in humans after controllable and predictable stress (Kammeyer-Mueller, Judge, & Scott, 2009). However, the increase in responding to highly novel stimuli suggests possible pitfalls of such a bias, and may account for the higher probability for impulsive aggression in some populations (Pavic et al., 2003). In humans, reactive aggression is often co-morbid with anxiety, although in community and clinical based samples, aggression has been reported to precede anxiety (Bubier & Drabick, 2009). Beyond enhanced defense behaviors, we found no additional evidence for anxiety. The potentiation of reactive aggression after exposure to repeated threat shares some parallels with epidemiological literature on aggression; children exposed to neighborhood violence are more likely to have behavior disorders (Youngstrom, Weist, & Albus, 2003), including higher aggression (Guerra, Huesmann, & Spindler, 2003). Ironically, the bias toward active coping may have additional pitfalls, including impairments in working memory (Kim et al., manuscript in preparation) like those previously described after

stress (Conrad, 2010). Therefore adaptions do not simply produce resilience, but instead are better described as a double-edged sword.

Overall, the stress group exhibited facilitated active coping with no change in passive coping behaviors, a pattern that may be suggestive of the site of plasticity. Burying was not a specific response observed in the stress condition, therefore it is unlikely the stress condition induced plasticity at the level of motor generation for burying, which is dependent upon the periaquaductal gray (PAG) area in the midbrain. (Bernard & Bandler, 1998; McNaughton & Corr, 2004; Vianna, Graeff, et al., 2001). Stimulation of select columns of the PAG produce burying (Bernard & Bandler, 1998). The generalization of increased burying to novel stimuli argues against the possibility that stress solely produced sensitization to aversive stimuli in the tunnels. Enhancement of coping was restricted to active defensive responses, and did not generalize to passive coping (i.e., avoidance of open arms in the EPM), therefore we hypothesize that the unpredictable threat condition produced plasticity in a location where active and passive coping behaviors or approach avoidance behaviors are already segregated. Separate cell populations in the cingulate cortex are associated with approach and avoidance behaviors in non-human primates (Amemori & Graybiel, 2012). In mice, cells with activity that is associated with the decision to stay or move on during foraging have been described in the anterior cingulate cortex (Kvitsiani et al., 2013). The likely activation of these sites during our foraging manipulation, make the anterior cingulate cortex a candidate site for plasticity. Future studies will aim to empirically test sites of activation and search for plasticity.

Foraging serves as an excellent model of the risks humans take to gain rewards. By stripping environments down to the fundamental features of life's sustaining activities it may be possible to systematically study the role of key factors in the environment (e.g., control,

prediction and availability of resources) and the interactions they play in shaping behavioral tendencies such as biases toward active coping, helplessness and resilience. Here, we present the first step, a study of unpredictable, but controllable threats. After cessation of the treatment conditions, the unpredictable threat stress group displayed potentiated defensive behaviors to both predator odor (despite repeated presentations during the treatment phase), and in later cohorts to highly novel stimuli. There were no group differences in responses to diffuse cues, a feature of anxiety, or differences in passivity associated with helplessness, a feature of depression. The pattern of results suggests that the subjects in the threat condition were more sensitive to risk and more likely to response to threat. This selectively enhanced active coping should increase the likelihood that the threat stress group would successfully defend themselves in the face of clear threat, a feature of resilience.

We hypothesize that the controllable feature of the unpredictable threat manipulation likely played a key role in the selective enhancement of active coping, and that the introduction of uncontrollable features would bias the system away from active coping and toward passive coping behaviors. Direct tests of these hypotheses can be conducted in the future with our foraging platform. If successful, systematic comparisons should lead to a greater understanding of how to refine the categorization of human stressors and enhance our understanding of the role environmental factors play in shaping behavior. Such an understanding would open the possibility of engineering whole environments (e.g., therapeutic environments) to optimize behavioral choices and reverse stress-related disorders.

Table 1. Food and water consumption

	Group	n	Habituation	Week 1	Week 2	Week 3
Food Consumption (grams)	CT	41	24.04 ± 0.99	26.90 ± 0.84	26.98 ± 0.92	33.53 ± 1.60
	ST	40	23.35 ± 1.27	25.23 ± 1.24	26.09 ± 1.05	31.90 ± 1.98
Water Consumption (grams)	CT	41	27.38 ± 2.21	35.56 ± 1.01	32.45 ± 1.58	41.56 ± 2.38
	ST	40	26.52 ± 1.71	34.17 ± 1.60	33.99 ± 0.96	37.61 ± 2.00

Table 2. Measures of symptoms associated with anxiety

		n	Avoidance (latency)	Avoidance (time)	Exploration (number)
Elevated Plus Maze			Latency to Open Arm (sec)	Open Arm Time (sec)	Total Crossing 17.50 ± 1.89
	CT	10	27.04 ± 8.45	109.90 ± 21.31	17.58 ± 1.30
	ST	12	20.24 ± 6.09	111.74 ± 10.21	
Open Field Test			Latency to Enter Inner (sec)	Inner Field Time (sec)	Total Crossing 90.08 ± 9.92
	CT	12	92.32 ± 25.63	2.66 ± 1.39	95.92 ± 4.14
	ST	12	183.42 ± 39.32 #	5.16 ± 2.13	
Novelty Suppressed Feeding			Latency to Approach (sec)		Total Crossing 142.50 ± 11.96
	CT	10	195.40 ± 29.26		177.36 ± 12.08 #
	ST	11	148.25 ± 19.50		
			Latency to Eat (sec)		
	CT	10	318.60 ± 17.63		
	ST	11	315.10 ± 33.99		

Table 3. Measures of symptoms associated with depression

		n	Helplessness	Anhedonia
Forced Swim Test			Immobility (sec)	
	CT	29	20.54 ± 4.97	
	ST	23	20.34 ± 4.56	
Sucrose Preference Test				Sucrose Preference (grams)
	CT	12		0.73 ± 0.05
	ST	11		0.83 ± 0.03

Figure 1

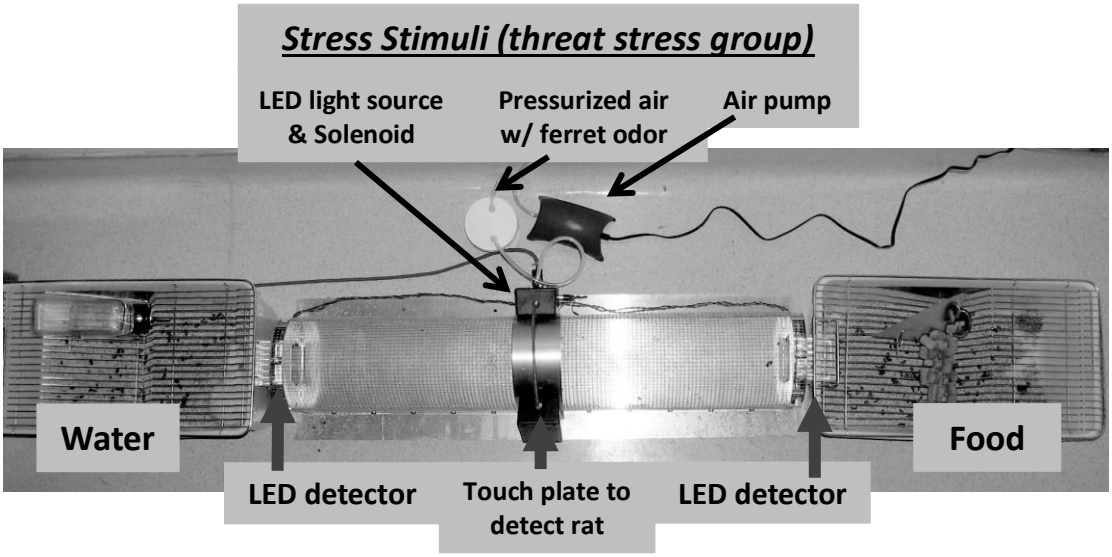


Figure 1. Unpredictable threat stress tunnel. Two tubcages were separated by a 3 feet wire mesh tunnel. Food and water were available ad libitum separately for each tubcage. The unpredictable threat stress tunnel condition included unpredictable presentation of ferret odor, abrupt solenoid, and abrupt flash of lights located at the center of the tunnel.

Figure 2

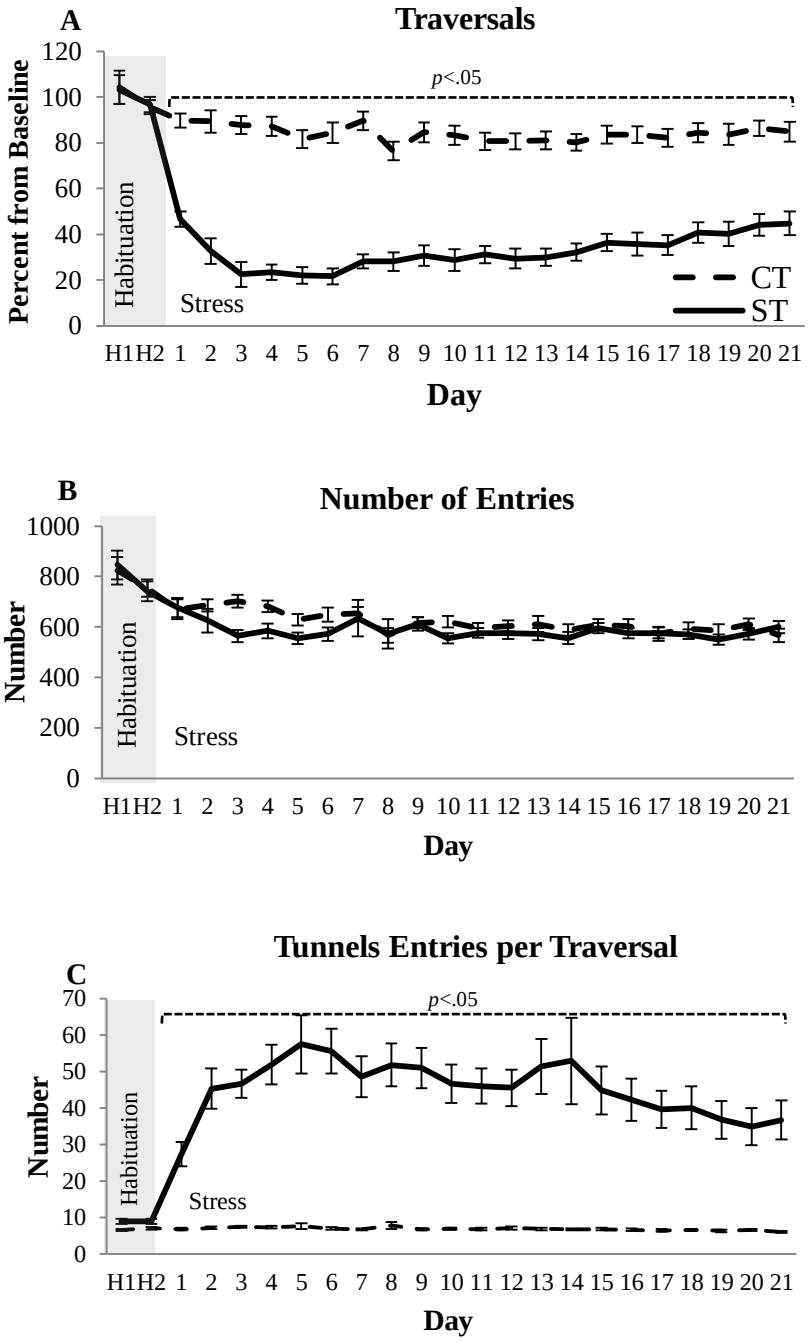


Figure 2. Tunnel behaviors were measured during the habituation period and the 3-week stress manipulation period. (A) Traversals during the treatment conditions relative to the number made during the habituation period. ST dramatically reduced traversals during the treatment conditions, whereas the control group did not ($p<.05$). (B) The frequency of tunnel entries was similar for the two groups ($p>.05$. (C) The tunnel entries per whole traversal did not differ across groups during habituation period (baseline), but differed during the treatment phase. The

Figure 3

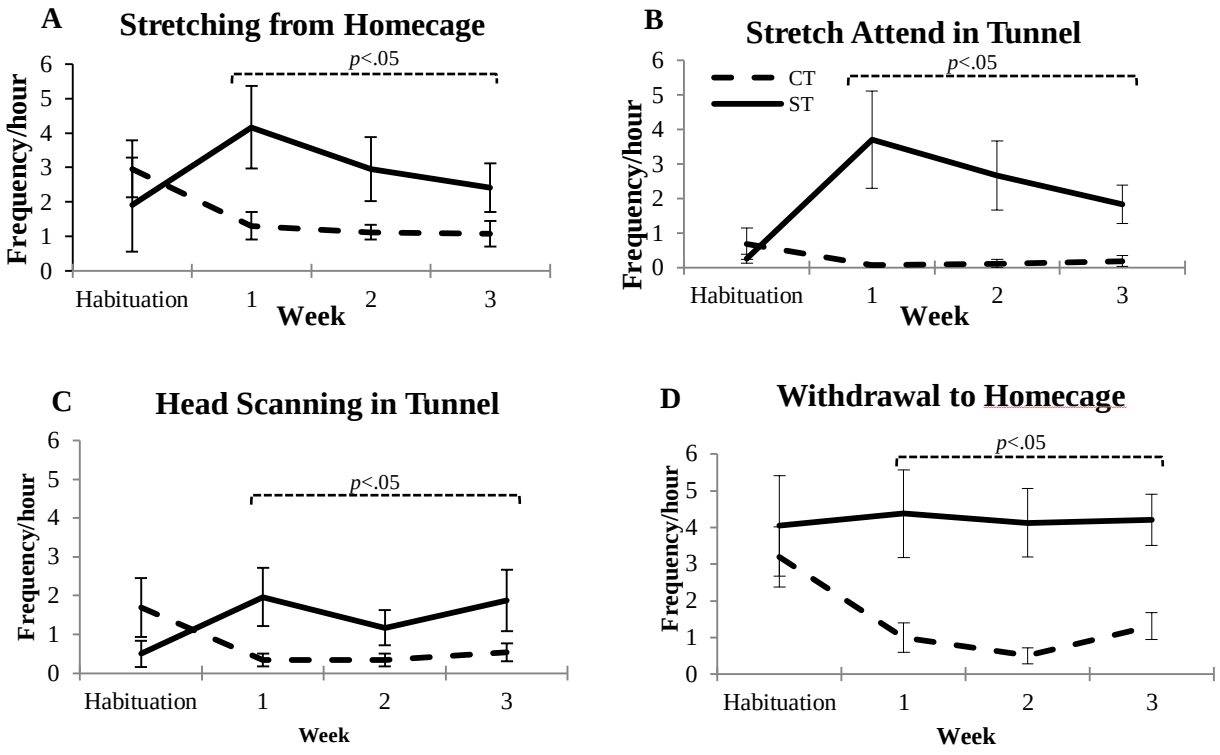


Figure 3. During habituation, there were no group differences in the frequency of risk assessment behaviors. During the treatment conditions, the ST group had more defensive behaviors, including (A) stretching out to look from within the tubcages, (B) stretched-attends, C) head-scanning, and (D) withdrawals from center of the tunnel back into the nearest tubcage.

Figure 4

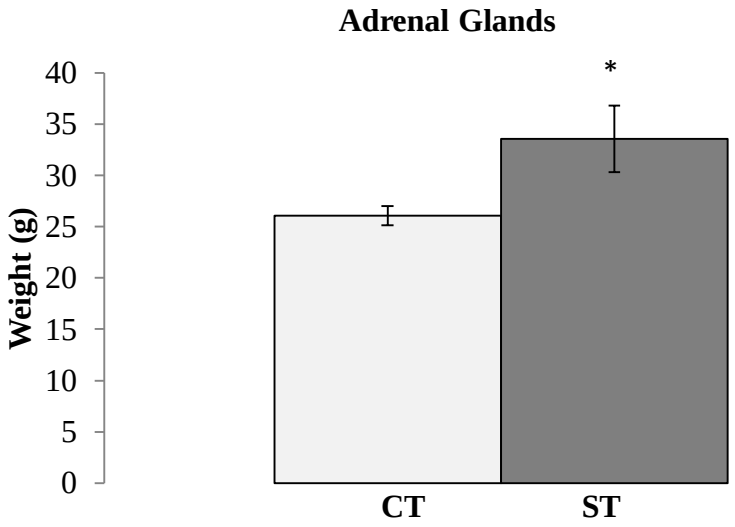


Figure 4. The stress group had a higher adrenal gland weight (p<.05) measured after the cessation of the 3 week stress manipulation.

Figure 5

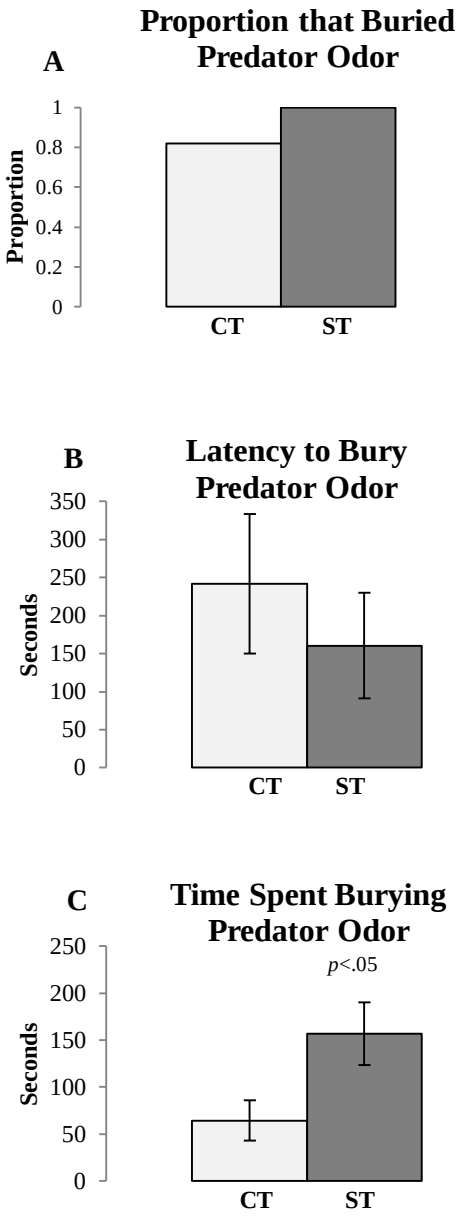


Figure 5. After cessation of the treatment conditions, there were no group differences in the proportion that buried in each group (A), and no difference in latency to bury the ferret rag (B) but the ST group spent more time burying the ferret dander rag than the CT group (C). A control rag was not buried by rats in either group.

Figure 6

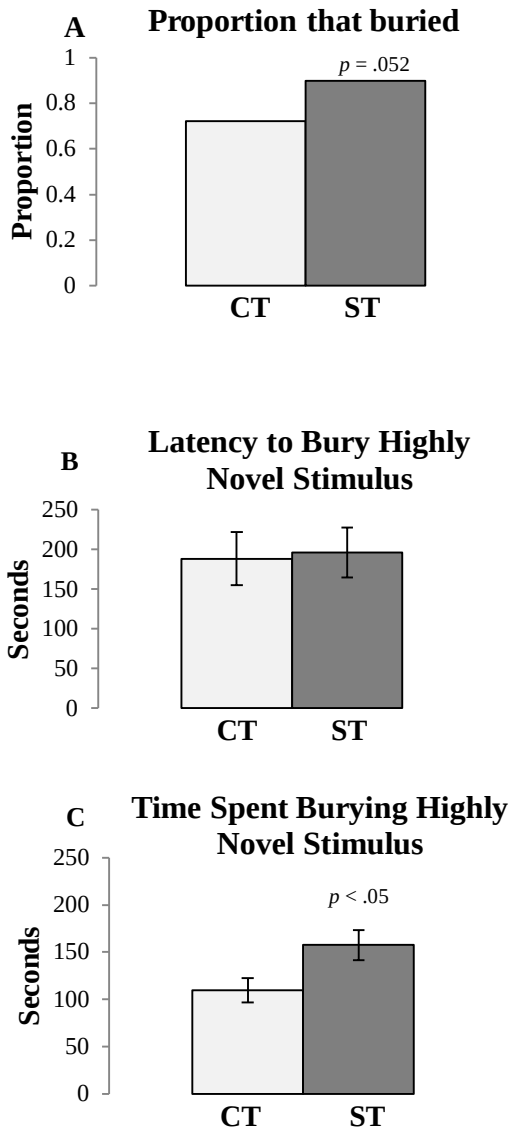


Figure 6. There was a tendency for a group differences in the proportion that buried in each group (A), but no group differences in the latency to bury a highly novel stimulus (a 6V motor with a moving propeller). (B). The ST group spent more time burying the novel stimulus (C).

Chapter 4

Experiment 2: Spatial Working Memory Impaired by Chronic Unpredictable Threat

IV. 1. Introduction

An organism attempts to uphold a steady equilibrium despite a constantly changing environment. When that equilibrium is at risk, a general physiological response, the stress response, serves to restore it. In humans, the most basic stress response is a *reactive* response to *physical stressors,* which are direct challenges to homeostasis (Herman, Prewitt, & Cullinan, 1996; Sawchenko, Li, & Ericsson, 2000). Direct challenges include, but are not limited to extreme temperatures, hypoxia, hypoglycemia, dehydration, and injury (Ulrich-Lai & Herman, 2009a). Indirect threats such as stress related to work (i.e. job insecurity, role overload, etc.) (Gilboa, Shirom, Yitzhak, & Cooper, 2008) or threats of traumatic events (i.e. terrorism, violence, and natural disasters) (Cancro, 2004; Cohen & Eid, 2007) or social conflict (T. W. Miller, 2007) require anticipation of challenges to homeostasis, and therefore are *proactive* responses that require cognitive interpretation of exteroceptive stimuli. Whether innate or learned, exteroceptive cues associated with physiological challenges independently initiate stress responses. These *anticipatory* responses are referred to as *psychological/proactive stress.*

During psychological stress, the remoteness of the physical stressor allows for preparatory and preemptive physiological and behavioral responses (e.g., risk assessment and coping), that serve to counteract the potential threat. The behavioral, endocrine and autonomic output are coordinated (Kerman, 2008) during physical stress, and since psychological stress modulates systems that react to physical stress, psychological stressors also elicit coordinated

responses. Because of the remote nature of the anticipated stressors, these anticipatory responses can be prolonged or intermittent, with no resolution. Although preparations for challenges are adaptive in response to immediate threat, chronic stress can prioritize action over tissue restoration, leading to accumulating damage (Lupien et al., 2007; McEwen, 1999, 2000b). Environments with multiple threats, despite low physiological challenges, have been proposed to be damaging through the chronic and accumulating effects of anticipatory stress. In humans, the greater ability to plan and anticipate future events, including negative events, brings with it a vulnerability to psychological stress and its potential consequences even when physical stressors fail to materialize. Yet, there is little research to test whether environments with highly anticipated threat and low physical challenge produce damaging effects of stress.

To study the behavioral consequences of psychological stress in animals, a number of models have been successfully employed. Physical stressors such as a water bath or foot shock (Mizoguchi et al., 2000; Yoshioka et al., 1996), restraint (Conrad et al., 1999; Magarinos & McEwen, 1995), and social defeat (Buwalda et al., 2005; Calvo et al., 2011) are used to induce psychological stress. In the chronic unpredictable stress model, random exposure to adverse conditions has been used in an attempt to induce the anticipation of threat (Roth & Katz, 1981). Repeated restraint has also been used to induce psychological stress (Conrad et al., 1996; Luine et al., 1994; Sapolsky, Krey, & McEwen, 1985). It is widely accepted that these models induce psychological stress, and produce behavioral deficits, but many of these models include a physical stress component, leaving open the possibility that psychological stress alone is insufficient to produce the variety of effects reported. Since psychological stress in humans often occurs as an event completely isolated from physical stressors, a full understanding of the behavioral consequences of psychological stress without accompanying physical stress would be

beneficial. Therefore, we set out to develop a basic rodent platform for inducing psychological stress without physical stress. We further sought to develop a platform that can be used in future studies to systematically manipulate the key factors associated with psychological and physical stress.

Previous research has shown that chronic stress and chronic elevations in corticosterone, which is elevated during stress, impair spatial working memory (Coburn-Litvak, Pothakos, Tata, McCloskey, & Anderson, 2003; Conrad et al., 1996; Kleen, Sitomer, Killeen, & Conrad, 2006; Luine, Spencer, & McEwen, 1993; Luine et al., 1994; McLay et al., 1998). The deficits have been attributed to psychological stress, which is believed to be of a longer duration and therefore more harmful than physical stress (McEwen & Gianaros, 2011). As noted above, in many models of psychological stress, physical stressors are used. Since human subjects often experience psychological stress without physical stress, in this experiment we tested whether psychological stress alone without direct harm is sufficient to impair spatial working memory.

Since chronic stress has fewer effects on memory when memory is tested in aversive conditions, but affects spatial memory in neutral conditions (Conrad, 2010), we tested spatial memory in neutral conditions. The Barnes Maze allows for testing of spatial memory without the physical challenge of deprivation (with food as reinforcement) or exposure to extremely aversive conditions (i.e. shock) (Barnes, 1979). The maze consists of a large white platform with holes. The holes lead to a dark box under the maze as rats naturally seek dark enclosed areas when exposed to an open environment. Although the Barnes Maze is not highly aversive, the rats are motivated to find the dark goal box due to escape from the open circular platform. Chronic stress has also been shown to inhibit novelty-induced elevations in locomotion and exploration (R. J. Blanchard, M. J. Kelley, & D. C. Blanchard, 1974; Conrad et al., 1999; Katz et

al., 1981), although the effects are mixed (Wright and Conrad, 2005). Since exploration may contribute to the measures in error and time duration to complete the memory task, we also measured their average mobility on the Barnes Maze trials. This would help further explain the difference between true errors due to memory impairment versus locomotor activity. We hypothesize that the unpredictable threat condition will decrease tunnel crossings during the 3 week duration. After the stress manipulation, we hypothesize that there will be impaired working memory in the group assigned to the unpredictable threat stress condition.

IV. 2. Methods

Subjects. Twenty-two male Sprague-Dawley rats were born in the local animal facility (Charles River) and transferred to our colony. Animals were housed in a reversed light-dark cycle. Each litter was weaned on postnatal day 21. The rats were handled for one week and were then randomly assigned to two separate conditions: control tunnel (CT)(n=13) and unpredictable threat tunnel (ST)(n=9).

Apparatus and experimental conditions. Tunnels (3 feet long) consisted of an aluminum lane (6 ¼" wide) covered with hardware cloth separating two standard tub cages, one containing food and one with water. The tub cages were attached to the tunnel by square aluminum tubes (3" X 3"). The tunnels were interfaced with a computer that recorded behavioral activity and controlled stimulus presentations. Infrared LEDs placed in the entry tubes on each end of the tunnel detected when rats poked heads or passed through the tunnel. When breaks of the LEDs at separate entrances were sequential, a traversal was recorded. To time stimulus presentations in the center of the tunnel to the presence of the rat, the rat was detected at the central position by a touch plate. At the center, a valve attached to a container of pressurized air containing a cloth with ferret dander odor. Cloths were obtained by placing a hammock or cloth in a ferret cage for

at least 2 weeks (Masini et al., 2005). A patch of cloth (6 X 8") was placed in a 500 mL plastic bottle connected to an air pump (Whisper 40 Air Pump 120 Volts AC 60 Hz 2.9 Watts, Tetra, Melle, Germany). On random crossing with a probability of 0.25, detection of the rat at the center of the tunnel triggered the opening of a valve. To reduce habituation, we increased stimulus complexity by presenting ferret dander odor with two additional mildly aversive stimuli, a solenoid that hit the side of the tunnel, and a flash of 10 ultra-white LEDs, 5 on each side (3500 millicandles of light of 600 nanometer wavelength) for 1 second duration. The tunnel used for the control condition were an exact replica of the stress tunnel, but animals received no presentations of any mildly aversive stimuli or odor, and were housed in an identical, but separate room to avoid exposure to any stress stimuli.

At the beginning of any experiment, all rats were habituated to the tunnels for 3 days. After habituation, the ST condition was initiated with the induction of the center stress stimuli while the CT condition remained unchanged. The experimental conditions lasted three weeks. Food and water measurements were taken at the end of the habituation period, and after each week of the experimental conditions. Body weight was also measured before rats were randomly placed into the tunnels and at the end of the experimental conditions. The number of tunnel entries and full traversals were recorded hourly (12 hours/12 hours). Since disruptions in circadian rhythms could affect memory by affecting sleep patterns, we also analyzed circadian patterns of activity over the course of the day during the experimental conditions. Rats were exposed to either control or psychological stress for 21 days before behavioral testing.

Behavioral testing after the treatment conditions

Barnes Maze. The Barnes maze (Barnes, 1979) consists of a large round platform (1.22 m in diameter) with a white surface in a brightly lit room with visuo-spatial cues in the maze periphery.

In such an open environment, rats naturally seek a dark enclosed surrounding, which is provided in the form of a dark box under one of 12 evenly spaced 10-cm round holes around the perimeter of the platform. Rats were habituated to the platform and tested using procedures previously described (Coburn-Litvak et al., 2003; McLay et al., 1998). On the 3rd day after removal from the tunnels, animals were habituated to the goal box and maze over three trials. One day later, rats were placed into a start box (27.9 cm tall and 24 cm in diameter) in a random orientation. After 30 seconds, the box was raised by the investigator blind to the groups who stepped away from the maze to stand in the same position for each trial. Rats were allowed to explore the maze until they entered the new goal position or 3 minutes elapsed. Once the goal box was entered, rats stayed there for 2 minutes, after which they were returned to their home cage for a 15' inter-trial interval. The maze and goal box were cleaned between trials to avoid use of any odor cues, and the maze was rotated randomly to prevent rats from using unintended intra-maze marks to find the goal position. The goal location was used for eight test trials, with four trials each over two days. The second day of trials commenced between 22-24 hours later.

Statistics. To compare behavior in the tunnels during the experimental manipulations we used a repeated measures ANOVA with group as the between subjects factor and day as the within subjects factor. The proportion of traversals, entries and the ratio of entries per traversal across the full day were analyzed with a one way repeated ANOVA. The circadian patterns of activity were analyzed with a two way ANOVA (group by light period). A subsequent one way ANOVA was used to analyze group effects during the dark cycle. Food consumption and water consumption were compared with a repeated measures ANOVA with group as the between subjects factor and week as the within subjects factor. Body weight at the end of the condition was compared with a one-way ANOVA with group as the between subjects factor. To compare

behavior tested after the experimental conditions, which included latency, error number, repeat error number and holes per minute on Barnes maze working memory trials, we used a repeated measures ANOVA (SAS 9.0, Cary, NC) with group as the between subjects factor and trials as the within subjects factor**.** The same measures from trial 1 and trial 5, which measured motivation and reference memory, respectively were analyzed with a one-way ANOVA with group as the between subjects factor.

IV. 3. Results

Tunnel Data

Traversals. During the habituation trials, the stress and control condition produced similar numbers of traversals ($F(1,20)=2.5$, $p=0.13$) The stress group made significantly fewer traversals throughout the three week experimental condition than the control group ($F(1,20)=44.04$, $p<.0001$) (see Fig. 7A). The traversals changed over time ($F(22,440) = 8.75$, $p < .0001$.) There was an interaction ($F(22,440) = 4.91$, $p < .0001$). The number of traversals for the control group was consistent over time. The stress group initially had traversal numbers similar to controls, but dramatically reduced traversals upon commencement of the stress condition, and then slowly increased traversals over time, but never reached control values.

To assess vigilance, we calculated the number of times the beams at the tunnel entrance were broken by either whole body entry or head insertions into the tunnel. There were no differences in the number of tunnel entries between the two groups ($F(1,20)=.81$, $p=.38$) (see Fig. 7B) and no interaction ($F(22,440) = 1.15$, $p = .28$). The ratio of entries per traversal differed by group with the ST group making 57 entries per full traversal in contrast to 7 by the CT group ($F(1,20)=37.90$ $p<.0001$) (see Fig. 7C), suggesting that the stress group was monitoring the tunnel, despite making fewer crossings. There was a day effect ($F(22, 440) =$

4.04, p<.0001) and an interaction ($F(22,440) = 3.95$, $p < .0001$). The stress group decreased their tunnel entries and ratio of entries as compared to their habituation levels in contrast to the steady values in the control group.

Food and Water Intake and Body Weight. Physical stress would include inadequate energy supply and dehydration. Since our model was designed to induce psychological stress without physical stress, it is imperative that food and water consumption was adequate. We measured both groups (CT=13 and ST=9) at four time points during the stress manipulation and found no group differences or interactions with time in food and water consumption (Food, $F(3,20)=.14$, $p=.7129$; Water, $F(3,20)=.01$, $p=.9109$) (see Table 4). All animals ate more food as the experimental condition progressed ($F(3,60) = 9.29$, p <.0001), but water consumption remained consistent ($p > .05$). Body weight data is missing from 2 cohorts. Of the cohorts measured, both groups gained equal amounts of body weight ($F(1,15)=.56$, $p=.4647$).

Circadian Rhythms. To determine if the psychological stress paradigm induced changes in daily activity rhythms, the percent of traversals and tunnel entries in the dark and light cycles were compared. The percent traversals and entries over the light and dark periods were averaged across each of the three weeks of the experimental condition. A two way repeated ANOVA with group as the between subject factor indicated that for both traversal and tunnel entries there was a time of day effect and an interaction between group and time (Traversal time of day: $F(1,40)= 74.00$, $p < .0001$; interaction $F(1,40)=20.00$, $p < .0001$; Entries time of day $F(1,40) = 108.00$, $p < .0001$)) (Fig. 8A and 8C). Post-hoc analyses revealed that the ST group made proportionally more traversals and entries during the dark period than controls (Traversals, $F(1,20) = 5.527$, $p =0.029$; Entries, $F(1,20) = 11.21$, $p =0.003$) (Fig. 8B and 8D). There is no indication that they lost sleep, because their activity reflected a dark/light rhythm, but they made proportionally

fewer traversals and entries during the inactive period. To confirm the persistence of these rhythms, we further tested whether there was a shift only at the end by analyzing the distribution of traversals on day 20, the last full day of data collection. There were no differences in proportion of total daily traversals in the dark cycle $F(1,21) = 0.176$, $p = 0.682$) and light cycle $F(1,21) = 0.173$, $p = 0.682$)

Behavioral Consequences of Psychological Stress

Working Memory. During the first trial, when the goal location is first encountered, there were no group differences in the latency to find the escape hole ($F(1,21)=.64$, $p=.43$), suggesting similar motivation for finding the goal location. Trials 2- 4 and Trial 6- 8 tested working memory. For latency, there was a group effect with the CT group taking less time to find the goal on working memory trials ($F(1,20)=7.44$, $p=.013$).,There was no trial effect or interaction ($p>.05$). Reference memory is tested on the first trial on day 2. There was no group difference ($F(1, 21)=.33$, $p=.57$), suggesting that impairments were limited to working memory (see Fig 9A).

Errors were counted when the animal persistently investigated a specific hole for more than two seconds and/or placed their nose into or over the hole. The ST group had a tendency to make more errors than the CT group on all working memory trials (2-4 and 6-8) ($F(1,20)=3.89$, $p=.063$). There was no trial effect or interaction ($p > .05$, see Fig. 9B). There were no significant differences in the number of errors between the two groups on the first trial with the new goal position (1st trial, $F(1,21)=2.53$, $p=.13$). Likewise, there was no difference on the 5th trial, which tested reference memory ($F(1,21)=.02$, $p=.90$). Errors were further divided so that we could test repeat errors within a trial. On the first trial the ST group had a tendency to revisit incorrect holes ($F(1,21)=3.67$, $p=.07$), but was no different on the 5th trial ($F(1,21)=.001$,

p=.972). On working memory trials, the ST group made significantly more revisits to incorrect holes ($F(1,20) = 5.32$, p = .032) (see Fig. 9C). There was no trial effect or interaction ($p>.05$).

To determine whether working memory errors were solely accounted for by an increase in the number of repeat errors, we calculated non-repeated errors by finding the difference between the total amount of errors minus the amount of repeated errors. The ST group made significantly more non-repeat errors during the working memory trials ($F(1,20) = 5.32$, p = .032) (see Fig. 9D), but there were no differences between the groups for trials 1 and 5 ($p>.05$). There was no trial effect or interaction ($p>.05$). This suggests that the ST group had a tendency to make more errors overall, with significantly more repeated and non-repeated errors.

To assess general movement and activity level, we calculated the number of holes visited per minute. On the first trial, the ST group had a tendency to be more mobile ($F(1,21)=4.12$, $p=.056$). On working or reference memory trials, there were no group differences in activity rate (working memory; $F(1,20)=.005$, $p=.94$; reference memory trial ($p>.05$)), suggesting that the ST group was equally motivated to find the goal box (see Fig. 9E).

IV. 4. Discussion

The fundamental goal of the present experiment was to a) develop and test a platform that allows us to induce chronic psychological stress, and b) test the ability of one set of parameters, unpredictable/semi-controllable presentations of aversive stimuli, to induce psychological stress without physical/reactive stress. By using ferret dander odor paired with lights and sound, challenges to homeostasis were threatened but never paired with direct physiological challenge or pain. The intermittent presentations delivered over 21 days produced persistent avoidance of the location of stimulus presentations as seen by fewer tunnel crossings in the ST group relative to the control group, results that are consistent with previous observations that sensory signals

representing nearby predators are associated with avoidance (D. C. Blanchard, Canteras, Markham, Pentkowski, & Blanchard, 2005). Despite the reduced number of crossings, the number of entries per traversal was significantly greater in the stress than control group, suggesting heightened vigilance, which is associated with awareness of a predator. In a separate study (Kim et al., submitted) of the quality of behaviors in the tunnel, ST rats exhibited significantly more risk assessment behaviors, consistent with behaviors produced in response to predators or predator cues (R. J. Blanchard et al., 1998; R. J. Blanchard, Yang, Li, Gervacio, & Blanchard, 2001). The low probability of stimulus presentations was designed to allow sufficient numbers of crossings for adequate food and water consumption, which was equivalent across groups, therefore excluding the possibility of physical stress. Our model appeared to succeed in producing psychological stress exclusively, allowing us to test the effects of psychological stress on working memory, and activity.

Although biological stress was defined by Selye (1936) as a systemic defensive response to physiological challenge, it was quickly expanded to include responses to the anticipation of such a challenge (Mason, 1968a) , through higher order processing of sensory stimuli. A cue of impending threat suggests that threat is remote in space and/or time, and through descending projections can elicits a preemptive response through control over the reflexive stress response circuitry (Herman et al., 1996; Sawchenko et al., 2000). Since humans are particularly adept at anticipating future events, they are particularly vulnerable to psychological stress. Despite the initiation of a preemptive response, the resolution may take some time, leading to a prolonged stress response. To develop a flexible model of chronic psychological stress, we created a living environment in which rats were required to move through a tunnel to obtain necessary resources. The awareness of a predator captures the essence of a threat to homeostasis, and therefore

psychological/processive/exteroceptive stress. Therefore, our model is built around the ability of predator dander odor, specifically that of ferret dander, to signal impending threat (Masini et al., 2005; Masini, Sauer, White, Day, & Campeau, 2006), and activate the HPA axis (Masini et al., 2005). This odor not only elicits defensive responses in rats, but also initiates c-Fos expression in brain regions activated during psychological stress (Masini et al., 2005). By using ferret odor, the model incorporates the dependency on exteroception, higher order processing and remoteness of a direct homeostatic challenge in space and/or time.

Our model was intended to study the effects of chronic rather than acute stress, therefore odor was presented repeatedly over twenty one days. The stimuli persistently reduced traversals over the full duration of the treatment condition, and increased risk assessment behaviors over the full duration (Kim et al., submitted). The persistence supports the ability to test working memory deficits after psychological stress. The equivalence of body weight at the end of the experiment in addition to similar rats of food and water consumption support our ability to test the effects of psychological stress selectively.

In previous studies of working memory deficits after stress, stressors have included over 24 hours of food and water deprivation, restraint for several hours, and shock lasting more than several seconds (Conrad et al., 1999; Katz et al., 1981; Luine et al., 1994). The variability of these physical stressors produces the anticipation of stress, but does not necessarily exclude physical stress. Therefore, the latter cannot be discounted for its potential contribution to the subsequent behavioral deficits. These models have played a key role in identifying behavioral deficits produced by chronic stress, and potentially by psychological stress. Our model provides the opportunity to test the premise that psychological stress alone is sufficient to produce behavioral deficits associated with spatial working memory.

Despite a lack of direct challenges to homeostasis, our model produced working memory deficits. The deficits are similar to those reported in previous studies of chronic stress, and support assertions that memory deficits resulted from chronic psychological stress. The stress group performed significantly worse than the control group throughout the working memory trials. In our study, both the control and stress group were capable of learning the position of the goal box in the Barnes Maze, but the control group reached their peak performance on fewer trials. Both groups reduced errors and latency over trials. The deficits did not reflect differences in motivation, because both groups took similar time to find the goal box the first time it was encountered. If anything, the stress group had higher motivation reflected by their tendency toward higher rates of goal visits per unit time. There were no group differences on trials reliant on memory from the day before, similar to the failure to find reference memory deficits after restraint stress (Mika et al., 2012). The deficits on working memory trials reflected both repeat visits to incorrect holes within trials, as well as non-repeat errors reflecting impaired memory over the 15 minute inter-trial interval. The hippocampus has cells that encode positions in space and successive moments over a temporal gap, suggesting that cells in this region disambiguate moments in time as well as overlapping spatial sequences (Devito & Eichenbaum, 2011). Both the hippocampus and frontal cortex are necessary for working memory over long delay intervals in rodents (Churchwell & Kesner, 2011). Therefore, the greater number of non-repeat errors on working memory trials suggests that either structure may have adapted to the high threat condition to affect non-repeat working memory. Both structures are affected by chronic corticosterone elevations or stress models that utilize both physical and psychological stressors (Conrad, 2006; Dias-Ferreira et al., 2009; Sousa & Almeida, 2002; Tata, Marciano, & Anderson, 2006; Wellman, 2001), opening the possibility that psychological stress alone may

lead to adaptations in both structures as well. Both regions are individually capable of accommodating working memory demands over short delay intervals and therefore repeat visits within trials, which reflect deficits over relatively shorter delays, is consistent with the possibility that both structures have adapted in our model (Churchwell & Kesner, 2011). Future studies will attempt to directly test this possibility.

Chronic stress and corticosterone administration have produced mixed effects on locomotion in a novel environment (Bowman, Zrull, & Luine, 2001; Coburn-Litvak et al., 2003; Conrad et al., 1999; Fernandes, McKittrick, File, & McEwen, 1997; Katz et al., 1981; van den Buuse, van Acker, Fluttert, & de Kloet, 2002; Wright & Conrad, 2005). Thus, it is important to rule out the possibility that impairments in working memory reflected differences in locomotion or exploration rather than memory deficits. To avoid this potential confound, rats were habituated to the testing environment over three trials on the day before the first test. On test days, there were no differences in the frequency of holes visited per minute, with the exception of a tendency on trial 1. The failure to observe differences in locomotion in the Barnes maze on the trials testing working memory suggests that the memory deficits cannot be explained by differences in locomotor activity.

If the vigilance in the tunnel disrupted circadian cycles, it is possible that sleep deprivation accounts for the spatial memory deficits. Therefore, we analyzed behavior over light/dark cycles. The ST group had typical circadian cycles driven by the light/dark cycle, much like the control group, but with significantly more activity in the dark than light cycle. The ST group had proportionally more traversals and entries in the dark period, suggesting that they were more conservative during the inactive period. The similar performance on the 5th trial, reflecting memory of the goal position from trials on the previous day, argues against the

possibility that altered sleep patterns could account for the deficits. Although the ST group appeared more vigilant, the enhanced circadian activity patterns fails to support the possibility that altered sleep patterns could account for impairments in working memory.

The Barnes maze was chosen as a test for spatial memory, because it provided the ability to avoid potential group differences in reactivity to a stressful testing condition. When inherently stressful tests of spatial memory are used, chronic stress produces no deficits or superior performance (Conrad, 2010). Thus, the deficits in this study may be selective to tests like the Barnes maze that avoid stressful or aversive testing conditions. The role that differences in environmental adversity plays during testing have been proposed to reflect altered hippocampal sensitivity to corticosterone, which would be elevated in adverse testing environments. If chronic psychological stress elevates plasma corticosterone, receptors are expected to decrease, leading to less binding in tests of control conditions. In contrast, corticosterone elevations during arousal may compensate for the insensitivity developed in a chronic stress condition, but impair function in controls as a consequence of greater binding (Conrad, 2008). Thus the deficits in spatial working memory could reflect a downregulation of corticosterone receptors in the hippocampus and frontal cortex, areas that play an integral role in working memory (Churchwell & Kesner, 2011). This explanation of behavioral deficits is problematic, however, because plasma elevations in glucocorticoids take approximately three minutes, too long to account for effects within trials. Stress is expected to elevate plasma glucocorticoids. Corticosterone, the primary glucocorticoid in rodents has been shown to produce impairments in spatial working memory in the Barnes Maze when administered chronically. However, across published reports, only when corticosterone was administered for 56 (Coburn-Litvak et al., 2003) and 80 days (McLay et al., 1998), was spatial working memory

impaired. When administered for only 21 days, the duration of the current stress manipulation, corticosterone impaired reference, but not working memory (Coburn-Litvak et al., 2003) and impaired body growth. Our stress manipulation for 21 days was both shorter and had subtler systemic effects (no impairments in body weight gain), and yet impaired spatial working memory. This raises the possibility that corticosterone acting alone may not account fully for the spatial working memory deficits reported here, or that downregulation occurs sooner when corticosterone is elevated with other feature of stress (e.g., activation of the SNS). If the deficits reported here reflect receptor downregulation to accommodate for the chronically high level of stress, then we predict that such adaptations should produce optimal learning in high stress environments rather than deficits regardless of the level of adversity during testing.

A number of studies have demonstrated impairments in spatial learning (Kim, Lee, Han, & Packard, 2001; Packard & Wingard, 2004; Schwabe, Dalm, Schachinger, & Oitzl, 2008), and a preference for habit learning (Schwabe et al., 2008) during stress. Thus, it is also possible that the impairments in spatial working memory reflect a shift in preference for habits at the expense of flexible, but time-intensive, learning systems such as the PFC (Arnsten, 2009). This interpretation is consistent with our finding that the stress group had enhanced defense responses (Kim et al., submitted). However, stress has previously been shown to impair spatial learning while having no effect on maze performance when intra-maze cues are available (Wright & Conrad, 2005). Any shift in preference for S-R responses may represent adaptation to a threatening environment that requires rapid and therefore pre-existing responses like habits (Broadbent, 1971; Schwabe, Wolf, & Oitzl, 2010). In contrast, flexible learning requires calculation of strategy and prediction of outcome, which requires time. What is strikingly different about our findings relative to others reporting such a shift is that our rats were removed

from the stressful environment for several days before testing. Furthermore, they were habituated to the testing environmentwhich avoided novelty-related stress during testing. Thus, any shift away from a flexible spatial learning system appeared to be a longer-term change in response strategy, not simply a change reflecting the immediate testing, or pre-testing environment, but instead longer lasting forms of adaptation to stress.

Earlier interpretations of memory deficits after stress and corticosterone treatment have also attributed effects to altered hippocampal cell structure, including cell loss (Sapolsky, 1994). Corticosterone alone has not been fully established to produce cell loss; when unbiased counting methods are used there is no evidence for cell loss (for review see Tata & Anderson, 2010). Dendritic atrophy and synapse loss have been described after corticosterone administration or stress (Conrad, 2008; Conrad et al., 1999; Sandi et al., 2003; Sousa, Lukoyanov, Madeira, Almeida, & Paula-Barbosa, 2000; Tata et al., 2006), but may be partially reversible (Sandi et al., 2003; Sousa et al., 2000). The morphological alterations are not accompanied by ultrastructural indicators of tissue damage (Tata et al., 2006), suggesting that any anatomical plasticity may reflect adaptive re-wiring. Dendritic atrophy is not a perfect predictor of working memory impairments (Conrad, 2010). It is possible that the role of the hippocampus is reduced in order to bias toward habit based systems (White & McDonald, 2002). If the system is biased toward habits and away from spatial learning and working memory, then we predict that the stress group will continue to have impaired performance when tested in highly aversive conditions.

The food and water delivery in our model was predictable and consistent. Failure to acquire resources expected in a location causes rats to elicit odors that signal cohorts to avoid the empty location, odors that can produce pronounced avoidance behavior (S. F. Davis, Nash, Anderson, & Weaver, 1985). In our model, the consistently available resources meant that risk

was always rewarded, which may have moderated the impact of psychological stress. Consequently, it is somewhat surprising that psychological stress in our model was capable of impairing working memory. We strongly suspect that behavioral outcomes will be more pronounced if we reduce the reinforcement ratio in future studies. Nevertheless, the parameters of the current model are sufficient to significantly alter behavior.

To study the effects of psychological stress in a controlled environment, we sought to develop a rodent model of chronic psychological stress. In our model, rats controlled the approach to the location of aversive stimuli but could not predict the timing of the stimuli. Risk taking was always rewarded with access to resources (food or water). We found that the stimuli presented were sufficiently aversive to persistently reduce unnecessary crossings while maintaining enough crossing to allow sufficient food and water consumption. The stressful events in our model were unpredictable, but controllable, and yet still were sufficient to impair spatial working memory. The impairments were not explained by differences in general locomotion or exploration. Since spatial working memory deficits after psychological stress occurred after a shorter treatment duration than after corticosterone administration tested with the same maze (Coburn-Litvak et al., 2003), these deficits likely depend on the complex set of responses during psychological stress (endocrine, autonomic and behavioral responses), not just corticosterone. The platform we have developed to induce stress will allow us to systematically manipulate controllability, predictability and reinforcement in the rats' living environment to better understand the role key factors of psychological stress play in producing behavioral outcomes, and neural re-wiring.

Table 4. Food and water consumption for Barnes Maze cohorts

	Group	Habituation	Week 1	Week 2	Week 3
Food Consumption (grams)	CT	98.38±11.70	105.77± .50	95.15±10.25	143.85±15.12
	ST	74.78± 4.52	89.00±10.82	98.56±9.94	162.44±34.40
Water Consumption (grams)	CT	131.8±14.30	149.54± 9.12	105.00±15.78	128.00±15.10
	ST	111.78 ±16.66	139.38±16.14	133.78±16.62	134.44±14.37

Figure 7

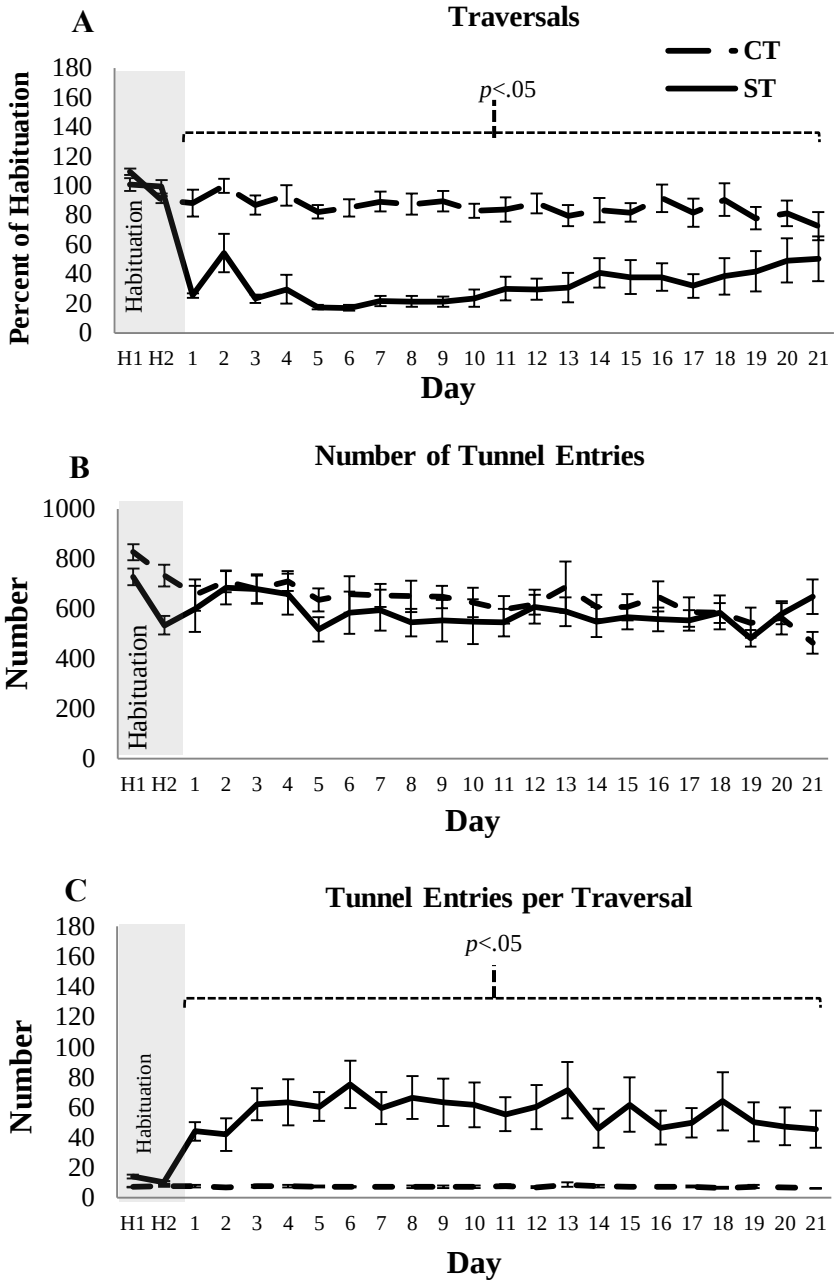


Figure 7. Tunnel behaviors were measured during the habituation period and the 3-week stress manipulation period. (A) Percent of traversals made during the manipulation period relative to the traversals made in the habituation period was measured (baseline). ST rats made significantly fewer traversals during the condition than during the habituation period ($p < .05$). (B) The two groups made similar numbers of tunnel entries ($p > .05$). (C) The tunnel entries per hole did not differ across groups during habituation period (baseline), but during the condition, the ST group made significantly more entries per full traversal ($p < .05$). They were monitoring events in the tunnel without crossing.

Figure 8

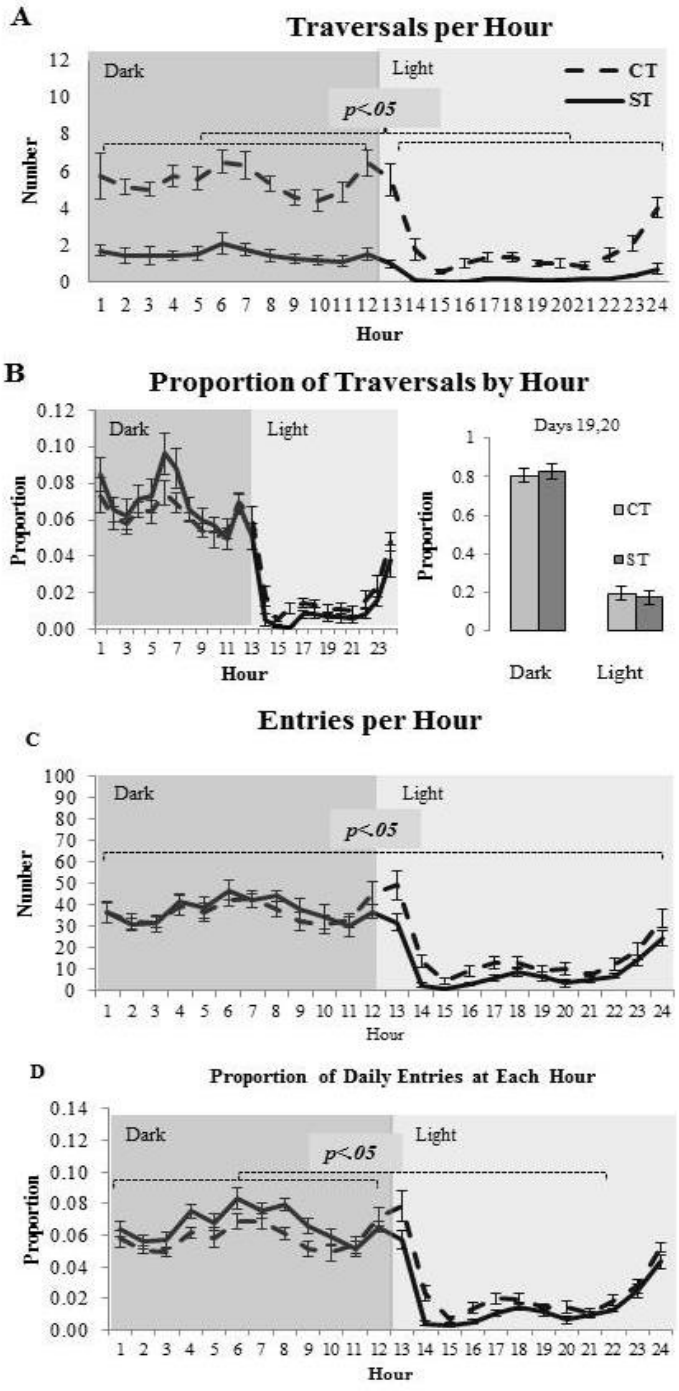


Figure 8. Tunnel behavior was measured during 24-hour periods to assess circadian rhythm. Both the ST and CT group made proportionally more traversals (A) and entries (C) during the dark (active) period. For both traversals and entries, the ST group had proportionally more activity (traversals (B) and entries (D) in the dark period than controls.

Figure 9

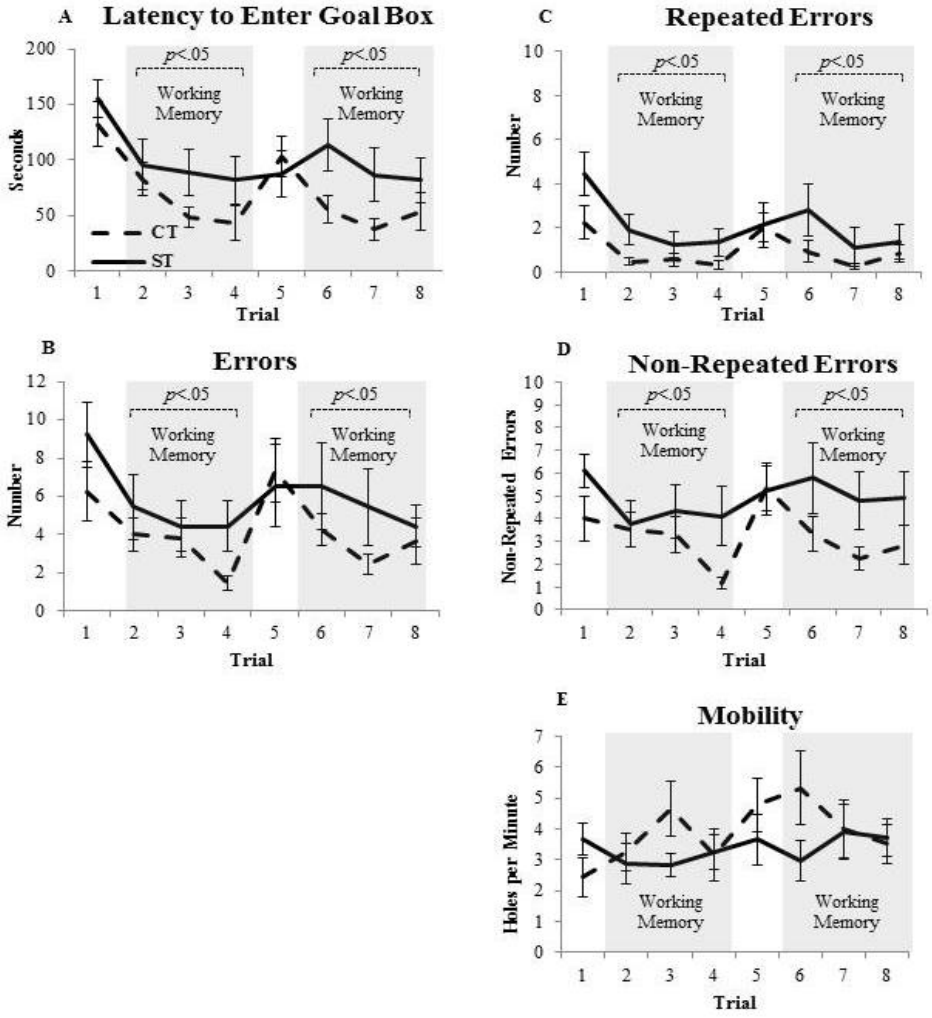


Figure 9. The goal position was introduced on trial 1 of Day 1. Three subsequent trials, within 2 hours of the introduction of the goal position, tested spatial working memory. On the following day (Day 2), four additional test trials (trials 5-8) were given. Trial 5 tested reference memory, whereas trials 6-8 tested working memory. (A) The ST group had higher latency throughout the working memory trials (trials 2-4 and 6-8). (B) The ST group had a tendency for more errors throughout the 8 working memory trials. (C) The ST group made significantly more repeat visits to incorrect holes on working memory trials,and a tendency for more repeate errors on trial 1. (D) The ST group made significantly more non-repeatead visits to incorrect holes on working memory trials, and a tendency for more repeate errors on trial 1. (E) Barnes maze activity measure. There were no differences in the number of holes visited per minute on the working memory trials ($p>.05$).

Chapter 5

Experiment 3: Metabolic Mapping of Regions Associated with Chronic Presentation of Unpredictable Predator Odor Threat

V. 1. Introduction

Using a living environment that included repeated unpredictable threats consisting of predator odor and abrupt lights and sounds presented, we previously found that rats exhibited more risk assessment behaviors than rats living in an environment without threat. After removal from the stress condition, the threat group exhibited greater defensive responding (Kim et al., submitted), and enhanced startle (manuscript in preparation), consistent with threat-related vigilance, but impaired memory (Kim et al., submitted). The shift toward enhanced sensitivity to risk and defense behaviors but impaired working memory when tested in neutral conditions is presumably adaptive, and is dependent upon neural plasticity. Our next goal in this series of studies is to identify the neurobiological substrates of these adaptations, but first we need to identify candidate sites of plasticity, which is the goal of this project.

Neurobiological plasticity underlying behavioral adaptation will be located in sites that are activated by the environmental factors that drive adaptation. In our model, the three simultaneous threat stimuli are expected to activate regions processing and integrating the key sensory cues as well as activating responses systems. Predator odors activate the accessory olfactory bulb, which in turn projects to the medial amygdala (Canteras, Chiavegatto, Ribeiro do Valle, & Swanson, 1997) (see Fig 10). Other non-odor cortical sensory and association areas involved in potential predator presence project to the basal medial and lateral nucleus of the

amygdala (Canteras et al., 1997; Walker et al., 2003), potentially subserving the integration of lights, sounds and predator cues, but abrupt lights and sounds also project directly from the thalamus to the amygdala, bypassing the cortex (M. Davis, Walker, & Lee, 1997; Leitner, Powers, & Hoffman, 1980). Anatomically, the amygdala, in turn, projects to a number of nuclei in the hypothalamus, either directly or indirectly through the bed nucleus of the stria terminalis (BNST), to activate a number of subnuclei, including those implicated in defense control circuitry and nuclei that play an integral role in activation of the HPA axis. Consistent with known anatomical pathways, activation and lesion studies have shown that predator odor is processed through the hypothalamic defensive system. These previous studies have shown that the medial hypothalamic zone is a critical region for expression of innate defensive behavior to both predator and predator odor threat (Canteras, 2002; Canteras et al., 1997). The three key regions associated with the medial hypothalamic defensive system are the anterior hypothalamic nuclei (AHN), ventromedial hypothalamic nucleus, dorsomedial part (VMHdm), and dorsal premammilary nucleus (PMD) (see Fig 10). The latter projects to the PAG which contains cells that activate autonomic and motor neurons Our model of unpredictable stress utilized ferret odor, which, like predator odors in general, produces innate fear responses (Masini et al., 2005). Measuring c-Fos mRNA expression, a marker of early gene activation, ferret dander odor specifically activates cells in the orbitofrontal cortex, prelimbic cortex, lateral septum, BNST, dorsomedial part of the ventromedial nuclei of the hypothalamus, and dorsal premammillary nucleus (Masini et al., 2005). Based on the known anatomical pathways and regional activation patterns following exposure to predator odor, any of these regions may be candidates for sites of plasticity (see Fig 11). Previous activation studies utilized a single prolonged exposure to predator odor. Since we use three simultaneous stress stimuli presented briefly but repeatedly,

we seek to confirm these sites as candidates for sites of plasticity by confirming the activation of these areas with the stimulus parameters of our model.

To study regional activation, we can use metabolic mapping methods. Metabolic mapping methods include 2-DG, fMRI and cytochrome oxidase (COX). These methods are useful indicators of neuronal activity because of the tight coupling between energy metabolism and the activity of neurons (M. T. Wong-Riley, 1989). Energy will not be generated unless the previous energy has been used (M. T. Wong-Riley, 1989). Methods such as 2-DG and fMRI measure glucose utilization and blood flow to assess immediate neuronal energy demands (McIntosh & Gonzalez-Lima, 1992). These methods would be appropriate for single intense exposures, but not intermittent acute exposures over many days. In order to measure energy demands over a longer duration, requires measurement of the basal metabolic capacity. This capacity can be upregulated due to increases in energy demands. With higher neuronal activity, the higher the energy to sustain the activity is demanded. The cytochrome oxidase enzyme is critical for its role in cellular respiration needed for any activity in the nervous system. (M. T. Wong-Riley, 1989). By observing this enzyme with COX histology, we can measure the basal metabolic demands associated with basal increases in neuronal activity and therefore regions associated with our environmental condition.

It is well-established that chronic changes in neural activity can be represented by up or down-regulation of the cytochrome oxidase enzyme (M. T. Wong-Riley, 1989), which is tightly coupled with the production of ATP (Skou, 1965), the currency for biological work. Cytochrome oxidase (COX) is a mitochondrial enzyme. Sustained changes in COX activity reflects changes in energy demands (Skou, 1965). Its activity is a useful indicator of the regional functional activity of the brain (M. T. Wong-Riley, 1989). Previous studies by Wong-Riley &

Carroll (M. Wong-Riley & Carroll, 1984) have blocked sensory information to the lateral geniculate nucleus and striate neurons using tetrodotoxin (TTX). The complete deprivation of a sensory system decreased cytochrome oxidase activity after 3 days. Therefore, when nerve cells are more active or inactive, it leads to increased or decreased metabolic activity, respectively.

Early studies of cytochrome oxidase regulation by neural activity used complete sensory blockade. To be useful for identifying sites activated by repeated acute stimuli, however, requires that cytochrome oxidase regulation can be identified after behavioral changes and environmental alterations. COX activity increased after 6 months of exercise behavior. Voluntary wheel running increased COX activity in regions of the motor cortex, as well as the dorsolateral caudate putamen, both of which contain limb representations (McCloskey et al., 2001). In a discrimination task, learning to distinguish between a positive stimulus which signals a reward and a negative stimulus, signaling no reward using 2 arms in a Y-maze was correlated with increased COX activity. Learning consisted of increased head turning at a choice point in the Y-maze and choosing the positive stimuli correctly resulting in increased rewards (Hu, Xu, & Gonzalez-Lima, 2006). Both acute and chronic neural activity drives metabolic change, including changes in the activity of cytochrome oxidase. As it is demonstrated in both acute and chronic behavioral studies, we aim to utilize this method to identify regions activated by unpredictable threats and that are likely candidates for plasticity related to the behavioral adaptations resulting from threat. The histochemical method of Wong-Riley (M. Wong-Riley, 1979) will be used to observe metabolic activity resulting from our stress manipulation.

In the current study, rats were exposed for three weeks to one of two living conditions, one with unpredictable threat condition and the other without threat. The three stimuli comprising threat were abrupt sound, flash of light, and ferret odor presented randomly with an

average rate of once per four crossings. In previous studies of identical conditions, the subjects in the threat condition produced significantly more risk assessment behaviors and avoidance of the location of threat presentation throughout the 3 week duration. After rats were returned to standard colony conditions, they exhibited adaptations in arousal responses, defense responses and learning. To identify the sites of plasticity we will use the COX histochemistry, which is sufficiently sensitive to reveal up or down regulation of the COX enzyme from behavioral manipulations, to identify regions activated differentially by unpredictable threat. We will focus our investigation on brain regions implicated in predator odor processing and regions that initiate defense responses. These include anterior cingulate cortex, medial amygdala, anterior hypothalamic nucleus, basolateral amygdala, prelimbic cortex, and lateral septum (Masini et al., 2005). Additionally, the hypothalamic defensive circuit is involved along with its afferents and efferents (Canteras, 2002; Canteras et al., 1997). These structures include dorsomedial part of the ventromedial nuclei of the hypothalamus, dorsal premammillary nucleus and their target, the periaqueductal gray. These regions are also known to be activated by predator odor (Canteras, 2002; Canteras et al., 2008).

V. 2. Methods

Stress manipulation

Twenty rats were handled for a week after weaning and placed in their respective conditions (unpredictable threat condition or control condition) for 3 weeks. After an initial 3 day habituation period in the tunnel conditions, rats in the unpredictable threat condition were presented with an abrupt sound of a solenoid hitting the side of the tunnel, a flash of light, and a puff of air containing ferret dander odor randomly (p=.25) for 3 weeks. The control group was housed in identical tunnels but never experienced threat stimuli. Food, water, and weight

differences were measured every week to confirm similarity of intake and consumption between the 2 conditions.

Tissue processing

After the 3 week stress condition, the rats were injected with sodium pentobarbital (100 mg/kg) and decapitated. Brains were removed and rapidly frozen in isopentane at -40°C and stored at -75°C. Thirty µm coronal sections were collected with a cryostat maintained at -20°C. Three sets of alternate coronal sections were mounted on subbed slides and stored at -75°C. Standard homogenates were made from 5 additional rats. The homogenate paste was stored in Eppendorf tubes at -75°C. The homogenates were sectioned with thickness of 10-, 15-, 30-, and 60- um to serve as standards run in parallel with sections in all incubation solutions. Slides were incubated in 10 batches.

Staining and histochemistry

To react for COX enzyme activity, slides were immersed in 0.1 M phosphate buffer (0.5% glutaraldehyde, pH 7.4) for 5 minutes to affix sections to slides and then placed in 0.1M phosphate buffer (4% sucrose) four times for 5 minutes each. After the phosphate buffer rinse, the slides were incubated in the COX histochemical solution (50mg diaminobenzidine, 20mg cytochrome c, 100mL 0.1 M phosphate buffer (4% sucrose) per 100 mL solution). The solution was agitated and maintained at 37°C for 1.5 hours. After incubation, the sections and homogenates were transferred to 0.1M phosphate buffer (4% sucrose) and 0.1M phosphate buffer for 5 and 10 minutes respectively. The slides were then dehydrated in a series of increasing concentrations of ethanol (50%-75%-95%-three 100% EtOH) followed by immersion in two solutions of xylene, each for 10 minutes and then coverslipped with Permount.

Regions of interest

The regions of interest included regions associated with sensory processing of predator threat and/or motor responses to threat (Dielenberg & McGregor, 2001; McGregor, Hargreaves, Apfelbach, & Hunt, 2004) (Canteras, 2002; Canteras et al., 1997; Cezario et al., 2008). Coordinates of the ROI were obtained from the *Atlas of the Rat Brain* by Swanson (1991) and *Paxinos and Watson (1997).* The anterior –posterior axis was measured relative to Bregma, the intersection of the sagittal and coronal skull sutures. The dorsal ventral position was measured from the ventral surface as if the section is resting on the platform (mm). The medial lateral axis was measured in mm relative to the midline. The control region is the central amygdala (IL), because it is not activated by unconditioned predator odor exposure (Canteras et al., 1997; Masini et al., 2005). Coordinates are listed in Table 5. The guidance on the locations of the PAG was from Paxinos and Watson, 1997.

Image processing and data analysis

The reacted tissue sections were scanned using Epson perfection 4870 Photo. For each scan, three steps from a Kodak Step tablet #2 were measured to ensure that values and scanning parameters were consistent across scans. At least 5 uniformly random samples were collected. Images were calibrated to a known optical density scale (Kodak Step Tablet #2). The homogenate measurements were used to standardize measure values across incubations solutions.

A regression analysis was used to determine the relationship between the mean optical density and the homogenate section thickness (standards). A one-way analysis of covariance was used to observe the group effects for each region of interest with the control region (IL) as the covariate. Using the control region as a covariate in the 1-way ANCOVA will reduce the

influence of individual differences on error and in turn increase the sensitivity to experimental effects.

V.3. Results

Regression analysis of the MOD from 10-, 15-, 30-, and 60 µm-thick homogenate sections revealed a significant linear trend (r=.982, p <.05; see Fig. 12), demonstrating that there was a linear relationship between the amount of COX in the tissue and the MOD.

One-way ANCOVA analyses with experimental condition as the between subjects factor, and the control region as a covariate, were run for each region of interest (CT n =9; ST n=11). In the forebrain and cortex regions, there was a significant effect of the experimental condition for the lateral septum (LS) $F(1,17)=4.233$ (p=.056), and a significant effect for the prelimbic cortex (PL) $F(1,17)=6.032$ (p=.026), and no significance in the dorsal anterior cingulate cortex (dACC) $F(1,17)=2.471$ (p=.13) (Fig. 13A).

In the amygdala regions, there were no group difference for the posteroventral part of the medial amygdala (MEApv) $F(1,17)=0.852$ (p=.37) and basolateral amygdala (BLA) $F(1,17)=0.011$ (p=.916) (Fig. 13B). The defensive motor circuit includes the hypothalamus and periaqueductal gray. In these regions, there was a significant effect for the premamillary nucleus (dorsal) (PMD), $F(1,17)=8.798$ (p=.01), a tendency toward significance for the premamillary nucleus (ventral) (PMV), $F(1,17)=3.638$ (p=.07), and no group differences for dorsomedial part of the ventral portion of the hypothalamus (VMHdm) $F(1,17)=0.17$ (p=.682) and anterior hypothalamic nuclei (AHN) $F(1,17)=0.192$ (p=.667) (Fig. 13C). There were no group differences for two subregions of the periaqueductal gray, the dorsolateral region (dlPAG) $F(1,17)=0.61$ (p=.445) and ventrolateral region (vlPAG) $F(1,17)=0.029$ (p=.867) (Fig. 13D).

V.4. Discussion

Predator odor is a good indicator of close proximity of a predator (D. C. Blanchard et al., 2003; Masini et al., 2005). As a result, rats appear to have evolved to innately fear the dander of predators such as ferrets (Masini et al., 2005). As shown by our previous behavioral data (Experiment 1), the 3-week exposure to unpredictable threats elicited risk assessment behaviors and produced behavioral changes in defensive behaviors. These behaviors are indicative of enhanced neural activity in brain regions associated with predator threat and defense. In the present study, 3 week exposure to repeated unpredictable threats increased COX activity in regions associated with defensive behaviors elicited in response to predator stress (Canteras, 2002; Canteras et al., 2008; Figueiredo, Bodie, Tauchi, Dolgas, & Herman, 2003), but not in regions associated with sensory processing of predator threat. The sustained COX activity may have been restricted to the regions for inhibiting motor programs in order to analyze risk and to regions receiving sensory and higher order information and integrating the information in response to predator threat.

Previous studies have shown that the medial hypothalamic zone is a critical region for expression of innate defensive behavior to both predator and predator odor threat (Canteras, 2002; Canteras et al., 1997). The three key regions associated with the medial hypothalamic defensive system are the anterior hypothalamic nuclei (AHN), ventromedial hypothalamic nucleus, dorsomedial part (VMHdm), and dorsal premammilary nucleus (PMD). Predator and predator-odor presentation that is sufficient to elicit defensive behavioral responses increase c-Fos expression in these regions (Canteras et al., 1997; Masini et al., 2005). The expression of immediate early gene c-Fos is a reliable marker for cellular activation to behavioral and environmental conditions, but dissipates once the stimulus is removed, making it a marker useful only for acute stimuli. To identify areas that change over a longer period, and as a result could be critical in the behavioral

adaptations identified, requires a marker of more sustained changes in neural activity. Cytochrome oxidase in an enzyme linked to ATP synthesis that is up or down regulated as neural activity increases or decreases (M. T. Wong-Riley, 1989). The metabolic COX activity in the PMD was significantly higher for the stress group indicating that this region is responsive to the threat stimuli, with the change most likely driven by the predator odor. Lesions to the PMD lead to a decrease in fear and defensive responses to predator threat (Canteras et al., 1997; Cezario et al., 2008). Cytochrome oxidase histochemistry indicated there were no group differences in metabolic activity in AHN and VMHdm, and therefore a low probability for group differences in neural activity in these regions. Although there was no difference in the AHN, tracing studies have shown that AHN projects strongly to the PMd (Comoli, Ribeiro-Barbosa, & Canteras, 2000). The subfornical region of the lateral hypothalamus (LHAsf), is similar to AHN, as it integrates information from higher order regions and has bilateral projection with the PMD (Goto, Canteras, Burns, & Swanson, 2005). The VMHdm, like the AHN, is another region that is activated in response to a predator and not predator contextual cues (Canteras, Ribeiro-Barbosa, & Comoli, 2001). In previous studies measuring c-Fos, the exposure to either a live predator or predator cues presented in enclosed cages or enmeshed wires where there was no escape for a set amount of time (D. C. Blanchard et al., 2005; Masini et al., 2005). In these studies, predator threat was presented in inescapable or unavoidable conditions, whereas the threat stimuli in the current study were avoidable and escapable. Because of the avoidability, the metabolic activity of the VMHdm and AHN may not have had sustained neural activity in our stress manipulation. Predator odor threat may selectively continuously activate specific regions of the medial hypothalamic zone. Not all areas previously shown to respond to inescapable predator had metabolic plasticity in this study using repeated acutely presented predator odor threat. The PMD has bilateral projections with the VMHdm and AHN and this region works as

an amplifier for hypothalamic neural processing of predator-related cues and is a critical region for anti-predator related behaviors (Canteras, Resstel, Bertoglio, Carobrez Ade, & Guimaraes, 2010; Cezario et al., 2008). Interestingly, there was a tendency for an increase in metabolic activity in the PMV, another subregion of the hypothalamus, for the stress group. It is associated with exposure to aggressive conspecific threat (Gross & Canteras, 2012; Silva et al., 2013). In the present study, the stress group may have been exposed to aggression displayed by conspecifics' reactions to the predator odor threat. Conspecific alarm pheromones (Kiyokawa, Shimozuru, Kikusui, Takeuchi, & Mori, 2006), possible ultrasonic vocalizations, and defensive behaviors towards the predator threat may have produced sustained activity in the PMV. Together, the regions of the hypothalamic network may integrate information from higher order regions and coordinate an output of motor response behaviors.

The medial hypothalamic defensive system receives inputs from cortical areas such as the prelimbic (PL), infralimbic (IL) cortex, and dorsal anterior cingulate cortex (ACC). There were group differences in the PL which has is also affected by chronic uncontrollable 6-hour restraint stress (Radley et al., 2004) and chronic unpredictable stress (Cerqueira, Mailliet, Almeida, Jay, & Sousa, 2007). These stress manipulations included unpredictable timing of the stress presentation. In the current study, the PL may have also been consistently activated due to the chronic unpredictable nature of the stimuli. There were no group differences in the IL, which may be associated in response to systemic stressors (Crane, Ebner, & Day, 2003). Previous studies have shown that the IL may play a regulatory role on the HPA axis. When IL was lesioned, the HPA axis increased in response to a physical stressor of an immune challenges but not to psychological auditory stress (Crane et al., 2003). Our stress manipulation did not involve direct harm, but instead was restricted to psychological stress. The stress group was consistently risk assessing and avoiding the stress

threat location indicating that psychological stress alone was sufficient to produce the behavioral changes. The stress group assessed risk and avoided the stress location but was motivated to cross when necessary to obtain resources. The dACC is a region critical for assessing risk and reward for reinforcement guided behavior (Walton, Croxson, Behrens, Kennerley, & Rushworth, 2007) and was leaning towards significance with higher activity in the stress group. This indicates that the increased metabolic capacity in these cortical regions may be resulting from the approach/avoidance conflict arising from the need to forage for resources in the face of unpredictable threat.

Defense behaviors are modulated by context, which is controlled in part by the lateral septum (D. C. Blanchard et al., 2005), and areas with a tendency toward higher metabolic capacity in the threat group. Contextual learning is expected in this living environment due to the specific location of the stress stimuli presentation. The lateral septum located in the forebrain region may have a top-down modulatory role on aggressive behavior (Roeling et al., 1994) and anxiety (Hakvoort Schwerdtfeger & Menard, 2008) through its interconnections with the hypothalamic areas involved in goal-directed behaviors (Risold & Swanson, 1997; Sheehan, Chambers, & Russell, 2004). LS sends output the hypothalamic defense control structures such as the AHN. Although there was a tendency for more neural activity in the LS for the stress group, lesions to the lateral septum have produced conflicting results on defense behaviors, including hyper-defensiveness (Albert & Chew, 1980) anxiolytic effects, (Menard & Treit, 1996) and less freezing (Albert & Chew, 1980). The LS may have been suppressing aggressive defense behaviors (Albert & Chew, 1980), as the stress group was assessing risk more than aggressively defending themselves. Instead, risk assessment behaviors were increased towards the potential threat location and contextual cues were likely used. We are currently seeking metabolic activation measurements in the hippocampus, which is well-known for

its role in processing context (Smith & Mizumori, 2006), and sends projections to the lateral septum (Risold & Swanson, 1997).

The PMD receives input from the lateral septum and target the dlPAG and vlPAG, which are associated with motor responses to predator odor and potential predator detection. Previous studies have shown PAG c-Fos activation in response to defensive behaviors to acute threat such as live conspecific presentation (Motta et al., 2009), predator threat in an enclosed cage with no escape (D. C. Blanchard et al., 2005; Canteras et al., 1997; Masini et al., 2005), live predator presentation (D. C. Blanchard et al., 2005; Qi et al., 2010) and electrical stimulation in this region invokes defensive behaviors (Depaulis, Keay, & Bandler, 1992; Keay & Bandler, 2001). Lesions of the dlPAG block flight, freezing, and risk assessment behaviors (Gross & Canteras, 2012; Sukikara, Mota-Ortiz, Baldo, Felicio, & Canteras, 2010). Since repeated threat increased the probability of eliciting defense responses, and the duration of defense responses in Experiment 1, regions of the PAG were candidate sites for metabolic effects. However in this study, there were no group differences shown for either defense-related region of the PAG. The findings of PAG c-Fos activation in previous studies may be dependent up specific features of the manipulations; although the manipulations were acute, they were not avoidable or escapable. In contrast, our current study was a foraging environment that included predator threat that was both avoidable and escapable. The dlPAG and vlPAG may have been activated acutely in which c-Fos measures will be able to detect the activity, but the regions may not have sustained neural activity leading to no differences in baseline metabolic activity.

Predator odor has been known to activate the medial amygdala (MEA), and damage to this area leads to reduced defense behavior. Therefore I expected the predator odor in our manipulation to drive MEA cell activity, and therefore increase COX activity (D. C. Blanchard et al., 2005;

Takahashi, Nakashima, Hong, & Watanabe, 2005). The basolateral amygdala (BLA) is a sensory integration region incorporating sensory cues in the environment such as light and sound that may signal for predator or threat (M. Davis, Walker, Miles, & Grillon, 2010). However, there were no differences in MEApv and BLA activation. Instead, it is possible that the MEA and BLA were activated acutely at the time of each stimulus presentation, but the presentations were too transient and infrequent to upregulate COX activity. Similarly, sensory processing of the abrupt lights and sound in the BLA may not have been repeatedly activated for a sustained duration. Without sustained neural activity, COX would not be upregulated. Given the sustained avoidance of, and risk assessment of the central threat location, the failure to find effects at the level of the amygdala are likely to reflect the low frequency and duration of the threat stimuli rather than habituation to the stimuli.

The low frequency and duration of the threat stimuli have ethological relevance, but may limit metabolic plasticity to low levels that are difficult to detect. The unpredictable and abrupt presentation was sufficient enough to produce changes in behavior during and after the 3 week stress manipulation. The stress manipulation and foraging components in this study are multidimensional taking into account the multifaceted dimensions of motivations while facing threat stimuli that are also complex. It is possible that subtle plasticity was distributed throughout a broad network, making it difficult to detect plasticity in areas expected. To overcome this possibility, future studies could explore the possibility of increasing intensity (e.g., two puffs of predator odor) or the frequency of the threat stimuli (e.g., p=.5) within the tunnel. Alternatively, a bigger sample size may be needed in order to detect further changes. Currently, the observed power is .41. We measured regional metabolic capacity, which reflects the whole set of cells and neuropil. While this has the advantage of measuring COX activity in dendrites, the site of

the computational action in the brain, it also includes both excitatory and inhibitory neurons and glial cells. If the plasticity is restricted to neuronal populations, or dendritic compartments, then the inclusion of all cells and cellular compartments could wash out effects. Subregions, such as layers (e.g., cortical layers I to VI, etc.) were difficult to be delineated with this stain. Although the subtlety of stress presentation may not have produced robust metabolic changes, the complexity of the environment and unpredictable repeated threat without harm produced a unique pattern of brain regional differences.

The results from the present study indicate that neuronal activity in PMD, PMV and perhaps LS and PL are altered by the stress manipulation. Chronic threat activated the PL and neural activity may have increased due to the unpredictability of threat leading to chronic anticipation. The PMD and PMV regions are known to integrate defensive behaviors towards threat while higher order regions may modulate the defensive behaviors such as the lateral septum. To understand how these structures might account for the increased probability and duration of burying, and memory deficits, future studies should test for changes in receptor density or synapse numbers. Specific neurotransmitter receptors may have been up or down-regulated in response to the continued repeated threat exposure. The risk assessment behaviors are associated with increased attention and arousal to threat location. Future studies should also measure neurotransmitters associated with arousal such as noradrenaline and norepinephrine (NA/NE). These measures will provide more sensitive measures of plasticity and neurobiological signals associated with the behavioral changes and response to the unpredictable threat stimuli.

Unpredictable environmental conditions shape behavioral tendencies towards enhanced attention and threat detection (Neuberg, Kenrick, & Schaller, 2011; Stein & Nesse, 2011). While

quickly identifying threatening cues may be advantageous, the heightened threat anticipation produces inability to appropriately monitor and respond to ambiguous cues in a dynamic environment. The current study provides evidence suggesting that repeated threats create changes in neural activity that drive metabolic plasticity to repeated unpredictable threat. Unpredictable environments and chronic exposure to threat may be key factors that shape information processing, such as hyper-attentiveness and expectancy to potential risk. The PMD integrates motor response to predator threat and expression of defensive behavior (Canteras et al., 1997; Cezario et al., 2008). It also received input from the LS that modulates the defensive responses (Albert & Chew, 1980). The increased risk assessment behaviors are indicative of increased attention towards the threat location (Kavaliers & Choleris, 2001) that is modulated from the LS and behaviors need to be inhibited in order to assess risk (Canteras et al., 2010). These regions may be candidate sites of the plasticity that underlie the potentiated defensive burying measured in our previous study. After the cessation of the stress manipulation, the stress group had a higher proportion of animals that engaged in defensive burying and also increased defensive burying to potentially threatening stimuli. This indicates potentiated defensive behaviors to threat. It is intriguing that the LS, known for inhibiting aggressive and defensive behaviors, had increased metabolic activity. The stress group did not display aggressive and defensive behaviors to the threat location. Instead, risk assessment behaviors were significantly displayed throughout the 3 week stress manipulation. The unpredictable stress stimuli produced heightened vigilance and increased threat expectancy that led to later hyperreactivity to potential threat. There was a higher proportion of the stress group that defensively buried and for longer duration; this indicates that there may be a lower threshold for generating defensive behaviors and hyperreactivity to potential threat.

The heightened threat expectancy, hypervigilance, and hypperreactivity are common subset of symptoms associated with anxiety disorders such as Generalized Anxiety Disorders (GAD) (Grupe & Nitschke, 2013) and PTSD (Adenauer et al., 2010; Shalev et al., 2000). Here, we have repeatedly presented threatening stimuli that were not paired with harm, stimuli that in other studies (Experiment 1) were capable of driving subfeatures of these disorders, including hyper-reactivity, which is common to GAD and PTSD. In other studies, not yet published, the same stimuli produced evidence for hyper-vigilance. Testing for differences in metabolic capacity provides the opportunity to survey the brain for regions that have likely had sustained changes in neural activity (M. T. Wong-Riley, 1989), and therefore regions that are associated with hyper-reactivity and hyper-vigilance. The unpredictable threat environment drove these behavioral and metabolic changes. Increased probability of defensively burying a highly novel stimulus may be driven by higher threat expectancy that is processed in higher order regions such as the PFC as it processes multidimensional information from the environment such as unpredictability of threat. The prefrontal cortex (M. Davis, 1992) is involved in the top-down control mechanisms involved with association, attention, and interpretive processes (Bishop, 2007) that can contribute to anxiety (Grupe & Nitschke, 2013).There was no prediction of threat presentation leading to consistent attention and vigilance. This enhances threat expectancy (Grupe & Nitschke, 2013) and drives later generalized behaviors to potential threat (Grupe & Nitschke, 2013; Nelson & Trainor, 2007). Hence, the stimuli may not be intense or explicit, but the threat expectancy may be heightened due to chronic unpredictable threat exposure (lower threshold), leading to increased defensive responses or hypperreactivity.

In summary, to identify sites activated by threat, I measured metabolic capacity with COX histology. COX activity reflects long-term energy demands, which changes in response to

altered sensory input (M. T. Wong-Riley, 1989). After repeated threats, specific regions of the medial hypothalamic zone, particularly the PMD, had increased basal metabolic activity in the stress group. The PMD integrates motor response to predator threat and expression of defensive behavior (Canteras et al., 1997; Cezario et al., 2008). It also received input from the LS that regulates the defensive responses (Albert & Chew, 1980). Specifically the LS may inhibit defensive behavior. No defensive behaviors such as attack were displayed in the tunnels, but risk assessment behaviors were displayed continuously towards the threat location utilizing contextual cues. Cortical regions, the IL and PL, send projections to areas of the medial amygdala and BLA, respectively (Vertes, 2004). The IL may be more involved in systemic or physical stressors (Crane et al., 2003) and PL with chronic psychological stressors (Cerqueira et al., 2007). The two subregions of the amygdala did not have increased metabolic activity, possibly due to the transient presentation of threat. Yet the stress group continued to avoid the threat location and risk assessment behaviors were maintained throughout the 3 week condition. The pattern of behaviors and metabolic activity is associated with the unpredictable presentation of threat.

Table 5. Regions of Interest

Region	Bregma	Distance from platform (mm)	Lateral from the midline (mm)
Forebrain Cortex	+1.70	90-175mm	3-17mm
Lateral Septum (LS)	+1.45	98-150mm	3-35mm
Lateral Septum (LS)	+1.20	110-164mm	5-38mm
Lateral Septum (LS)	+0.95	118-260mm	5-41.1mm
Lateral Septum (LS)	+0.45	120-189mm	5-40mm
Lateral Septum (LS)	+.10	123-197mm	8-34mm
Lateral Septum (LS)	0.00	123-198mm	10-48mm
Lateral Septum (LS)	-0.11	146-201mm	24-47mm
Lateral Septum (LS)	-0.26	143-201mm	28-53mm
Lateral Septum (LS)	-0.46	130-205mm	25-51mm
Prelimbic cortex (PL)	+4.85	144-183mm	6-28mm
Prelimbic cortex (PL)	+4.20	128-196mm	5-32mm
Prelimbic cortex (PL)	+3.60	133-184mm	7-35mm
Prelimbic cortex (PL)	+3.20	145-190mm	6-35mm
Prelimbic cortex (PL)	+2.80	157-180mm	7-36mm
Prelimbic cortex (PL)	+2.15	155-179mm	15-30mm
Infralimbic cortex (IL)	+3.20	110-144mm	6-35mm
Infralimbic cortex (IL)	+2.80	120-152mm	7-36mm
Infralimbic cortex (IL)	+2.15	125-155mm	15-30mm

Infralimbic cortex (IL)	+1.70	173-180mm	12-21mm
Anterior cingulate cortex (dorsal) (dACC)	+1.70	203-220mm	7-37mm
dACC	+1.45	203-223mm	20-42mm
dACC	+1.20	210-228mm	9-48mm
dACC	+0.95	213-235mm	10-40mm
dACC	+0.45	218-238mm	8-38mm
dACC	+.10	227-248mm	7-35mm
dACC	0.00	235-252mm	8-35mm
dACC	-0.11	235-260mm	7-35mm
dACC	-0.26	240-260mm	6-35mm
dACC	-0.46	240-258mm	6-30mm
dACC	-0.51	245-255mm	6-30mm
dACC	-0.83	245-258mm	6-30mm
dACC	-1.05	250-265mm	6-25mm
Anterior cingulate cortex (ventral) (vACC)	+1.70	180-203mm	7-37mm
vACC	+1.45	182-202mm	10-42mm
vACC	+1.20	210-228mm	9-48mm
vACC	+0.95	213-235mm	10-40mm
vACC	+0.45	218-238mm	8-38mm
vACC	+.10	227-248mm	7-35mm

vACC	0.00	235-252mm	8-35mm
vACC	-0.11	235-260mm	7-35mm
vACC	-0.26	210-240mm	6-35mm
vACC	-0.46	215-240mm	6-30mm
vACC	-0.51	215-246mm	6-30mm
vACC	-0.83	215-245mm	6-30mm
vACC	-1.05	220-250mm	6-25mm
Hypothalamic areas			
Anterior hypothalamic nuclei (AHN)	9.11	55-72mm	16-37mm
AHN	9.31	53-94mm	12-43mm
AHN	9.47	55-88mm	10-46mm
AHN	9.67	60-84mm	13-43mm
AHN	9.85	68-86mm	16-31mm
Ventromedial Hypothalamus (dorsal medial) VMHdm	-2.00	56-73mm	10-21mm
VMHdm	-2.45	74-94mm	14-34mm
VMHdm	-2.85	76-90mm	8-26mm
Premammillary nucleus (dorsal) (PMD)	-3.90	89-98mm	24-32mm
PMd	-4.20	51-67mm	5-27mm
Premammillary nucleus (ventral) (PMV)	-3.90	39-56mm	18-46mm

PMv	-4.20	43-50mm	12-23mm
Amygdala			
Medial Amygdala (posteroventral) MEApv	-2.45	35-50mm	83-108mm
MEApv	-2.85	68-92mm	84-101mm
Basolateral Amygdala (BLA)	-1.33	55-70mm	140-155mm
Basolateral Amygdala (BLA)	-1.53	43-80mm	135-150mm
Basolateral Amygdala (BLA)	-1.78	42-79mm	155-170mm
Basolateral Amygdala (BLA)	-2.00	39-80mm	140-168mm
Basolateral Amygdala (BLA)	-2.45	39-60mm	130-170mm
Basolateral Amygdala (BLA)	-2.85	47-67mm	130-174mm
Basolateral Amygdala (BLA)	-3.25	37-65mm	150-170mm
Basolateral Amygdala (BLA)	-3.70	30-55mm	160-175mm
Periaqueductal gray			
Periaqueductal gray (dorsolateral) (dlPAG)	-7.64	115-124mm	6-23mm
dlPAG	-7.80	106-126mm	7-21mm
dlPAG	-8.00	105-123mm	7-23mm
Periaqueductal gray (ventrolateral) (vlPAG)	-8.72	87-109mm	3-22mm
vlPAG	-7.80	85-105mm	3-22mm
vlPAG	-8.00	91-106mm	5-25mm

Figure 10

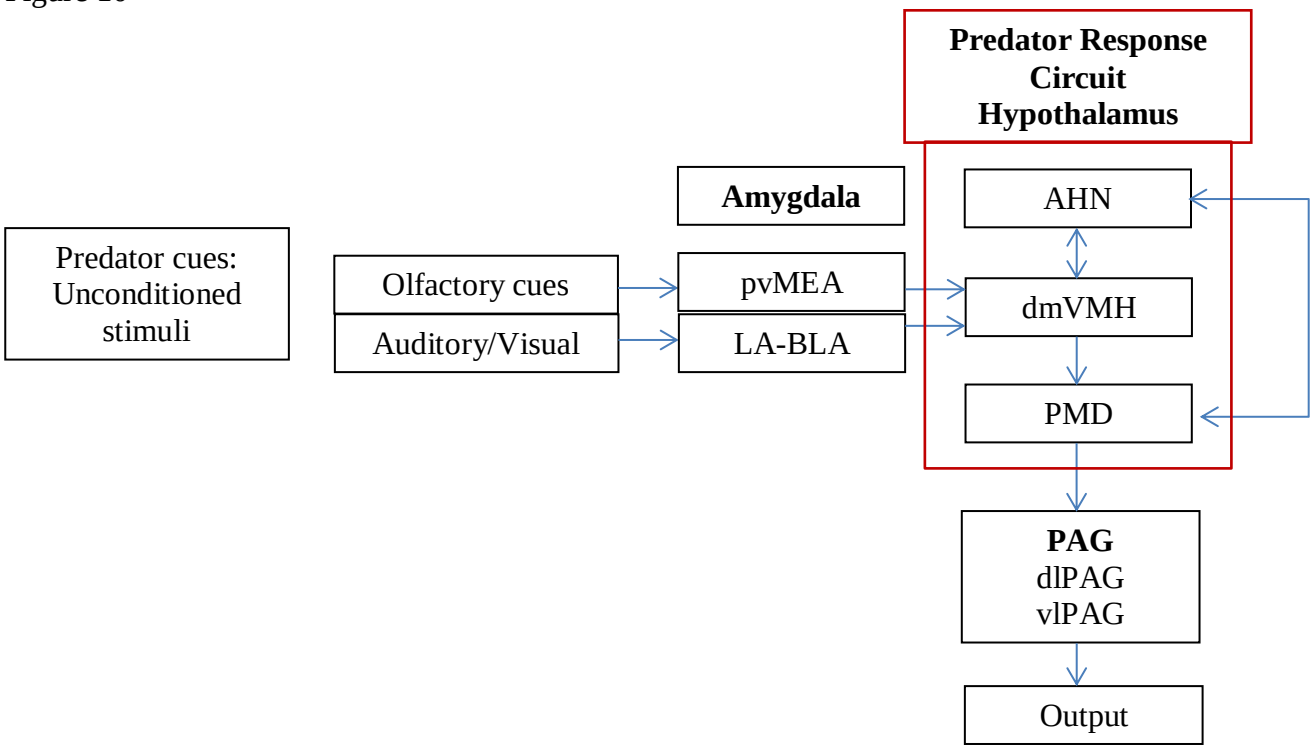


Figure 10. Predator threats activate subregions of the amygdala. The circuitry involves the predator response pathway of the hypothalamus. The dorsal premammillary nucleus (PMD) sends input out the dorsolateral part of the PAG (dlPAG). pvMEA, posteroventral part of the medial amygdala; LA, lateral amygdala; BLA, basolateral amygdala; AHN, anterior hypothalamic nucleus; dmVMH, dorsomedial part of the ventralmedial hypothalamus; vlPAG, ventrolateral part of the PAG.

Figure 11

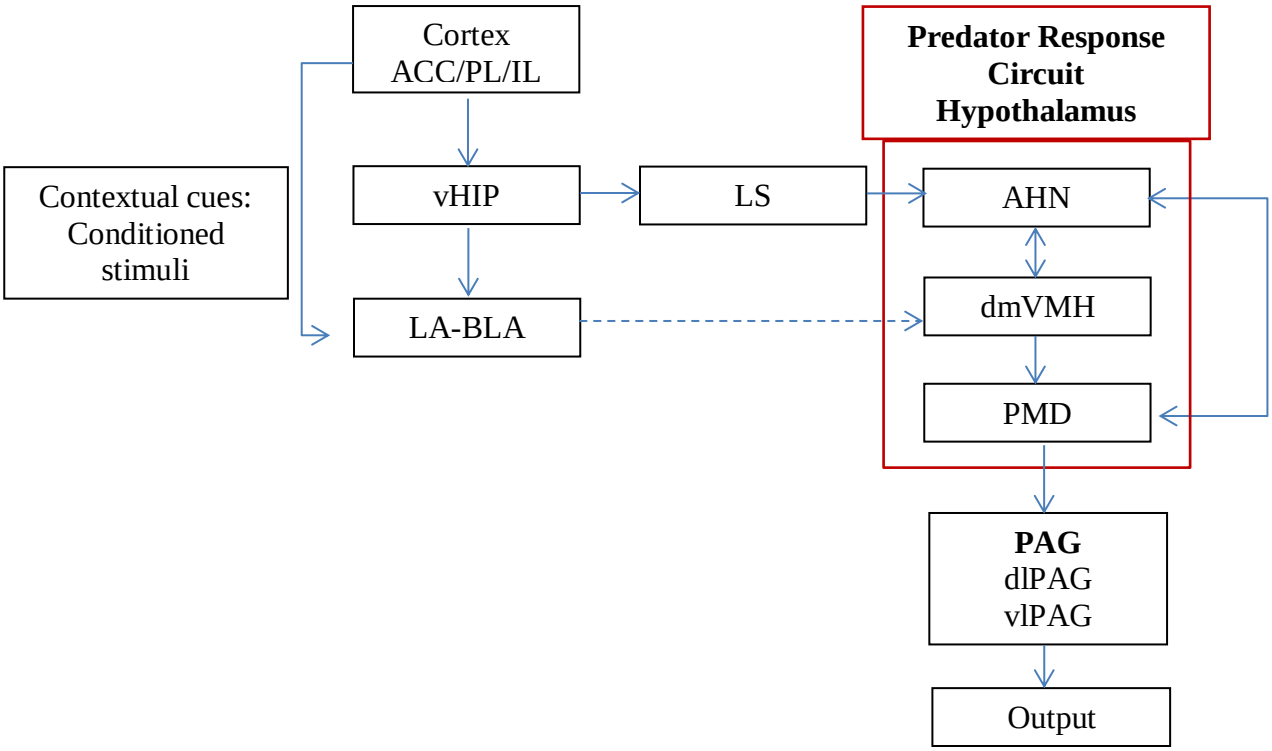


Figure 11. The cortex and hippocampus are involved in higher order processing of contextual cues. ACC, anterior cingulate cortex; PL, prelimbic cortex; IL, infralimbic cortex; vHIP, ventral hippocampus; LA, lateral amygdala; BLA, basolateral amygdala; AHN, anterior hypothalamic nucleus; dmVMH, dorsomedial part of the ventralmedial hypothalamus; dlPAG, dorsolateral part of the PAG; vlPAG, ventrolateral part of the PAG.

Figure 12

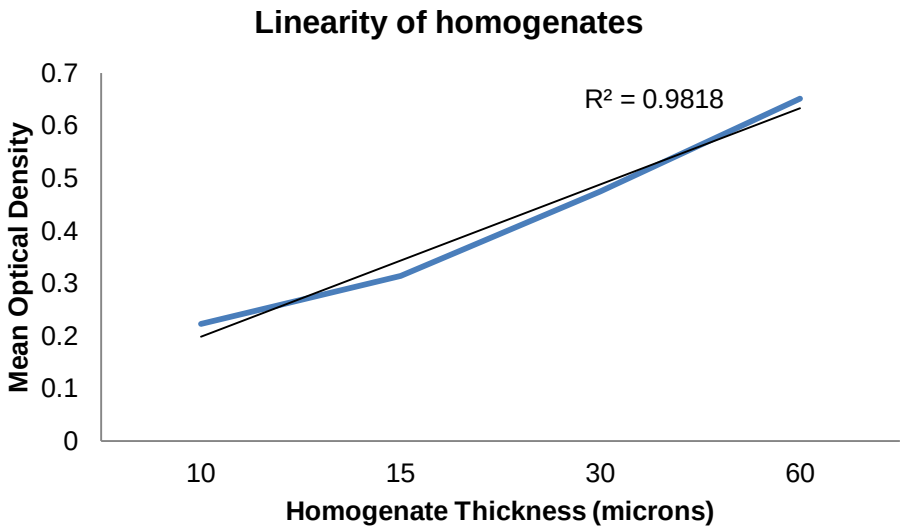
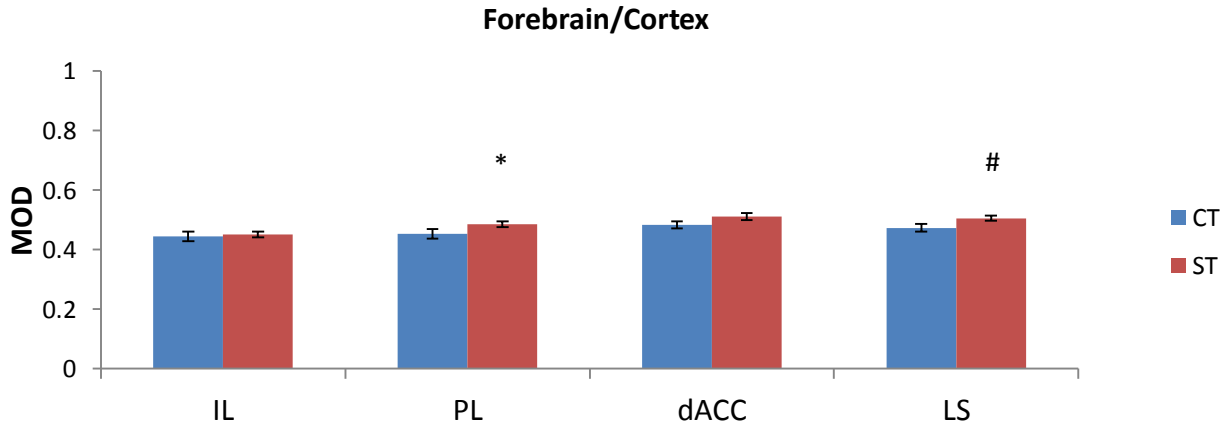


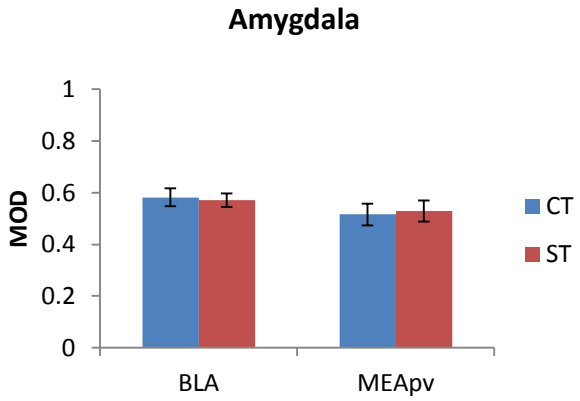
Figure 12. Regression analysis of the Mean Optical Densities (MOD) from 10-, 15-, 30-, and 60 µm-thick homogenate sections revealed a significant linear trend (r=.982, p <.05).

Figure 13

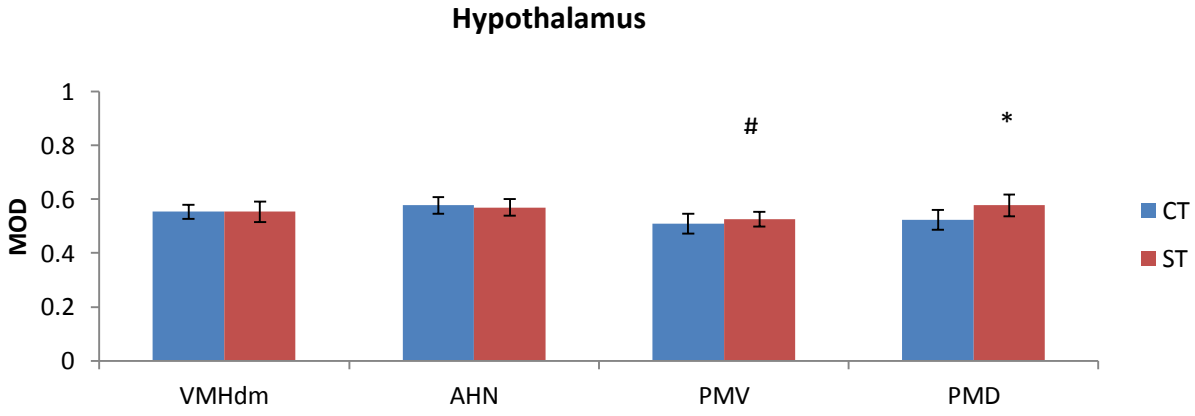
A



B



C



D

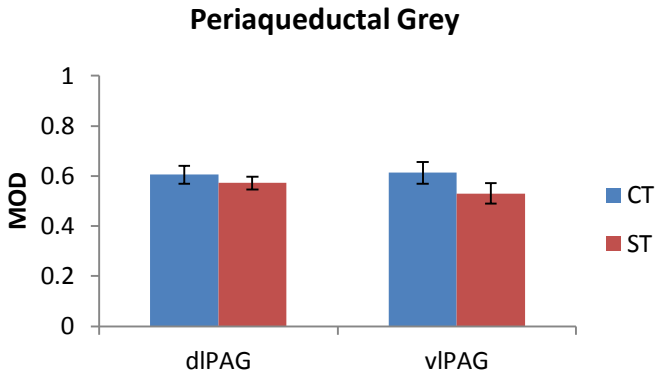


Figure 13. Mean optical densities (MOD) were measured for each region of interest. (A) Forebrain/Cortex, there was a group difference for the prelimbic cortex (PL) and a tendency towards significance for the lateral septum (LS) (B) Amygdala, there were no group differences in either the basolateral (BLA) or posteroventral part of the medial (MEApv) of the amygdala (C) Hypothalamus, there was a group difference for the dorsal premammillary nucleus (PMD) and a tendency towards significance for the ventral premammillary nucleus (PMV) and (D) Periaqueductal Grey, there were no group differences in either the dorsolateral PAG (dlPAG) or ventral lateral PAG (vlPAG).

Chapter 6

Conclusions and Implications

In humans, stressors are often psychological and anticipatory in nature rather than directly harmful. Current animal models utilize random exposure to adverse conditions, but they rely on physical challenges (i.e. foot shock and food deprivation) to induce psychological stress. In order to understand the consequences of chronic psychological stress, we developed a rodent model of psychological stress that restricts stressors to those environmental factors that induce only the anticipation of stress and avoid direct challenges to physiology. Predator odor indicates impending threat that is a direct precursor for potential physiological challenge (D. C. Blanchard, Blanchard, & Rosen, 2008; D. C. Blanchard et al., 2011; Takahashi et al., 2005). This model is the first to utilize chronic psychological stress stimuli in a foraging environment, providing an ethologically relevant context while excluding direct challenges to homeostasis.

The studies conducted aimed to identify the behavioral and metabolic adaptations to unpredictable threat. I hypothesized that chronic exposure to unpredictable threat, without the presentation of harm, should increase risk assessment behaviors during the manipulation. There was significant avoidance of the center confirming the aversive nature of the stimuli and decreased foraging behavior, measured as crossings through the potentially threatening location. In contrast, the control group did not reduce their crossings. The stress group entered the tunnels at frequencies similar to the control group, but did not cross the center location and to the opposite side, suggesting that they were monitoring and assessing the tunnel. The unpredictable threat stimuli presentations were effective in reducing crossings and the persistence of the

reductions demonstrates that there was no habituation to the stimuli. The rats in the unpredictable threat condition risk assessed and approached the potentially harmful center location of the stimuli in order to traverse and obtain resources at the other end, consistent with earlier reports that abrupt and non-specific stimuli elicit vigilance and risk assessment behaviors that serve to detect predators and harm (Bednekoff & Lima, 1998; Kavaliers & Choleris, 2001). Rather than avoid the central location altogether, the rats in the threat condition faced risk in order to obtain resources crucial for survival. The unpredictable threat was highly ambiguous in terms of its actual presentation and harm. Preparatory responses such as vigilance were increased in the anticipation that the threat could be an actual danger. In the current living environment, the unpredictability of threat shifted behavioral tendencies towards greater attention and assessment towards the threat location. After the cessation of the 3 weeks stress manipulation, the adrenal glands were weighed for a subset of animals. The stress group had higher adrenal gland weight compare to the control suggesting increased HPA axis response to the chronic stress manipulation. The avoidance of the central stimuli, increased risk assessment, and adrenal gland weight all served as evidences of an effective model of chronic unpredictable threat stress.

To develop a model that induces only psychological stress requires exclusion of direct harm or deprivation. There were no differences in weekly measures of food/water consumption indicating that the stress group crossed just enough to obtain necessary resources and avoid significant challenges to homeostasis. The ability to maintain body weight with fewer crossings also signifies that the stress group sufficiently crossed to obtain resources while the control group may have crossed more than necessary. There was no evidence that sleep cycles were altered. Both groups crossed the tunnel more in the dark than light period indicating a similar circadian

pattern. The unpredictable threat stress provided only the anticipation of harm with no direct challenges to homeostasis.

Unpredictable environmental conditions can shape behavioral tendencies towards enhanced attention and threat detection (Neuberg et al., 2011; Stein & Nesse, 2011). The current stress condition used in the study consisted of unpredictable threats that contributed to uncertainty. The anticipatory responses occur under conditions of threat uncertainty and are common subset of symptoms associated with anxiety disorders (Grupe & Nitschke, 2013). The five key processes that are likely contributors to responses under uncertainty, or anxiety, are the following: inflated estimates of threat cost and probability, hypervigilance, deficient safety learning, behavioral and cognitive avoidance, and heightened reactivity to threat uncertainty that forms the uncertainty and anticipation model of anxiety (UAMA) (Grupe & Nitschke, 2013). Inflated estimates of threat cost and probability includes biased assessments which lead to heightened threat expectancies (Grupe & Nitschke, 2013; Mogg, Mathews, & Weinman, 1989). Conditions of uncertainty create increased estimates or heightened threat expectancies (Grupe & Nitschke, 2013). Although the current study did not measure inflated estimates of threat cost, heightened threat expectancies may have also be driven by increased attention and vigilance towards potential threat. Increased attention was measured by risk assessment behaviors including headpokes from the tub cages and stretched-attends (front limbs moving close to the threat location while hind legs are stretched back for quick retraction). These behaviors were maintained throughout the 3 week manipulation and suggest increased threat attention, vigilance, and heightened threat expectancy.

Conditions of uncertainty increased threat expectancy, attention and vigilance towards the potential threat which may drive heightened reactivity to threat uncertainty. After cessation

of the treatment conditions, rats were tested for defensive behaviors as measures of reactivity to threat uncertainty. The stress group, when exposed to ferret dander odor spent more time burying the rag, demonstrating the sustained power of predator odor to induce threat. To test whether the increased burying to the ferret odor was driven by sensitization of the odor or a generalization to any potential threat, a highly novel stimulus was used in a different set of rats. When exposed to the highly novel stimulus a higher proportion of the stress group buried, and for those animals that buried, the stress group spent more time burying. Overall, the stress group exhibited greater likelihood and intensity of defensive responding. These behaviors function to remove and protect the organism from danger but also prepare the organisms for an actual direct defense response when escape is not possible. Active defensive behaviors, such as fighting and burying are displays of aggression (Gilbert, 2001). Aggression can take the form of reactive or motivated aggression (Gilbert, 2001; Nelson & Chiavegatto, 2001; Neumann, Veenema, & Beiderbeck, 2010). Reactive aggression reflects impulsivity including heightened and sustained aggressive responses once provoked (Nelson & Chiavegatto, 2001). Whereas, motivated or instrumental aggression is considered to be more controlled and proactive which includes planning and preemptive strikes (Gilbert, 2001; Nelson & Chiavegatto, 2001). Reactive aggression occurs as a response to harm while motivated aggression is displayed to the expectation of harm. However, these two types of aggression should not be seen as two separate categories at opposite ends of the spectrum. Rather, aggression should be viewed as a continuum. Heightened anticipation for threat may provoke reactive-like aggression as if there is an actual stimulus. Defensive burying, in particular, is a behavior intended to spray dirt or sand in the face of a competitor (R. J. Blanchard & D. C. Blanchard, 1989b), a clear act of reactive aggression. If defensive behavior is intended to pre-empt an attack, then it falls into the category

of motivated aggression. However, it is elicited in close proximity to threat, suggesting high arousal conditions, and therefore has characteristics closely linked to impulsive aggression. There was a tendency for a higher proportion of the stress group to engage in burying and sustained burying, reflecting both motivated and reactive aggression, respectively. In this study, both the ferret odor and motor stimuli were threats and not actual direct harms, yet the stress group displayed proactive, motivated aggression with preemptive strikes (burying). The stress group also had sustained duration of the burying response indicating higher reactive aggression although they were not provoked. Increased expectation of threat produced both reactive and motivated aggression to the potential threat.

Due to conditions of uncertainty, both increased threat attention/vigilance and heightened threat expectancy may have also been driven by deficient safety learning (Grupe & Nitschke, 2013). After removal from the unpredictable threat condition, the stress group had increased startle response to acoustic stimuli after repeated sessions (4 sessions) (manuscript in preparation). Although there were no differences between the 2 groups during the first session consisting of 30 trials of acoustic stimuli spaced 30 seconds apart, the stress group increased startle responses during sessions 2-4 compared to the first session while the control group had a tendency for decreased startle amplitude during sessions 2-4 compared to the initial session. This indicates that while the control group habituated to the acoustic stimuli (no harm), the stress group had impaired safety learning and perhaps even sensitized to the acoustic stimuli. Both increased threat attention and heightened threat expectancy may cause sensitization. The current stress condition may have impaired safety learning leading to increased threat attention and expectancies.

For many mental health disorders, such as anxiety, the cognitive and behavioral symptoms are shifts in normal cognition and behaviors. Therefore, we asked whether the behaviors in the tunnel might generalize to other conditions. In the unpredictable threat condition, the stress group approached the center location of the threat stimuli, but generally inhibited further movements from crossing the location; daily crossings were sparse. There were behavioral avoidance and escape from the threat location. After the cessation of the stress manipulation, three standard tests of anxiety by measuring avoidance were employed. There were no differences in the Elevated Plus Maze (EPM). Within two open arms and two closed arms, rodents will generally restrict most of their activity to the closed arms (D. Treit, Engin, & McEown, 2010) indicating passive coping (Koolhaas et al., 1999). Two other tests were employed in the Open Field (OF) Box with different set of animals, so that no one animal was tested in both Open Field Test and Novelty Suppressed Feeding Test (NSF). As standard protocol, latency and mobility in the Open Field Test were measured. There was a tendency for the stress group to have higher latency to enter the middle region of the Open Field. This may suggest avoidance, but the stress group may have had higher risk assessments prior to entering the middle and future studies will investigate risk assessment behaviors in the Open Field test. In the second study using the Open Field in the NSF, food was placed in the center of the box and latency and mobility were measured. There were no differences in latency to enter the middle, but the stress group had a tendency for higher mobility. This may indicate that food provides a motivating factor for mobility and exploration. Passive coping is not indicted by measuring avoidance in the three standard tests of anxiety. The lack of effect cannot be explained by methodological flaws of one test. Although avoidance is a subset of symptoms

associated with anxiety, the current unpredictable threat manipulation did not create generalized avoidance when tested in later conditions.

Our current study was able to restrict stressors to those environmental factors that induce only the anticipation of stress and avoid direct challenges to physiology. There was also a degree of control in that the stress group was able to avoid the center location and transverse only when resources were needed; the stress group was also able to escape quickly to the other end of the tunnel leading to more control. Previous studies that use chronic variable stress (Katz et al., 1981; Papp et al., 1991) utilize random exposure to adverse conditions as unpredictable conditions, but they rely on physical challenges (i.e. foot shock and food deprivation) to induce psychological stress. Furthermore, there is no level of control in regards to avoiding the stressful stimuli. Controllability may be a critical factor in determining the gradient between passive coping and active coping strategies. After the cessation of the stress manipulation in the current study, the stress group did not respond differently than their matched controls when tested for sucrose preference and immobility in the forced swim test. Hence, there were no differences in passive coping behaviors associated with depression, measured by anhedonia and immobility, respectively. The results suggest that a high degree of control in conditions of threat do not produce symptoms associated with depression.

Although the potentially aversive environment enhanced active coping of defensive and aggressive behaviors which are advantageous to the organism's survival, there was a deficit in learning during higher cognitive tasks such as working memory. The current studies confirm that psychological stress in the form of unpredictable threats without harm is sufficient to produce behaviors associated to potential harm, but at a cost with spatial working memory impairment. The condition produced both acute and long term behavioral shifts to potential

threat. Many rodent models of stress include both physical and psychological stressors, yet deficits, like those reported for working memory (Mika et al., 2012; Wright & Conrad, 2005), are often attributed to psychological stress, because it is presumed to last longer. Our model of chronic psychological stress without physical challenge produced deficits in spatial working memory in the Barnes maze as measured by the latency to find the goal box. Also the stress group accumulated a greater number of both repeat and non-repeat errors within trials. Groups did not differ in motivation to find the goal box as measured by locomotor or exploratory activity in the Barnes maze. Another exploratory behavioral measure was obtained through the Open Field Box test, which is a novel environment for both stress and control groups. There were no differences in locomotor behavior in the Open Field Box, but there was a tendency towards a difference in mobility when food was available as a motivating factor. The data suggest that our model produced chronic psychological, but not physical stress that led to impairments in spatial working memory, but no differences in locomotion or exploration.

Spatial working memory was tested in a relatively neutral, non-aversive environment in the Barnes Maze as compared to more aversive apparatuses such as the Morris Water Maze (MWM) and Radial Arm Maze (RAM) using food reinforcement. The water maze engages the animals to swim and energy is expended in order to find a platform hidden in one of the 4 quadrants. The memory task may be confounded by the energy demands. The RAM uses food reinforcement which is also confounding when testing for memory processing and not motivational conflicts. When the test apparatus is stressful, chronic stress may produce no impairments or better performance in the memory task (Conrad, 2010). The task indicates an inverted-U shaped function reflecting the relationship between the degree of stress and performance and memory (Conrad, 2008; Roozendaal, 2002). The inverted-U shaped function

suggests an optimal peak performance relating to an optimal degree of stress and arousal (see Fig 14). Too little (left of x-axis) or too much stress (right of x-axis) will impair performance and memory. The current working memory impairments may reflect chronic stress effects on altering hippocampal sensitivity to corticosterone such as downregulation of receptor density. If corticosterone release is low in a neutral test condition, then there is less binding of corticosterone receptors, and impaired working memory. The neutral Barnes Maze condition may have been less stressful leading to less corticosterone release. With less corticosterone release, there is less binding which impaired performance for the stress group. Hence, the stress group is left of the optimal inverted-U shaped curve while the control group may have performed on the peak curve. If the current working memory impairments reflect receptor downregulation due to chronic stress living conditions, then optimal learning should occur in more high stress environments for the stress group. The inverted-U function may be shifted to the right for the stress group where more adverse testing conditions may elevate corticosterone counterbalancing hippocampal receptor deficits with more binding potential (Conrad, 2008). This will allow for optimal performance for the stress group as opposed to performance in the neutral condition.

Spatial working memory also requires flexible learning including strategizing and identifying contingency-outcome associations, in which both are time-intensive. Other studies have shown that stress may alter preferences for habit learning (Schwabe et al., 2008) instead of flexible learning dependent upon brain regions such as the PFC (Arnsten, 2009). The preference may adjust in response to threatening environments that require rapid responses, such as hard-wired defensive responding or habits (Oitzl, Champagne, van der Veen, & de Kloet, 2010). This is in line with our previous finding that the stress group had increased defense behaviors. If, these types of behaviors are prioritized, then flexible learning processes may suffer just as we

saw with the impairments in spatial working memory. These shifts in behavior and rapid response learning may be adaptive in unpredictable, high threat environments but may be maladaptive in safe environments. Future studies will test for working memory performance in high stress environments. If selective prioritization has occurred in the stress group, then the stress group should perform poorly in a spatial task even when tested in high stress conditions. In contrast, if the rats adjusted for optimal performance in high stress conditions, the stress group should perform better in a spatial task when tested in high stress conditions.

In order to detect the neuroplasticity produced from behavioral shifts, basal metabolic activity was measured in brain regions associated with predator threat. Sustained behavioral changes and neural activity associated with anti-predator/threat behaviors increased metabolic capacity. The corresponding brain regions associated with top down modulatory roles in goal and reinforcement oriented behaviors, such as the lateral septum, and regions associated with defensive behaviors, such as the dorsal premammillary nucleus (PMD), maintained higher energy capacity. The PMD is a region of the hypothalamus that integrates motor responses to predator threat and the expression of defensive behavior (Canteras et al., 1997; Cezario et al., 2008). Although the location of threat was predictable, the timing of the stimuli presentation was unpredictable, and the animals had to cross the threat location in order to obtain resources. There is a conflict between the need to approach the resources while avoiding and risk assessing the center location eliciting arousal and attention. The LS modulates and inhibits defensive motor behavior of the PMD and is reflected as increased metabolic activity due to neural activation. Both regions may have sustained activation indicated by the increased risk assessment while some defensive behaviors may have been inhibited with increased avoidance. The unpredictability of the threat presentation produced chronic activation of the prelimbic

cortex (PL) which is involved in top down modulatory roles on lower brain regions such as the amygdala and hypothalamus. Predator odor presentations were transient. Therefore, regions of sensory processing such as the amygdala may not have sustained neural activation. Whereas the contextual information for the location of threat (center of the tunnel as opposed to the home tubcages) was pertinent, behaviors associated with risk assessment was sustained throughout the 3 week manipulation to that threat location. The unpredictable threat environment produced a pattern of sustained behavioral and metabolic activity associated with anti-predator behaviors including the simultaneous inhibition of behaviors during resource approach.

Facing uncertainty such as unpredictable threat environments may produce adaptive behaviors to threat such as vigilance and enhanced learning and response to potential risk (McCrory et al., 2011; Pollak, 2008) but may serve as a cost to complex, time-intensive learning. In safe and non-threatening environments, these behaviors are viewed as hyperarousal and hypervigilance, and are symptoms associated with anxiety (Grupe & Nitschke, 2013). It is important to note that adaptive mechanisms likely underlying symptoms of psychopathology (Gilbert, 2001). The current unpredictable threat condition enhances increased attention and vigilance to potential threat during the 3 week manipulation as the potential threats are highly unpredictable in its timing and presentation. After the cessation of the threat condition, there were both increases in threat expectancy and hyperreactivity as measured with defensive burying. There was both a higher proportion of the stress group that engaged in burying. This may reflect a lower threshold of threat expectancy leading to motivated aggression (preemptive striking). And once burying was initiated, the duration of burying was longer than the control group. The current unpredictable threat stress condition is a model that likely produced both motivated and reactive aggression in response to increased threat expectancy.

Just as in rodents, humans face threat or potential harm and modify physiological and behavioral systems to detect and response to danger. Early life stressors that are unpredictable such as emotional neglect, child abuse and maltreatment (Gunnar, Fisher, Early Experience, & Prevention, 2006), witnessing family violence (Graham-Bermann, Howell, Lilly, & Devoe, 2011; Towe-Goodman, Stifter, Mills-Koonce, Granger, & Family Life Project Key, 2012) and neighborhood violence (Blair & Raver, 2012) may lead to heightened levels of vigilance so that the individual can react quickly to threat (Towe-Goodman et al., 2012). This increases threat expectancy bias implicated in anxiety. Threat expectancy is also associated with increased threat attention/hypervigilance, disrupted expected value calculation, heightened reactivity to threat uncertainty, behavioral and cognitive avoidance, and deficient safety learning (Grupe & Nitschke, 2013). Although the current study did not observe indications of generalized avoidance, there are other indicators that may drive symptoms of anxiety such as increased threat expectancy, increased threat attention and vigilance, and heightened reactivity (Grupe & Nitschke, 2013). This suggests that subset of symptoms may drive symptoms of anxiety (GAD). The subset of symptoms parallels other forms of anxiety such as PTSD. Following traumatic exposure, key behavioral symptoms of PTSD include intrusive recollections, persistent avoidant behavior, and hyperarousal (DSM-IV-TR 2000). Hyperarousal is associated with irritability, impaired concentration, hypervigilance, aggression, and exaggerated startle (DSM-IV-TR 2000) and is the first symptom cluster to emerge after a traumatic event (Bremner, Southwick, Darnell & Charney, 1996; Shalev et al., 2000). Although the persistent nature of symptoms is a likely feature of PTSD, it is equally important to note that the cluster of symptoms center upon hyper-arousal. The unpredictable threat manipulation, without trauma, was sufficient to drive clusters

of symptoms that may lead to vulnerabilities to symptoms associated with clinical disorders such as GAD and PTSD.

The current unpredictable feature of the environment possibly increased threat expectancy and threat attention. The increased hypervigilance and threat expectancies may drive the heightened reactivity that potentiated defensive burying, a measure of both reactive and motivated aggression. It seems likely that features of the environment, such as uncertainty, may enhance threat expectancy and increase the likelihood of aggression towards potential threat. To understand the vulnerabilities and risk factors that play key roles in the development clinical disorders, we need to understand environmental living conditions that shift behavioral tendencies. These shifts in behavior may be adaptive in unpredictable, high threat environments but may be maladaptive in safe environments. Future studies should measure adaptive behaviors to specific contexts and identify the parameters that tip the balance from resilience to vulnerability toward clinical disorders.

Figure 14

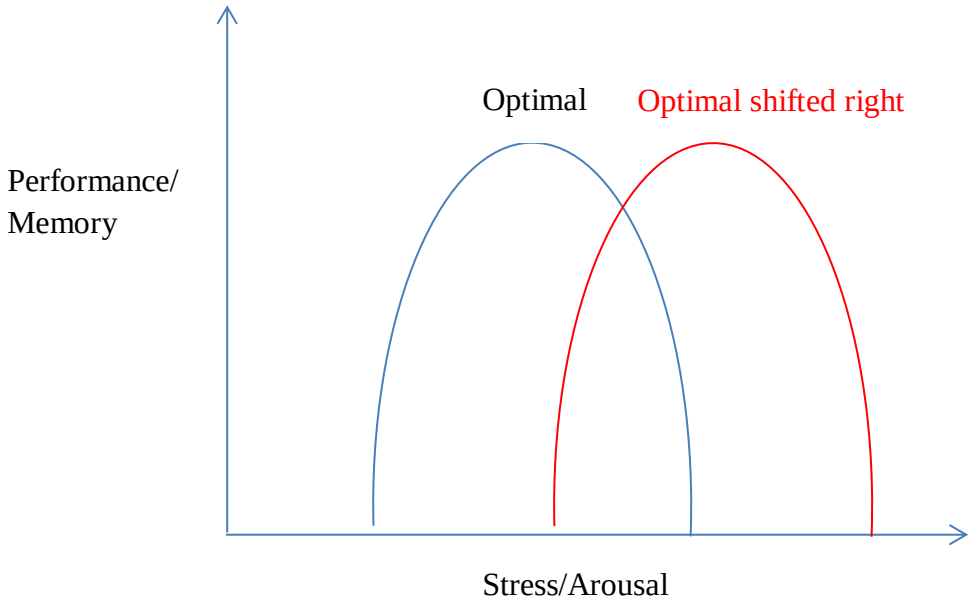


Figure 14. Inverted-U shaped function reflects the relationship between the degree of stress on performance and memory. The optimal peak performance related to an optimal degree of stress and arousal.

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