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Peritraumatic Interventions for Stress Resilience

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by

Traci Nicole Plumb

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ABSTRACT OF THE DISSERTATION

Peritraumatic Interventions for Stress Resilience

by

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University of California, Los Angeles, 2015
Professor Thomas R. Minor, Chair

Exposure to traumatic stress results in a number of physiological and psychological changes that interrupt attempts at active coping with further stress. These changes can lead to disorders such as Post-traumatic Stress Disorder (PTSD) and major depression. The learned helplessness model has long been used to model PTSD and comorbid depression following trauma in animals. Rats given traumatic stress show exaggerated fear responding and escape deficits following re-exposure to a relatively mild stressor 24 hours later. We have hypothesized that they transition during test from an anxious, agitated state to one of conservation-withdrawal, characterized by sensory unresponsiveness, cognitive dullness, and behavioral depression. This dissertation focuses on two methods of building resilience to trauma in the learned helplessness

procedure, as well as elucidates a potential mechanism of action of the conservation-withdrawal state normally observed following trauma.

Chapter One utilizes the concept of hormesis as a resilience-building technique. Hormesis is the process by which small stresses build resilience to large stresses. Rats were exposed to a number of parameters of hormetic stress in an effort to prevent the exaggerated fear conditioning and shuttle-escape deficits normally observed following traumatic stress. We examined stressor severity, pattern of rest, and number of pre-exposure stressors to define the most effective hormetic procedures at eliminating PTSD-like symptoms.

Chapter Two examines a second method of building stress resilience – post-stress glucose consumption. Rats received 18hr access to a glucose cocktail immediately following stress exposure. We found that post-stress glucose is a simple and effective method to prevent the deleterious effects that normally occur following trauma. Glucose eliminated the shift to conservation-withdrawal during shuttle-box testing, facilitated hormetic stress training, and built resilience to multiple traumas.

Lastly, Chapter Three discusses striatal adenosine signaling as a potential mechanism of action of glucose in preventing the transition to conservation-withdrawal during test. We discovered that blocking adenosine A_{2A} receptor activation in the core and shell of the nucleus accumbens before test eliminates conservation-withdrawal. Overall, the evidence suggests that the metabolic reaction to stress is essential to related pathology.

The dissertation of Traci Nicole Plumb is approved.

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2015

DEDICATION PAGE

This dissertation is dedicated to my husband, Nick.

His unwavering love and support made this possible.

And to my son, Scotty.

He brightened even my worst days.

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Portions of Chapter 2 are from a manuscript in preparation for *Stress*. Figures 5-8 are also from this manuscript. The authorship is as follows: Plumb, T.N. & Minor, T.R.

Portions of Chapter 3 are from a book chapter that was published in *Adenosine: A Key Link Between Metabolism and Brain Activity*. Figure 11 is also from this chapter. The full reference is as follows: Plumb, T.N., Sterlace, S.R., Cavanaugh, K.A., & Minor, T.R. (2013) Stress, brain adenosine signaling, and fatigue-related behavioral processes. In A. H. Avouris (Ed.), *Adenosine: A Key Link Between Metabolism and Central Nervous System Activity*. New York: Springer Publishers, pp 535-558.

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INTRODUCTION

Exposure to traumatic stress results in a number of physiological and psychological changes in both human and non-human species (Selye, 1942; see Minor, Huang, and Witt, 2006 for a review). These changes are often deleterious in nature and can endure throughout a lifetime. The factors that make one susceptible to the toxic effects of traumatic stress have been and currently are studied in great detail due to the profound impact of the exposure.

A number of psychological variables modulate the impact of trauma. Prediction and control are central to this analysis as they wield a large influence on the ability to properly adapt to ongoing stress (Seligman, Maier, and Solomon, 1971; Jackson and Minor, 1988; Mineka, Cook and Miller, 1984; Williams and Maier, 1977; Amat, Paul, Zarza, Watkins and Maier, 2006). Several animal models of trauma are regularly used to manipulate the effects of these variables on adaptive coping. One of the most commonly used models is the learned helplessness procedure. The learned helplessness procedure utilizes inescapable electrical shock as a means to induce traumatic stress and examine its impact on subsequent stress. The resulting behavior, termed the learned helplessness effect, mimics a number of symptoms of major depression, including anhedonia, insomnia, psychomotor retardation, fatigue, and anorexia or hyperphagia (American Psychological Association, 2000). As such, the learned helplessness procedure is traditionally used as an animal model of depression (Miller and Seligman, 1975; Overmier and Hellhammer, 1988).

Depression, however, is not typically a singular disorder and is often comorbid with other psychopathologies due to their similar etiologies. The most common of these is Post-traumatic Stress Disorder (PTSD) (Hammack, Cooper and Lezak, 2012). PTSD results from exposure to traumatic stress that is characterized by fear of injury or death (American Psychological

Association, 2000). Resulting symptoms mimic those of major depression. As such, the learned helplessness procedure has morphed into an animal model that more accurately reflects PTSD with comorbid depression (Minor, Plumb, Schell and Pham, 2011).

The Learned Helplessness Effect

The learned helplessness procedure was first described in a set of experiments by Overmier and Seligman (1967) and Seligman and Maier (1967). Three groups of dogs were suspended above the ground in cloth harnesses (also known as Pavlovian harnesses) that allowed each leg to hang below the body through four holes. The first group received escapable shock through electrodes attached to their hind paws. When the shock was initiated, they could terminate shock by pressing panels located on either side of their head, and in doing so, exert control on their environment. The second group received inescapable shock and was yoked to the escapable-shock group, or the master group. When the master dog made his response, shock was terminated for both the master and yoked dog simultaneously. This provided for the same shock onset and duration for both groups. The yoked animals were unable to terminate shock on their own, and exerted no control over their situation. A third group received simple restraint. All groups were tested 24 hours later in the shuttle-escape task. The shuttle-box consisted of two compartments separated by a short barrier. The dimming of the lights was used to signal the arrival of shock to the grid floor. The subjects could jump the barrier during this time to avoid shock. If the avoidance response was not made in time, shock would commence. The subject could then make an escape response by jumping the barrier to terminate shock. If no response was made within 60 sec of shock onset, the shock was automatically terminated.

Test results indicated that previous exposure to inescapable shock greatly impeded shock escape-avoidance responses in the shuttle-box when compared to the restraint group. Exposure to escapable shock during training prevents the shuttle-escape deficits seen in those exposed to inescapable shock; the escapable-shock group performed similarly to restraint controls. The shuttle escape deficits seen following exposure to traumatic, inescapable shock has been termed the learned helplessness effect.

Fear and Helplessness

The discovery of the learned helplessness effect sparked a vast debate as to the etiology of this phenomenon. A large number of biological and psychological theories were presented, each with its own unique take on this effect. The most well-known theory is likely the learned helplessness hypothesis, devised by the seminal experimenters (Maier and Seligman, 1976; Maier, Seligman and Solomon, 1969). This hypothesis uses a complex blend of motivational, emotional, and cognitive processes to attempt to explain the escape deficits seen after traumatic stress. Unfortunately, the evidence supporting the learned helplessness hypothesis has been controversial (see LoLordo and Taylor, 2001), and many alternative theories have been discussed in its place.

Most theorists explain the differential effects of escapable and inescapable shock as a product of controllability. Maier and Seligman (1976) stated that helplessness was due to the uncontrollability of the situation, and since then, situational control became the focus of research on helplessness. While this variable is certainly present in the learned helplessness procedure, closer examination points to another variable that coincides with controllability: prediction. It is extremely difficult to study controllability without prediction; having control over a situation

requires the ability to predict the events that are to be controlled. However, the same cannot be said for studying prediction without control. One can learn to predict an event regardless of the ability to control its onset or termination.

Much of the more recent research on helplessness has focused on the element of prediction as a mediator of fear. Decades of research have highlighted the crucial role of the amygdala in fear responses; manipulations of the nuclei of the amygdala have greatly modified behavioral indices of fear in rats (Fanselow and LeDoux, 1999). Fear is multifaceted in that it represents a complex pattern of neuronal activation resulting in neurochemical, emotional, and behavioral changes designed to help the organism escape from threat (Fendt and Fanselow, 1999). The task of measuring all of the physiological and psychological modifications associated with fear is daunting, so oftentimes researchers focus their studies on only one aspect of the fear response. For example, the defensive behavior of freezing is often used as a measure of fear in rats, with higher levels of freezing indicating a greater fear response (Bouton and Bolles, 1980). In the learned helplessness procedure, freezing following foot shock is measured during the first 5 trials of shuttle-escape testing to discern the fear-provoking nature of inescapable versus escapable shock. Rats have consistently shown higher levels of freezing during the first few trials after receiving inescapable shock than escapable shock or restraint, indicating a sensitized fear response in inescapably-shocked rats upon entering the test. Freezing levels are equated between groups by the fifth shock trial. Perhaps it is this disparity of fear between groups at the start of the testing phase that results in the discrepancy in escape latencies during later trials.

The fear response is implicated as the mediator for the cascade of changes seen following inescapable shock. Mowrer and Viek (1948) characterized this idea best when they found that escapable rats experience pain while inescapable rats experience pain plus fear. Inescapable

shock is likely to be more fear inducing due to the unpredictability of the situation. The animal cannot predict the onset or termination of shock, and therefore remains in a chronic state of fear throughout the stress session. Studies on safety signals and cessation conditioning during traumatic stress all share a similar outcome of fear reduction and result in lower escape latencies during testing (Mineka, Cook and Miller, 1984; Jackson and Minor, 1988; Minor, Trauner, Lee and Dess, 1990). Animals can use these cues during a stress session to predict the termination or absence of shock and no longer remain in a chronic fear state. Interestingly, the ability to control the situation did not change. Even though shock was still uncontrollable, prediction of shock was enough to reduce fear and the deleterious effects of the aversive event.

The data indicate that unsignaled, inescapable shock produces a chronic fear state that is then responsible for escape deficits during later shuttle-escape testing. The question remains as to why fear would persist 24 hours later in a context that is different than the one initially used for stress exposure. This fear generalization was studied in great detail and the results suggest that inescapable rats transfer a high level of fear of the training context to the testing context, likely due to stress odor cues present in both contexts. Escapable rats do not experience as much fear during training, and therefore have negligible fear transference (Minor and LoLordo, 1984; Minor, 1990). If helplessness is due to the greater level of fear experienced during inescapable shock, then blocking fear should eliminate the escape deficits seen in helpless rats. Drugan, Ryan, Minor and Maier (1984) tested this theory and found that injection of a benzodiazepine before training eliminated helplessness. These studies strongly indicate that the fear experienced during inescapable shock is critical to the formation of helplessness, likely triggering a cascade of events that ultimately leads to escape deficits when tested 24 hours later.

Conservation-Withdrawal

Exposure to inescapable shock in the learned helplessness procedure results in exaggerated fear responding and escape performance deficits. Unfortunately, no theory explains the physical mechanism of this relationship. It is evident that fear plays a critical role in producing helplessness, but the question remains as to how the fear experienced during unsignaled, inescapable shock affects later shuttle-escape learning? The theory of conservation withdrawal is likely the one to do just that. This theory states that the source of psychological and behavioral deficits following an aversive event is likely a shift in state during testing from one of anxiety and agitation to one of conservation-withdrawal (Engel and Schmale, 1972). This shift in motivational state occurs unconditionally after periods of intense neural activation or stress, and is critical for husbanding limited resources. A conservation-withdrawal state is characterized by sensory unresponsiveness, cognitive dullness, and behavioral depression, which are seen as adaptive mechanisms for recovering metabolic homeostasis.

The characteristics of conservation-withdrawal are reasonably good descriptors of the myriad of effects seen after exposure to inescapable shock. This change in physiological state mimics the behavioral state observed following aversive events. The behavioral depression symptom is of particular interest to the learned helplessness effect as it describes the escape deficits seen during test. Exposure to traumatic inescapable shock results in a chronic fear state, represented by intense catabolic output. Upon re-exposure to shock during testing, this fear response is sensitized and results in a disproportionately high catabolic rate. The subject quickly transitions from an anxious, agitated state to one of conservation withdrawal, resulting in the affectless symptoms of behavioral depression seen in the learned helplessness effect (Minor, Chang, and Winslow, 1994; Minor, Winslow, and Chang, 1994; Minor; Huang, and Witt, 2006).

Allowing for prediction or control of the inescapable shock session reduces fear and the transition to a conservation-withdrawal state is prevented, eliminating helplessness.

Stress Resilience

The majority of work on stress resilience has focused on early-life experiences and the subsequent effect on stress coping techniques as an adult. Exposure to intermittent mild stress during infancy builds resilience during adulthood only if the individual is allowed time to recover from the stressor prior to the next stress experience (Boyce & Chesterman, 1990; Denenberg, 1967; Hunt, 1965; Khoshaba & Maddi, 1999; Levine, 1960). Additionally, the more exposure to mild stresses during infancy, the greater the protection against stress as an adult (Denenberg & Haltmeyer, 1967). The benefits of the previous stress, however, are overwhelmed when the target challenge is too severe (Bateson et al. 2004; Macrì, Zoratto & Laviola, 2011; Minor, Chang & Winslow, 1994).

Whereas the available evidence indicates that mild-to-moderate stress early in life can benefit the individual in adulthood, it is less clear that adults are equally malleable. Adult resilience may be established during a critical developmental period (Dennenberg, 1967; Dennenberg & Haltmeyer, 1967) or may be subject to mother-offspring interactions that are only available during infancy (Bateson et al., 2004; Macrì & Wuerbel, 2006; Macrì, Zoratto & Laviola, 2011; Meaney, Aitken, Viau, Sharma & Sarrieau, 1989).

Some evidence that resilience is enhanced in adults comes from the classic work on "toughening-up" by Miller, Weiss, and their colleagues (Miller, 1976; Weiss, Glaser & Pohorecky, 1976; Anisman, 1978; Weiss et al., 1981). Rats were exposed to a progressively severe shock stressor over a two week period in these experiments. This initial training

eliminated symptoms of behavioral depression following exposure to uncontrollable traumatic stress in the learned helplessness procedure. Even though these data provide evidence that changes in resilience can be achieved in adulthood, the paradigm has limited value because of the severity of the pre-exposure stressor.

McEwen and his colleagues (McEwen & Stellar, 1993; McEwen & Gianaros, 2011) argue that rest is important in repairing the damaging effects of stress and building resilience. The rationale for this proposal is that tissue damage associated with a rise in catabolic hormones is repaired by a nocturnal rise in anabolic hormones. Failure to achieve adequate rest (or sleep) following stress results in an accumulation of stress-related damage (allostatic load) and impedes the ability to respond adaptively to future stressors. However, whether rest is necessary or sufficient for resilience is not clear.

Post-Stress Glucose Consumption

The brain does not store metabolic substrates and requires a constant supply of glucose and oxygen from blood for normal function. The brain is normally constrained to using glucose as the metabolic fuel for the anaerobic phase of respiration. Brain metabolic demand increases substantially during a fear or stress response (Clarke & Sokoloff, 1993), and metabolic homeostasis has been shown to be compromised following exposure to traumatic stress (Plumb, Sterlace, Cavanaugh & Minor, 2013; Minor, Chang & Winslow; 1994; Minor, Winslow & Chang, 1994; Horner, Packan & Sapolsky, 1990; Bliss & Sapolsky, 2001).

Minor and Saade (1997) originally hypothesized that simply replenishing the energy supply following trauma should restore brain metabolic homeostasis and prevent the sequelae that normally result from uncontrollable stress. They found that rats given 18-hour access to

100mL of a 40% (wt/vol) aqueous glucose solution immediately following traumatic shock stress showed reduced escape latencies during shuttle-box testing equal to that of restraint controls.

Rats given access to glucose following experience with severe, uncontrollable, and prolonged stress fail to develop the PTSD-like and depression-like symptoms that normally occur as a result of the trauma. Moreover, post-stress glucose changes what normally is a seriously debilitating experience into one that enhances resilience to and recovery from subsequent traumatic stress.

The evidence indicates that metabolic demand is exaggerated following traumatic stress. Fear is an intensely catabolic state and the consequences of maintaining this state appear to result in the loss of a variety of regulator mechanisms that render the rat highly fearful and vulnerable to subsequent stressors. Fortunately, the body is equipped with a neural circuit breaker known as adenosine that is released under intense neural activation and serves to inhibit further activity in an effort to prevent cell death. Perhaps it is the inhibitory action of adenosine that is responsible for the PTSD-like symptoms normally observed in the learned helplessness procedure.

Adenosine

Adenosine is a nucleoside that is derived from adenosine-5'-triphosphate (ATP) under intense neural activation. ATP is broken down into adenosine intracellulary by dephosphorylation of ATP and then extruded into extracellular space. It is also derived extracellularly by the activation of a family of enzymes known as ectonucleotidases (Burnstock, 2006; Vorhoff, Zimmermann, Pelletier, Sevigny and Braun, 2005). Extracellular adenosine binds to receptors both pre-synaptically and post-synaptically. Adenosine then potently inhibits further

neuronal activation in an effort to prevent the utilization of all available ATP and ultimately, cell death (Dunwiddie, 1985).

Adenosine exerts its homeostatic and regulatory actions by interacting with four G-protein coupled stereospecific P1 receptors: A_1, A_{2A}, A_{2B}, & A_3 (see Haas & Sesbach, 2000; Phillis, 2004 for reviews). A_1 receptors are widely distributed in the brain and mediate adenosine's inhibitory actions by coupling with a G_i protein that inhibits adenylyl cyclase (Burns, Lu & Pugsley, 1986). A_2 receptors mediate adenosine's excitatory actions by coupling a G_s protein that excites adenylyl cyclase (Calon et al., 2004; Sebastião & Riberio, 1996). The A_{2B} subtype is a low-affinity receptor that is widely distributed in most brain regions. The high-affinity A_{2A} subtype has a much more limited distribution, being localized primarily on enkephaline-containing GABAergic neurons in the striatopallidal tract of the striatum (Svenningsson, Le Moine, Fisone & Fredholm, 1999; Ishiwata et al., 2005). Limited concentrations of A_{2A} receptors also are found in thalamus (Mishina et al., 2007; Castillo-Meléndez, Krstew, Lawrence & Jarrott, 1994; Weaver, 1993), nucleus tractus solitarius (Scislo & O'Leary, 2006; Coleman, Baghdoyan & Lydic, 2006), and cholinergic neurons of the pontine reticular formation (Ferré, Fredholm & Morelli, 1997; Gessi et al., 2008). A_3 receptors are found primarily in the periphery, with high concentrations in testes and mast cells, and are not heavily expressed in brain. These receptors play an important role in regulating inflammatory reactions (Linden et al., 1993; Dantzer, 2001).

Adenosine A_{2A} receptors and Animal Models of Depression

Adenosine is implicated in a number of animal models of depression, including learned helplessness, tail suspension, forced swim task, and reserpine-induced depression. Minor and

colleagues have extensively studied the role of adenosine signaling in the learned helplessness paradigm (see Minor, Huang, and Witt, 2006 and Minor, Plumb, Schell, and Pham, 2011 for reviews). They found that pretest systemic injection of the nonselective adenosine antagonists caffeine and theophylline reversed shuttle-escape deficits in rats previously exposed to inescapable shock in a dose-dependent manner (Minor, Chang, and Winslow, 1994). This indicates an increase in adenosine signaling at the time of test in this paradigm. They were also able to mimic escape deficits by giving unshocked rats adenosine analogs (Minor, Winslow, and Chang, 1994). Restrained rats were systemically injected with a nonselective adenosine agonist or a highly-selective A_1 receptor agonist. Both agonists induced shuttle-escape deficits, but the nonselective agonist produced deficits to a greater degree. This finding provided the first evidence that helplessness may be mediated by adenosine A_2 receptors.

This view was narrowed down further when Vaugeois and colleagues discovered an active role of adenosine A_{2A} receptors in both the tail suspension and forced swim task models of depression (El Yacoubi, Costentin and Vaugeois, 2003; El Yacoubi et al., 2001). Blockade of A_{2A} receptors or genetic inactivation of the A_{2A} receptor subtype significantly decreased immobility time in inescapably-shocked animals in both paradigms, reversing the symptoms of behavioral despair. Hanff, Furst, and Minor (2010) discovered a similar finding in rats given reserpine-induced depression. Injection of an adenosine A_{2A} antagonist before the forced swim task reduced immobility and reversed behavioral depression. These data predict that blocking A_{2A} receptors in the learned helplessness paradigm should likely reduce shuttle-escape deficits in inescapably-shocked rats.

The first assessment of the role of A_{2A} receptors in learned helplessness used a combination of the nucleoside transport blocker S-(4-Nitrobenzyl)-6-thioinosine (NBTI) and the

adenosine A_{2A} antagonist 8-(3-Chlorostyryl)caffeine (CSC) (see Minor, Huang, and Witt, 2006). Rats given restraint plus an intracerebroventricular (i.c.v.) injection of NBTI show escape deficits comparable to inescapably-shocked rats (Minor, Rowe, Cullen, and Furst, 2008). This is likely due to the increased concentrations of adenosine in the synapse when the transporters are blocked. Rats given an i.c.v. injection of NBTI + CSC show reduced escape deficits similar to restrained controls (Minor, Huang, and Witt, 2006). Blocking adenosine A_{2A} receptor activation eliminates shuttle-escape deficits, indicating the critical role of adenosine, specifically the A_{2A} receptor subtype, at the time of test in learned helplessness.

Overall, considerable evidence from a variety of animal models of depression indicate the primary role of adenosine A_{2A} signaling in conservation-withdrawal. The characterization of adenosine's function in the central nervous system seems to follow closely to Engel and Schmale's (1972) concept of conservation-withdrawal; indeed, adenosine's function appears to be the cellular equivalent of this state. Exposure to inescapable shock produces a chronic fear state represented by intense neural activation. Adenosine is released and binds to A_{2A} receptors to inhibit further activation, potentially resulting in a motivational shift to one of conservation-withdrawal. Behavioral depression ensues. Blocking A_{2A} receptors prevents the shift to conservation-withdrawal and eliminates behavioral deficits. The question remains as to the locus of the adenosine A_{2A} receptor activity. As A_{2A} receptors are found primarily on the enkephalin-containing GABAergic neurons in the striatopallidal pathway of the striatum, this seems a likely starting point to begin to answer this question.

Dissertation Objectives

This purpose of this dissertation is to examine peritraumatic interventions that build resilience to trauma in the learned helplessness paradigm**.** Chapter One examines if stress resilience can be achieved in adult rats, and if rest is a necessary factor in facilitating resilience. Four experiments are provided in which resilience to stress is achieved utilizing the concept of hormesis, a procedure involving pre-exposure to small stresses before traumatic stress. These experiments examine stressor severity, pattern of rest, and number of pre-exposure sessions in the learned helplessness paradigm as factors in building resilience to trauma.

Chapter Two examines a second resilience-building technique that aims to restore metabolic homeostasis after trauma by consumption of a simple sugar — glucose. Three experiments will utilize post-stress glucose consumption in an effort to eliminate conservation-withdrawal, facilitate hormetic stress and build resilience to multiple traumas.

Chapter Three examines striatal adenosine signaling as a potential mechanism of action of conservation-withdrawal. Adenosine A_{2A} receptors have been implicated in the shift to this depressive-like state following trauma. Two experiments will attempt to narrow down the locus of action to the nucleus accumbens shell and core.

At the clinical level, these results have important implications for the treatment of Post-traumatic Stress Disorder (PTSD) and comorbid depression. These experiments focus on the psychological and neurobiological processes associated with the development of PTSD-like symptoms in a rat model of psychological trauma, emphasizing the brain metabolic consequences of an emotional reaction of extreme fear or terror. These experiments suggest that PTSD symptoms may arise from neural fatigue associated with excessive emotional output. By understanding the circuitry involved in helplessness, as well as how this circuitry can be altered,

the experiments presented here provide an important step in the formulation of more successful techniques for the treatment and prevention of fear-induced disorders.

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CHAPTER ONE

Parameters of Hormetic Stress and Resilience to Trauma in Rats

Abstract

Hormesis is the process by which small stresses build resilience to large stresses. We pre-exposed rats to various parameters of mild-to-moderate stress prior to traumatic stress in the present experiments to assess the potential benefits of hormetic training on resilience to traumatic, uncontrollable stress. Rats underwent varying stress pre-training parameters prior to exposure to uncontrollable traumatic stress in the learned helplessness procedure. The ability to prevent the exaggerated fear responding and escape deficits that normally follow experience with traumatic stress were used as a measure of the benefits of hormetic training. Four experiments examined the effects of number of training sessions, stressor severity, and pattern of rest between pre-training stress sessions. Repeated exposure to mild restraint stress or moderate shock stress eliminated both the enhanced fear conditioning and shuttle-escape deficits that result from exposure to traumatic, inescapable shock. The pattern of rest did not contribute to resilience when the pre-exposure stressor was mild, but was vital when the pre-exposure stressor was moderate, with an alternation of stress and rest being the most effective procedure. The data also suggests that the level of resilience may increase with the number of pre-exposure sessions.

Introduction

Hormesis is the process by which small stresses build resilience to large stresses. The pharmacologist Hugo Schulz originally coined the term in 1888 in a discussion of the immunity to poisoning that develops when an individual ingests a small amount of the toxin over an extended period of time (Southam & Erhlich 1943; Calabrese et al. 2007). The term has been used more recently to describe the benefits of exercise and oxidative stress in preventing bodily disease and improving emotional health (Radak et al. 2008; Li & He 2009). Hormesis is probably best conceptualized in modern parlance as an increased capacity for allostasis – the process of adapting to an environmental challenge – as the result of repeated exposure to uncontrollable, but otherwise mild stress (Sterling & Eyer 1988). Allostatic load refers to the cumulative damage that occurs as a consequence of allostasis when recovery is inadequate or incomplete (McEwen & Stellar 1993; McEwen & Gianaros 2011; Schulkin 2003).

The present experiments examined the potential hormetic benefits of stress pre-exposure in the learned helplessness paradigm. This procedure is a traditional method for analyzing the effects of acute, traumatic stress and modeling related symptoms of Post-traumatic Stress Disorder (PTSD) and comorbid major depression in rats (Basoğla et al. 1997; Hammack et al. 2012; Minor, Plumb, Schell & Pham 2011; Minor, Dess & Overmier 1991). The procedure consists of two phases. Rats initially are exposed to a large number of unsignaled, inescapable tail shocks in tubes over an extended period (2 to 4 hours). A control group is simply restrained in tubes for the same time period in the absence of shock. All rats are tested for shuttle-escape performance 24 hours later. Rats pre-exposed to inescapable shock enter the test situation in an anxious/agitated state and show exaggerated fear responding during initial escape testing. Inescapably shocked rats rapidly transition to an unresponsive, depression-like state, termed

conservation-withdrawal, as testing progresses. The transition to conservation-withdrawal is evidenced as a profound deficit in escape performance (Minor, Chang & Winslow 1994a; Plumb, Sterlace, Cavanaugh & Minor 2013). More generally, experience with uncontrollable shock results in disturbances in sleep (Kant et al. 1995), exaggerated startle (Servatius et al. 1995), hypervigilance (McAuley et al. 2009), anorexia (Dess, Minor & Brewer 1989), anhedonia (Zackarko & Anisman 1971), reinstatement of drug seeking (Figueroa-Guzman et al. 2011), and attentional/cognitive deficits in rats (Jackson, Alexander & Maier 1980; Minor, Jackson & Maier 1984; Shors 2004).

Experiments on the "immunization effect" have found that subjects exposed to escapable shock before being exposed to inescapable shock show a lack of interference during shuttle-escape testing (Seligman and Maier, 1967; Williams and Maier, 1977). Even more surprising, the situation in which the subject is exposed to escapable shock can be different from that during later inescapable shock exposure and testing, but still produce a similar immunizing effect against learned helplessness (Williams and Maier, 1977). The authors argued that the positive association between response and outcome learned during the escapable task protected the subject from the negative effects of uncontrollability in the inescapable task, eliminating learned helplessness.

While control is certainly present in the learned helplessness procedure, closer examination points to another variable that coincides with controllability: prediction. It is extremely difficult to study controllability without prediction; having control over a situation requires the ability to predict the events that are to be controlled. However, the same cannot be said for studying prediction without control. One can learn to predict an event regardless of the

ability to control its onset or termination. In fact, much of the more recent research on helplessness has focused on the element of prediction as a mediator of fear.

Minor, Trauner, Lee and Dess (1990) tested the ability of cessation cues to immunize against inescapable shock. It has been found that cues that predict the cessation of shock elicit relief (Mowrer, 1960). Minor et al. found that exposure to cessation cues during a traumatic, inescapable shock session was sufficient to immunize the subject against a subsequent traumatic stress session. These data indicate that prediction of shock termination is an essential feature in mediating escape deficits during shuttle-escape testing.

The data on predication and control during trauma can be interpreted in multiple ways. First, control and prediction may both be equally and independently protective to the traumatic event (Maier & Warren, 1988; Rosellini, Warren & DeCola, 1987). Second, prediction, rather than control, may be the critical factor in eliminating the deleterious effects of traumatic stress. Perhaps it is the signal features of the escape response that are beneficial, not the control that comes with being able to effectively respond (Mowrer & Viek, 1948; Minor, Trauner, Lee & Dess, 1990). Third, neither prediction nor control is critical in itself, but serves to support another mechanism. For instance, both predication and control reduce the severity of the stressor. This can be seen in the magnitude of fear conditioning during predictable or controllable shock. The magnitude of fear conditioning is determined by the magnitude or perceived magnitude of the unconditioned stimulus. Because fear conditioning is greatly reduced during predictable or controllable shock, this suggests that the functional magnitude of the unconditioned stimulus is substantially reduced relative to a stressor that is unpredictable or uncontrollable (Mineka, Cook & Miller, 1984; Minor, 1990; Maier, 1990).

Additionally, in one of the original papers on cessation conditioning, Segundo, Galeano, Sommer-Smith & Roig (1961) shocked the legs of cats over a 30min period. The shock stimulus elicited a defensive EEG and considerable vocalization. Cessation cues signaling the termination of shock completely eliminated the defensive EEG and the vocalization despite the shock still being on. Such data suggest that predication and control may reduce the functional severity of the stressor. It is plausible that pre-exposure to a milder stressor may underlie effects such as immunization.

We pre-exposed rats to various parameters of mild-to-moderate stress prior to traumatic stress in the present experiments to assess the potential benefits of pre-training stress exposure. . The most severe pre-training stressor used in the present experiments (i.e. 25 shocks) is not sufficient to induce the helplessness effect alone (Minor, Dess, Ben-David & Chang 1994). The enhanced fear conditioning (Maier 1990; Minor 1990) and escape deficits (Maier, Albin & Testa 1973) normally observed 24 hours after experience with inescapable shock should be greatly diminished by pre-exposure to comparatively mild stress.

Method

Experiment 1

Experiment 1 examined whether pre-exposure to a number of days (3 or 5) of simple restraint stress (30 minutes) mitigated the exaggerated fear conditioning and shuttle-escape deficits that are normally observed 24 hours after exposure to traumatic, uncontrollable shock.

Subjects. Forty-eight male Sprague-Dawley albino rats (290-320g) from Harlan Laboratories were housed in individual cages with free access to food and water in a room maintained on a 12:12-hour light/dark cycle for one week prior to experimental treatment.

Experimentation occurred during the light portion of the cycle. All protocols in this paper were pre-approved by the UCLA IACUC.

Apparatus. Restraint and tail shock pretreatments occurred in clear Plexiglas restraining tubes, measuring 23 cm in length and 6 cm in diameter. Adjustable front walls prevented the rats from moving forward in the tubes. A rat's tail extended through the rear door of each tube and was taped to a plastic rod. Unscrambled electric shocks were delivered from one of four constant-current shock generators (Lafayette Instrument Co., Model 82400) through electrodes attached to the rat's tail with electrode paste and tape. Each tube was housed in a sound-attenuating enclosure containing an exhaust fan that masked extraneous noises. A 7-W house light located in the center of the rear wall of the attenuating enclosure's rear wall provided constant illumination.

Escape testing occurred in a (45 cm x 20 cm x 20 cm) shuttle box (BRS-LVE model 146-40). The shuttle box was divided into two equal compartments by a metal barrier that had an 8 x 7 cm center opening flush with the grid floor. The floor consisted of 2-mm diameter stainless-steel rods spaced 1.1 cm apart center to center. Continuous scrambled shock was delivered to the grid floor from a Grason-Stadler (Series 700) shock generator. The floor pivoted in the center and a response was recorded when a 300-g rat's front paws touched the center grid in a compartment. Two 6-W lamps located in the center of each end wall provided constant illumination. The shuttle box was housed in a sound-attenuating chest, containing an exhaust fan that masked extraneous noise.

Procedure. Rats were assigned randomly to one of six groups of 8 rats each. Two groups received no pre-training (Groups S and R). They received either exposure to 100, 1.0mA variable-duration (mean = 8.0 s; range: 3 to 15 s) inescapable tail shocks on a variable-time 60-s

schedule (range: 20 to 150 s) in restraining tubes over 1.83 hours (Group S) or simple restraint in restraining tubes with no tail shock for the same amount of time (Group R) during the stress treatment session. These groups served to define the boundaries of the learned helplessness effect. Two other groups received either 3 or 5 30-minute sessions of restraint stress in tubes with a day of interpolated rest occurring after each of these sessions. These groups then were exposed to the traumatic shock stressor during the treatment session (Groups r-r-r-S and r-r-r-r-r-S). Two other groups (Groups r-r-r-R and r-r-r-r-r-R) also received either 3 or 5 30 minute sessions of restraint stress in tubes with a day of interpolated rest occurring between each of these sessions. These groups received simple restraint during the treatment session.

Shuttle-escape testing occurred 24 hours later. The test consisted of five trials during which a rat had to cross from one side of the central barrier to the other in order to terminate shock (FR-1 trials). These trials occurred on a fixed-time 60-second schedule. A trained observer scored defensive freezing, defined as the absence of all bodily and vibrissae movement except for that related to respiration, during each intertrial interval using a time-sampling procedure every 5 seconds. FR-1 trials were followed by 25 FR-2 trials during which a rat had to cross from one side of the central barrier and then return to terminate shock. Shock terminated automatically if the appropriate response contingency was not met within 40 seconds of shock onset on a given trial. Escape latencies were recorded on each trial. Shock intensity was set at 0.6 mA with FR-2 trials occurring on a variable time 60-second schedule (range: 20 to 230 seconds); however, three minutes intervened between FR-1 and FR-2 trials (cf., Minor & LoLordo 1984).

Experiment 2

Experiment 1 clearly demonstrated that as few as 3 days of restraint stress with interpolated rest has hormetic benefits. McEwen and his colleagues (McEwen & Stellar 1993; McEwen & Gianaros 2011) have long argued that rest is important in repairing the damaging effects of stress and building resilience. This experiment aims to determine if rest is necessary for stress resilience by manipulating the pattern of rest surrounding pre-exposure sessions.

Subjects and Apparatus. Forty male Sprague-Dawley albino rats (290-320g) were housed as in Experiment 1. The apparatus was the same as described above.

Procedure. Rats were randomly assigned to one of five groups of 8 rats each. Two groups received no pre-training prior to exposure to traumatic shock (Group S) or simple restraint (Group R) during the stress treatment session. The other three groups received 3 sessions of 30-minute restraint in tubes prior to exposure to traumatic stress. These groups differed with respect to the pattern of restraint and rest: Group rrr---S received three consecutive days of restraint stress followed by three consecutive days of rest; Group ---rrrS received three days of rest followed by three consecutive days of restraint stress; and Group r-r-r-S received three days of restraint stress with three days of interpolated rest. Shuttle-escape testing occurred 24 hours after the stress treatment session.

Experiment 3

Experiment 3 determined whether pattern of rest is critical when the pre-training stressor is more severe. This experiment utilized the same general design as Experiment 2; however, the pre-training stressor was 25 inescapable tail shocks rather than restraint.

Subjects and Apparatus. Forty male Sprague-Dawley albino rats (290-320g) were housed as in Experiment 1. The apparatus was the same as above.

Procedure. Rats were randomly assigned to one of five groups of 8 rats each. Two groups received no pre-training prior to exposure to traumatic shock (Group S) or simple restraint (Group R) during the stress treatment session. The other three groups received 3, 30-minute sessions of 25, 1.0 mA variable-duration (mean = 8.0s; range: 3 to 15s) inescapable tail shocks on a variable-time 60-s schedule (range: 20 to 150s) in restraining tubes prior to exposure to traumatic stress. These groups differed with respect to the pattern of shock and rest: Group sss---S received three consecutive days of shock followed by three consecutive days of rest; Group ---sssS received three days of rest followed by three consecutive days of shock; and Group s-s-s-S received three days of shock with three days of interpolated rest. Shuttle-escape testing occurred 24 hours after the stress treatment session.

Experiment 4

Experiment 1 provided some evidence that more stress pre-training yields greater resilience against traumatic stress. In Experiment 4, we used the same general design as in Experiment 1, but tried to amplify the benefits of stress pre-training by increasing the severity of the stressor.

Subjects and Apparatus. Thirty-two male Sprague-Dawley albino rats (290-320g) were housed as in Experiment 1. The apparatus was the same as above.

Procedure. Rats were randomly assigned to one of four groups of 8 rats each. Two groups received no pre-training prior to exposure to traumatic shock (Group S) or simple restraint (Group R) during the stress treatment session. Two other groups received either 3 or 5,

30-minute sessions of 25 inescapable tail shocks with interpolated days of rest prior to exposure to traumatic shock (Group s-s-s-S and Group s-s-s-s-s-S). Shuttle-escape testing occurred 24 hours after the stress treatment session.

Results

Experiment 1

The left panel of Figure 1 shows mean percent post-trial freezing in each group. Rats exposed to inescapable shock without prior training (Group S) showed substantial higher levels of freezing from the outset of training and generally increased over trials relative to the restrained control (Group R). All pre-training groups performed similarly to the restraint control, with some indication that a greater amount of pre-training yielded lower freezing levels.

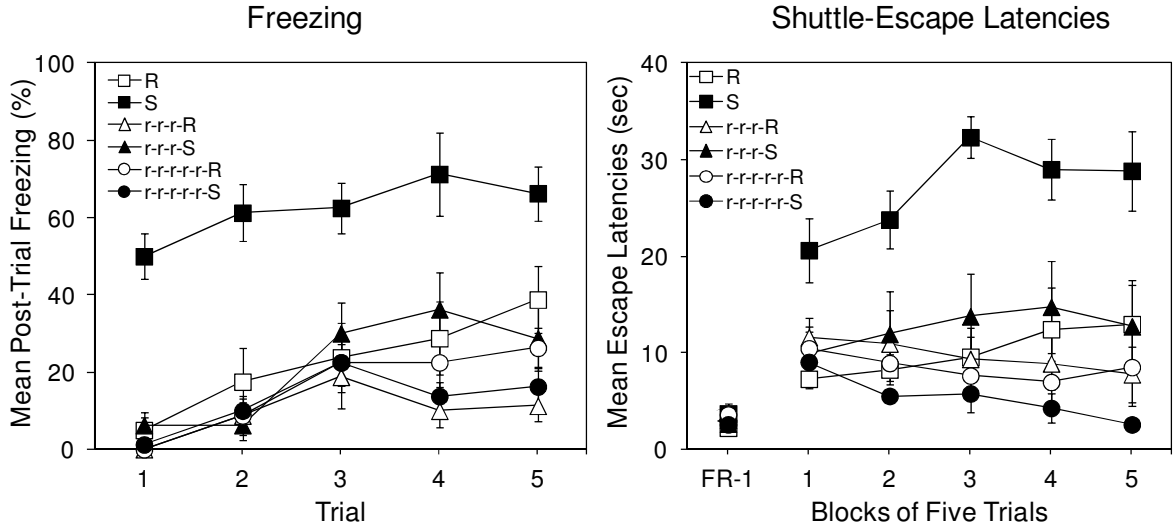

Figure 1. Percent freezing (left panel) and shuttle escape latencies (right panel) for 6 groups in Experiment 1. Two groups were exposed to traumatic shock stress (Group S) or simple restraint (Group R). Two other groups were pre-exposed to 3 days of restraint with interpolated days of rest followed by either restraint or traumatic shock (Groups r-r-r-R and r-r-r-S). Two other groups were pre-exposed to 5 days of restraint with interpolated days of rest followed by either restraint or traumatic shock (Groups r-r-r-r-r-R and r-r-r-r-r-S). Shuttle-escape testing occurred 24 hours later. Freezing was measured over 5 trials (FR-1) at the start of the shuttle-escape test. Impaired escape performance was assessed over the next 25 trials (FR-2).

A mixed-design analysis of variance (ANOVA: Stress Condition x Pre-training Condition x Trial) yielded significant main effects of Stress, $F(1, 42) = 17.87$, $p < 0.001$, Pre-training, $F(2, 42) = 23.49$, $p < 0.001$, and Trial, $F(4, 168) = 24.23$, $p < 0.001$, and significant interactions of Stress x Pre-training, $F(2, 42) = 10.95$, $p < 0.001$, and Stress x Pre-training x Trial, $F(8, 168) = 2.02$, $p = 0.05$. The interactions between Stress x Trial and Pre-training x Trial were not statistically significant. Newman-Keuls post-hoc contrasts ($\alpha = 0.05$) on grand mean freezing suggested the following ordered relation among group means: S > R = r-r-r-S = r-r-r-R = r-r-r-r-r-S = r-r-r-r-r-R.

The right panel of Figure 1 shows mean escape latencies across blocks of five trials in each group. FR-1 escape latencies did not differ among groups, $F < 1$. The standard helplessness effect is defined by the difference between FR-2 escape latencies of Groups S and R. All pre-training groups performed similarly to the restraint control, with some indication that a greater amount of pre-training afforded slightly greater protection.

A mixed-design ANOVA (Stress Condition x Pre-training Condition x Trial Block) on FR-2 escape latencies yielded significant main effects of Stress, $F(1, 42) = 6.89$, $p = .012$, and Pre-training, $F(2, 42) = 10.18$, $p < 0.001$, and significant interactions of Stress x Pre-training, $F(2, 42) = 7.75$, $p = 0.002$, and Pre-training x Trial Block, $F(8, 168) = 4.48$, $p < 0.001$. The main effect of Trial Block and the other potential interactions were not statistically significant. Newman-Keuls post-hoc contrasts ($\alpha = 0.05$) on grand mean FR-2 escape latencies suggested the following ordered relation among group means: S > R = r-r-r-S = r-r-r-R = r-r-r-r-r-S = r-r-r-r-r-R.

Experiment 2

The left panel of Figure 2 shows mean percent post-trial freezing in each of the five groups in Experiment 2. Group S showed excessive levels of freezing from the outset of testing relative to the restrained control (Group R). Stress training prior to traumatic stress mitigated fearfulness at the time of testing, regardless of the pattern of rest.

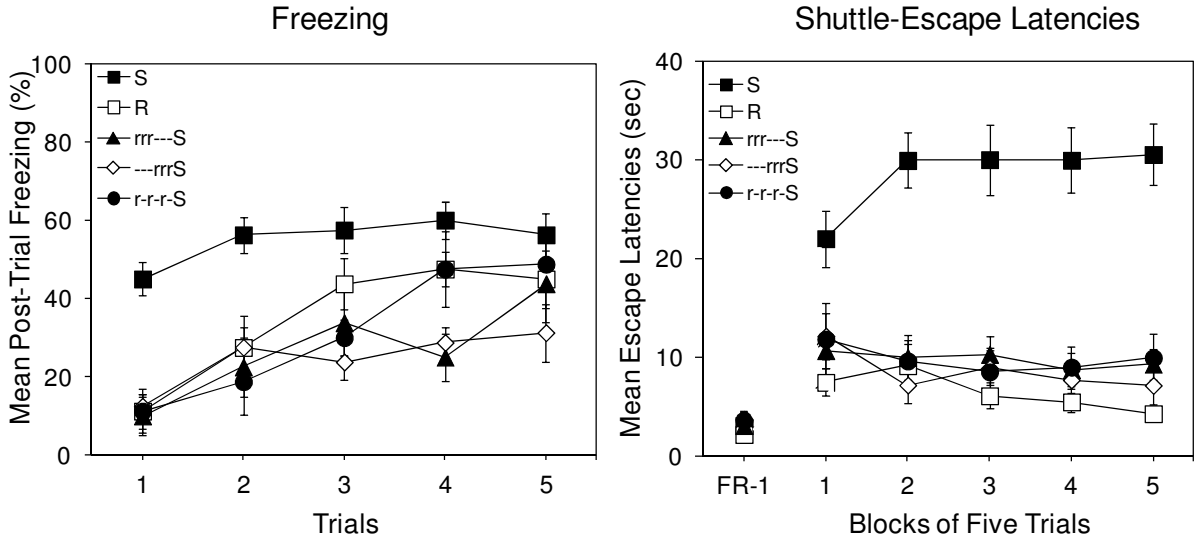

Figure 2. Percent freezing (left panel) and shuttle escape latencies (right panel) for 5 groups in Experiment 2. Two groups were exposed to traumatic shock stress (Group S) or simple restraint (Group R). Three other groups were pre-exposed to 3 days of restraint followed by traumatic shock. These three groups received 3 days of rest that either preceded training (Group ---rrrS), followed training (Group rrr---S), or was interpolated with training (Group r-r-r-S). Shuttle-escape testing occurred 24 hours later. Freezing was measured over 5 trials (FR-1) at the start of the shuttle-escape test. Impaired escape performance was assessed over the next 25 trials (FR-2).

A mixed-design ANOVA (Group x Trial) yielded significant main effects of Group, $F(4, 35) = 7.44$, $p < 0.001$, and Trial, $F(4, 140) = 23.32$, $p < 0.001$, and a significant Group x Trial interaction, $F(16, 140) = 1.93$, $p < 0.03$. Newman-Keuls post-hoc contrasts ($\alpha = 0.05$) on grand mean freezing suggested the following ordered relation among group means: S > R = r-r-r-S = rrr---S = ---rrrS.

The right panel of Figure 2 shows mean escape latencies across blocks of five trials in each group. FR-1 escape latencies did not differ, $F < 1$. Escape latencies were similar to freezing

behavior. A large deficit in FR-2 escape performance occurred in Group S relative to Group R. Stress pre-training dramatically improved escape performance, regardless of the pattern of rest.

A mixed-design ANOVA (Group x Trial Block) yielded a significant main effect of Group, $F(4, 35) = 22.15$, $p < 0.001$, and a significant Group x Trial Block interaction, $F(16, 140) = 3.045$, $p < 0.001$, indicating that escape latencies increased in Group S as they decreased in all other groups across trial blocks. The main effect of Trial Block was not statistically significant. Newman-Keuls post-hoc contrasts ($\alpha = 0.05$) on grand mean FR-2 escape latencies suggested the following ordered relation among group means: S > R = r-r-r-S = rrr---S = ---rrrS.

Experiment 3

The left panel of Figure 3 shows mean percent post-trial freezing in each of the groups in Experiment 3. There is considerable overlap in freezing behavior among all groups. A consistent pattern did not emerge among those that received stress pre-training and those that did not.

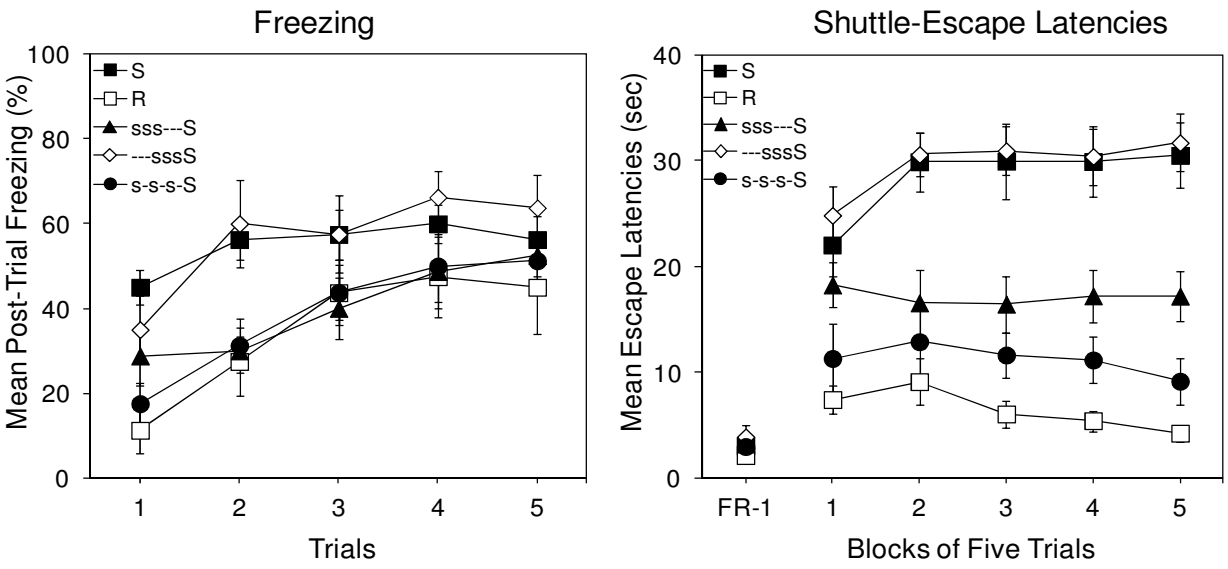

Figure 3. Percent freezing (left panel) and shuttle escape latencies (right panel) for 5 groups in Experiment 3. Two groups were exposed to traumatic shock stress (Group S) or simple restraint (Group R). Three other groups were pre-exposed to 3 days of 25 inescapable tail shocks followed by traumatic shock. These three groups received 3 days of rest that either preceded training (Group ---sssS), followed training (Group sss---S), or was interpolated with training (Group s-s-s-S). Shuttle-escape testing occurred 24 hours later. Freezing was measured over 5 trials (FR-1) at the start of the shuttle-escape test. Impaired escape performance was assessed over the next 25 trials (FR-2).

A mixed-design ANOVA (Group x Trial) yielded significant main effects of Group, $F(4, 35) = 3.46$, $p < 0.02$, and Trial, $F(4, 140) = 22.82$, $p < 0.001$. The interaction of Group and Trial was not statistically significant. Newman-Keuls post-hoc contrasts ($\alpha = 0.05$) on grand mean freezing identified a marginally significant difference ($\alpha = 0.054$) between groups and suggested the following ordered relation among group means: S = ---sssS > sss---S = s-s-s-S = R.

The right panel of Figure 3 shows mean escape latencies across blocks of five trials. FR-1 escape latencies did not differ, $Fs < 1$. A large deficit in FR-2 escape performance occurred in Group S relative to Group R. The benefits of stress pre-training clearly depended on the pattern of rest. Three days of pre-training shock stress prior to traumatic shock afforded no protection (Group ---sssS). By contrast, three days of shock stress with interpolated rest yielded the greatest protection such that Group s-s-s-S performed similarly to the restrained control. Three

consecutive days of shock stress followed by three days of rest yielded an intermediate amount of resilience (Group sss---S).

A mixed-design ANOVA (Group x Trial Block) yielded significant main effects of Group, $F(4, 35) = 22.86$, $p < 0.000$, and Trial Block, $F(4, 140) = 3.12$, $p < 0.02$, and a significant Group x Trial Block interaction, $F(16, 140) = 2.49$, $p < 0.01$. Newman-Keuls post-hoc contrasts ($\alpha = 0.05$) on grand mean FR-2 escape latencies suggested the following ordered relation among group means: S = ---sssS > sss---S > s-s-s-S = R.

Experiment 4

The left panel of Figure 4 shows mean percent post-trial freezing as a function of trial. Group S showed excessive levels of freezing from the outset of testing relative to the restrained control (Group R). Both stress pre-training groups performed similarly to the restraint control.

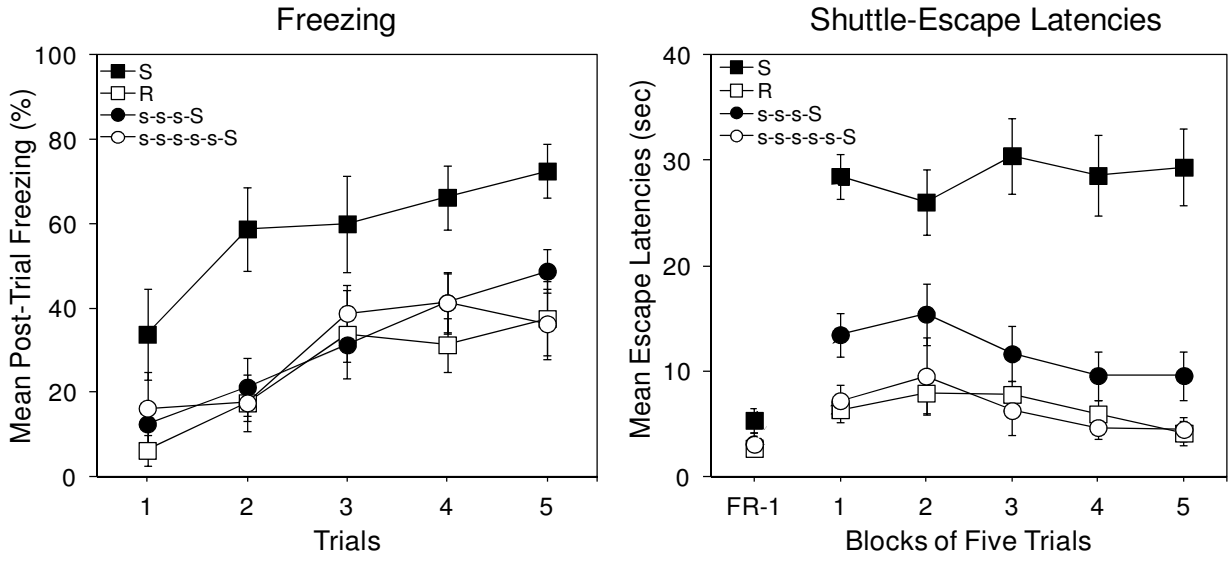

Figure 4. Percent freezing (left panel) and shuttle escape latencies (right panel) for 4 groups in Experiment 4. Two groups were exposed to traumatic shock stress (Group S) or simple restraint (Group R). Two other groups were trained with 3 or 5 days of 25 inescapable tail shocks with interpolated days of rest followed by traumatic shock (Groups s-s-s-S and s-s-s-s-s-S). Shuttle-escape testing occurred 24 hours later. Freezing was measured over 5 trials (FR-1) at the start of the shuttle-escape test. Impaired escape performance was assessed over the next 25 trials (FR-2).

A mixed-design ANOVA (Group x Trial) yielded significant main effects of Group, $F(3, 28) = 6.72$, $p < 0.002$, and Trial, $F(4, 112) = 22.35$, $p < 0 .001$. The interaction between Group x Trial was not statistically significant. Newman-Keuls post-hoc contrasts ($\alpha = 0.05$) on grand mean freezing suggested the following ordered relation among group means: S > R = s-s-s-S = s-s-s-s-s-S.

The right panel of Figure 4 shows mean escape latencies across blocks of five trials. FR-1 escape latencies did not differ, $Fs < 1$. A large deficit in FR-2 escape performance occurred in Group S relative to Group R. Both stress pre-training groups performed similarly to the restrained control, with evidence that 5 days of stress pre-training afforded slightly greater protection than 3 days.

A mixed-design ANOVA (Group x Trial Block) yielded a significant main effect of Group, $F(3, 28) = 34.93$, $p < 0.001$, and Trial Block, $F(4,112) = 3.13$, $p < 0.02$. The interaction between Group x Trial Block was not statistically significant. Newman-Keuls post-hoc contrasts ($\alpha = 0.05$) on grand mean FR-2 escape latencies suggested the following ordered relation among group means: S > R = s-s-s-S = s-s-s-s-s-S.

Discussion

The present experiments indicate that repeated exposure to severe stress is not necessary to build resilience in adult rats. Exposure to mild or moderate stress with interpolated rest is sufficient to block the exaggerated fear conditioning and shuttle escape deficits that normally follow experience with traumatic uncontrollable shock. The pattern of rest surrounding the mild pre-training stress sessions had no effect on escape latencies; however, the pattern of rest becomes critical when the initial stress sessions are more severe. When rest is allowed between

stress sessions, it allows the animal to recover physically from the damaging effects of each hormetic stress session (McEwen & Stellar 1993; McEwen & Gianaros 2011). Exposure to three days of shock stress immediately before traumatic stress did not provide adequate recovery time following each stress session, resulting in no benefit of stress pre-treatment and subsequent helplessness. Allowing for three days of rest following pre-training stress provided some benefit, but was less effective than if rest was allowed after each session. There is also some evidence that an increased number of pre-training stress sessions may be more beneficial, though this needs to be explored in greater detail.

The helplessness effect has both associative and nonassociative mediators – both are necessary, neither is sufficient (Minor, Dess & Overmier 1991; Weiss & Simson 1985). One way that pre-training stress sessions might impact the helplessness effect is by impacting one or the other set of mediators. For instance, repeated exposure to the treatment context prior to traumatic stress might facilitate discrimination between treatment and test contexts. This discrimination is severely impaired following traumatic stress and leads to a limited form of associative transfer based on common odors (see Minor & LoLordo 1984; Minor 1990). Manipulations that limit associative transfer across contexts eliminate the helplessness effect.

Pre-training stress sessions also might impact a nonassociative mediator. An early example was provided by Weiss, Glaser & Pohorecky (1976) in their studies of "toughening-up". These researchers attributed the helplessness effect to a depletion of brain catecholamines following traumatic stress. Repeated exposure to the traumatic stressor eventually upregulated the synthesis of tyrosine hydroxylase, the rate-limiting enzyme for catecholamine synthesis. The upregulation prevented catecholamine depletion and therefore behavioral impairment.

A more recent example of a potential nonassociative mediator involves brain metabolic regulation via adenosine signaling (Plumb et al. 2013). We have linked the onset of escape deficits in this paradigm to an increase in adenosine signaling in the nucleus accumbens. Adenosine is a critical modulator of neural activation that links cellular excitability to energy availability. The effect of enhanced adenosine signaling in this region is to uncouple dopamine from its receptor and undermine the motivation for ongoing behavior. Performance deficits ensue.

This hypothesis suggests that one way to mitigate the impact of traumatic stress is to increase metabolic capacity. Pre-exposure to mild or moderate stress may have such a function. In support of this, manipulations like exercise (Greenwood & Fleshner 2008) or treatment with methylene blue (Gonzalez-Lima & Bruchey 2004) increase metabolic capacity and eliminate the helplessness effect.

Stress pre-training also might impact processes that are orthogonal to the immediate causes of impairment. There is considerable interest in neuropeptide Y (NPY) as a potent antagonist of both the hypothalamic pituitary adrenal cortical axis (HPAC) and sympathetic adrenal medullary (SAM) axis (Heilig 2004). Stress pre-exposure might upregulate brain and peripheral concentrations of NPY, thereby reducing the overall impact of the traumatic stress session. Other mechanisms could have a similar impact. Repeated exposure to the pre-training stressor could result in habituation to shock and the resulting fear response, in which case the traumatic stress would be perceived as less severe and thereby eliminate the helplessness effect (Jackson & Minor 1988; Drugan et al. 1984; Minor et al. 1991; Minor et al. 1990; Mineka et al. 1984). Helplessness is usually observed only when the stressor is exceptionally severe (Minor et al. 1994).

Exposure to traumatic stress can be detrimental to one's physical and mental health, often resulting in psychological disorders such as Post-Traumatic Stress Disorder (PTSD) and major depression (see Minor et al. 2011; Plumb et al. 2013 for reviews). These disorders are often accompanied by the inability to effectively cope with subsequent stress. The learned helplessness procedure is an effective tool to study the deleterious effects of traumatic stress as the resulting behavior mimics a number of symptoms of PTSD and major depression, including anhedonia, insomnia, psychomotor retardation, fatigue, and anorexia or hyperphagia (Minor et al. 2011; Plumb et al. 2013). With this procedure, we have been able to show that repeated exposure to mild or moderate stress with interpolated rest builds resilience to traumatic stress and reduces the subsequent symptoms of PTSD and comorbid depression in rats.

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CHAPTER TWO

Post-Stress Glucose Consumption Eliminates Conservation-Withdrawal and Facilitates Hormesis and Resilience to Multiple Traumas

Abstract

Chapter One outlined various parameters of hormetic stress, a procedure that builds resilience to trauma and eliminates PTSD-like symptoms in rats. This chapter addresses a second resilience-building technique utilizing a simple sugar –glucose. Brain metabolic homeostasis is rapidly compromised following exposure to traumatic stress. Post-stress glucose consumption has been shown to be a simple and effective treatment for preventing the deleterious effects normally observed following trauma. Experiment 1 examines the ability of glucose to prevent the shift to conservation-withdrawal normally observed following trauma. Rats were exposed to inescapable, traumatic stress in the form of 100, 1mA tail shocks over 1.83 hours or simple restraint. Immediately following the end of traumatic stress exposure, rats were given 18 hr access to 100mL of an aqueous glucose solution or simple tap water. Shuttle-box testing occurred 24 hours after traumatic stress. During the test, rats were given 5 trials of escapable 0.6mA foot shock during which freezing and unconditioned shuttle crossings were recorded. Experiment 2 examines if post-stress glucose can facilitate hormetic stress. Rats were exposed to either an effective hormetic stress procedure or a previously ineffective procedure, as described in Chapter One. Following the pre-exposure stresses, rats were given access to glucose or water. They were then exposed to traumatic stress, followed by standard shuttle-escape testing 24 hours later. Experiment 3 determines if glucose is effective under a more severe situation. Rats were exposed to two traumatic stress sessions, 24 hours apart. These sessions were followed by either glucose or water in a 2x2 factorial design. Shuttle-box testing occurred 24 hours after the second trauma. The results from the three experiments show that glucose prevents the shift to conservation-withdrawal, facilitates hormetic stress training, and builds resilience to multiple traumas, respectively.

Introduction

Exposure to traumatic stress results in a number of physiological and psychological changes in both human and non-human species (Selye 1942; see Minor, Huang, and Witt 2006 for a review). These changes are often deleterious in nature and can endure throughout a lifetime. As such, there is an urgent need for practical interventions aimed at treating or preventing the damaging effects of traumatic stress.

Chapter One highlighted the benefit of hormetic stress on preventing the deleterious effects that follow traumatic shock. Hormesis is commonly used in toxicology where small doses of toxins build resilience to large doses of the same toxin, but has recently been extended to biological and medicinal fields (Southam & Erhlich 1943; Calabrese et al. 2007; Mattson 2008). Utilizing the concept of hormesis, our lab has demonstrated that pre-exposure to small stresses builds resilience to traumatic stress in the learned helplessness procedure (Plumb, Cullen & Minor 2014). A set of parametric studies in rats determined that exposure to mild or moderate shock before traumatic shock prevented the enhanced fear responding and shuttle-escape deficits normally observed in rats given no hormetic training. Furthermore, rest between stress sessions was not necessary if the hormetic stressor is mild; however, intermittent days of rest were critical if the hormetic stressor was more severe.

A second intervention aimed at treating the deleterious effects following trauma exposure utilizes simple glucose consumption. A number of findings suggest that metabolic homeostasis is challenged by exposure to uncontrollable, traumatic stress (Plumb, Sterlace, Cavanaugh & Minor 2013; Minor, Chang & Winslow 1994a; Minor, Winslow & Chang 1994b; Horner, Packan & Sapolsky 1990; Bliss & Sapolsky 2001). Minor and Saade (1997) hypothesized that simply

treating rats with glucose following traumatic stress would restore energy homeostasis and eliminate the helplessness effect. They found that rats given 18-hour access to 100mL of a 40% (wt/vol) aqueous glucose solution immediately following traumatic shock stress showed reduced escape latencies during shuttle-box testing equal to that of restraint controls. Simply replenishing blood glucose levels after a traumatic event eliminated the behavioral deficits normally observed following trauma.

The following experiments aimed to determine if post-stress glucose consumption eliminates conservation-withdrawal and facilitates hormetic stress training as well as resilience to multiple trauma exposures in the learned helplessness paradigm. This procedure utilizes acute, traumatic stress for studying Post-traumatic Stress Disorder (PTSD) and comorbid depression in rats (Basoğla et al. 1997; Hammack et al. 2012; Minor, Plumb, Schell & Pham 2011; Minor, Dess & Overmier 1991). Animals that received inescapable shock 24 hours earlier enter the test phase in a highly anxious/agitated state (Maier 1990; Minor 1990). Re-exposure to mild stress during testing provokes an excessive neural and behavioral fear response. Helpless rats rapidly transition from this catabolic state to one of *conservation- withdrawal*, characterized by sensory unresponsiveness, cognitive dullness, and behavioral depression (Engel & Schmale 1972).

Experiment 1 assesses if ingestion of glucose eliminates the shift to conservation-withdrawal normally observed during testing (Plumb et al. 2013; Minor, et al. 1994a; Minor, et al. 1994b). Experiment 2 examines if exposure to a glucose solution following previously-effective hormetic stress training, as described in Chapter One, further facilitates hormesis. Likewise, it examines if glucose consumption following previously-ineffective hormetic training now renders that training effective in eliminating helplessness. Experiment 3 expands the work

of Minor and Saade (1997) and examines if glucose consumption can protect against two consecutive days of traumatic stress.

Method

Experiment 1

A conservation-withdrawal state is an adaptive mechanism for conserving limited resources following trauma exposure and facilitating the eventual recovery of energy homeostasis (Engel & Schmale 1972). As glucose is the energy source of all cells, we postulated that supplementing available glucose following trauma would eliminate the necessity of the conservation-withdrawal state. Experiment 1 aims to determine if post-trauma glucose consumption prevents the shift to conservation-withdrawal normally observed during testing.

Subjects. Thirty two male Sprague-Dawley albino rats (290-320g) from Harlan Laboratories were housed in individual cages with free access to food and water in a room maintained on a 12:12-hour light/dark cycle for one week prior to experimental treatment. Experimentation occurred during the light portion of the cycle. All protocols in this paper were pre-approved by the UCLA IACUC.

Apparatus. Each metal rat cage was equipped with a standard glass (250 ml) water bottle with a rubber stopper and metal spout. Contacts with the spout were recorded when a rat completed a low-voltage circuit between the grounded metal cage and the spout. The glucose cocktail consisted of 40% glucose and 5% sucrose dissolved in tap water (weight/volume).

Stress pretreatment occurred in clear Plexiglas restraining tubes, measuring 23 cm in length and 6 cm in diameter. Adjustable front walls prevented the rats from moving forward in the tubes. A rat's tail extended through the rear door of each tube and was taped to a plastic rod.

Unscrambled electric shocks were delivered from one of four constant-current shock generators (Lafayette Instrument Co., Model 82400) through electrodes attached to the rat's tail with electrode paste and tape. Each tube was housed in a sound-attenuating enclosure containing an exhaust fan that masked extraneous noises. A 7-W house light located in the center of the rear wall of the attenuating enclosure's rear wall provided constant illumination.

Testing occurred in a (45 cm x 20 cm x 20 cm) shuttle box (BRS-LVE model 146-40). The shuttle box was divided into two equal compartments by a metal barrier that had an 8 x 7 cm center opening flush with the grid floor. The floor consisted of 2-mm diameter stainless-steel rods spaced 1.1 cm apart center to center. Scrambled shock was delivered to the grid floor from a Grason-Stadler (Series 700) shock generator. The floor pivoted in the center and a response was recorded when a 300-g rat's front paws touched the center grid in a compartment. Two 6- lamps located in the center of each end wall provided constant illumination. The shuttle box was housed in a sound-attenuating chest, containing an exhaust fan that masked extraneous noise.

Procedure. Rats were assigned randomly to one of four groups of 8 rats each. We exposed two groups (S: shocked) to 100, 1.0 mA variable-duration (mean = 8.0 s; range: 3 to 15 s) inescapable tail shocks on a variable-time 60-s schedule (range: 20 to 150 s) in restraining tubes during a 1.83 hour session. The other two groups (R: restrained) were restrained in tubes for the same time period and received no shock. One S and one R group received free access to water (W: Groups SW and RW) and one S and one R group received free access to the glucose cocktail (G: Groups SG and RG) for 18 hours beginning immediately following the traumatic stress session. We recorded total fluid consumption and licks at the delivery spout during this time period. All rats had free access to water over the next 6 hours.

Testing occurred 24 hours after exposure to traumatic stress in all groups. The test consisted of 5 trials of 5 sec, 0.6mA foot-shock in a shuttle-box. The inter-trial interval was fixed at 60 sec. The number of unconditioned shuttle crossings was measured during each trial. Defensive freezing, defined as the absence of all bodily and vibrissae movement except for that related to respiration, was measured during each inter-trial interval using a time-sampling procedure every 5 seconds.

Experiment 2

Our previous work on hormetic stress provided evidence that three days of moderate shock stress (25 tail shocks) with intermittent days of rest afforded the greatest protection against traumatic stress (100 tail shocks) (Plumb, Cullen & Minor 2014). Three days of moderate stress without rest immediately preceding traumatic stress afforded no protection. Experiment 2 aims to determine if post-stress glucose consumption facilitates hormetic stress training in two ways. First, will post-stress glucose facilitate an already effective hormetic stress procedure (3 days of shock with intermittent days of rest)? Second, will glucose exposure make a previously ineffective hormetic stress procedure (3 days of shock immediately before trauma) now effective in eliminating exaggerated fear conditioning and shuttle-escape deficits following traumatic stress?

Subjects and Apparatus. Thirty-two male Sprague-Dawley albino rats (290-320g) from Harlan Laboratories (Indianapolis, IN) were housed as described in Experiment 1. The apparatus was the same as described above.

Procedure. Rats were assigned randomly to one of four groups of 8 rats each. Groups given glucose underwent 3 days of pre-exposure to the glucose solution, with 7 days intervening between the end of pre-exposure and the start of hormetic training. This was done to establish a flavor preference, as rats often refuse to drink novel substances following stress (Minor and Saade, 1997). All groups received hormetic training in the form of 3, 30-minute sessions (1 session per day) of 25, 1.0 mA variable-duration (mean = 8.0s; range: 3 to 15s) inescapable tail shocks on a variable-time 60-s schedule (range: 20 to 150s) in restraining tubes prior to exposure to traumatic stress. These groups differed with respect to pattern of rest (0 or 3 rest days) and what fluid was available to drink (water or a glucose solution) following each 30-min shock session in a 2x2 factorial design. Each subject was given *ad libitum* access to either 100mL of tap water or 100mL of a concentrated 40% (wt/vol) aqueous glucose solution (with 1 drop of artificial vanilla extract added for a distinct odor) upon immediate return to the home cage at the end of each shock session. The glucose solution was available for a period of 18 hours immediately following the session, and then replaced with tap water for the remaining 6 hours. Two of the four groups were given no days of rest between the shock sessions (one session per day for 3 days): one group was given water following each session (Group swswswS) while the other received 18 hours of the glucose solution (Group sgsgsgS). The remaining two groups were given one day of rest between each session and given either water (Group sw-sw-sw-S) or glucose (Group sg-sg-sg-S) at the end of each session. All four groups received traumatic shock at the end of hormetic training. Traumatic shock consisted of exposure to 100, 1.0mA variable-duration (mean = 8.0 s; range: 3 to 15 s) inescapable tail shocks on a variable-time 60-s schedule (range: 20 to 150 s) in restraining tubes over 1.83 hours.

Shuttle-escape testing occurred 24 hours after traumatic stress. The test consisted of five trials during which a rat had to cross from one side of the central barrier to the other in order to terminate shock (FR-1 trials). These trials occurred on a fixed-time 60-second schedule. A trained observer scored defensive freezing, defined as the absence of all bodily and vibrissae movement except for that related to respiration, during each inter-trial interval using a time-sampling procedure every 5 seconds. FR-1 trials were followed by 25 FR-2 trials during which a rat had to cross from one side of the central barrier and then return to terminate shock. Shock terminated automatically if the appropriate response contingency was not met within 40 seconds of shock onset on a given trial. Escape latencies were recorded on each trial. Shock intensity was set at 0.6 mA with FR-2 trials occurring on a variable time 60-second schedule (range: 20 to 230 seconds); however, three minutes intervened between FR-1 and FR-2 trials (cf., Minor & LoLordo 1984).

Experiment 3

Minor and Saade's (1997) original experiment showed that glucose consumption following traumatic stress prevented PTSD-like symptoms. Experiment 3 investigates post-stress glucose following exposure to two inescapable, traumatic stress sessions 24 hours apart in an effort to push the envelope and assess the boundaries at which glucose is effective.

Subjects and Apparatus. Thirty-two male Sprague-Dawley albino rats (290-320g) were housed as in Experiment 1. The apparatus was the same as described above.

Procedure. All groups received two traumatic shock sessions, with the second session occurring 24 hours after the first. Each session consisted of 100, 1.0mA variable-duration (mean = 8.0 s; range: 3 to 15 s) inescapable tail shocks on a variable-time 60-s schedule (range: 20 to 150 s) in restraining tubes over 1.83 hours. Following each session, rats were returned to their home cage where they had *ad libitum* access to either 100mL of tap water or 100mL of a concentrated 40% (wt/vol) aqueous glucose solution (with 1 drop of artificial vanilla extract added for a distinct odor). The glucose solution was available for a period of 18 hours immediately following trauma, and then replaced with tap water for the remaining 6 hours.

Rats were randomly assigned to one of four groups of 8 rats each. Each group differed on what was available to drink following the two stress sessions (water or the glucose solution) in a 2x2 factorial design. The first group received water following both traumatic shock sessions (Group SwSw). The second group received the glucose solution following both sessions (Group SgSg). The third group received water following the first stress session, and the glucose solution following the second stress session (Group SwSg). The fourth group received the glucose solution following the first stress session, and water following the second stress session (Group SgSw). Shuttle-box testing occurred 24 hours after the second traumatic stress session for all groups.

Results

Experiment 1

Baseline glucose consumption for individual rats ranged between 24.5 and 30 ml. Mean intake was similar among groups and across pre-exposure days. A mixed-design analysis of

variance (ANOVA: Group x Pre-exposure Day) yielded no statistically significant main effects or interactions (Fs < 1.1).

The left panel of Figure 5 shows mean lick totals in each group across 3-hour bins following stress pretreatment. Groups with access to the glucose cocktail (RG & SG) consumed more fluid during the first 3 hours post stress than did their water counterparts (RW & SW).

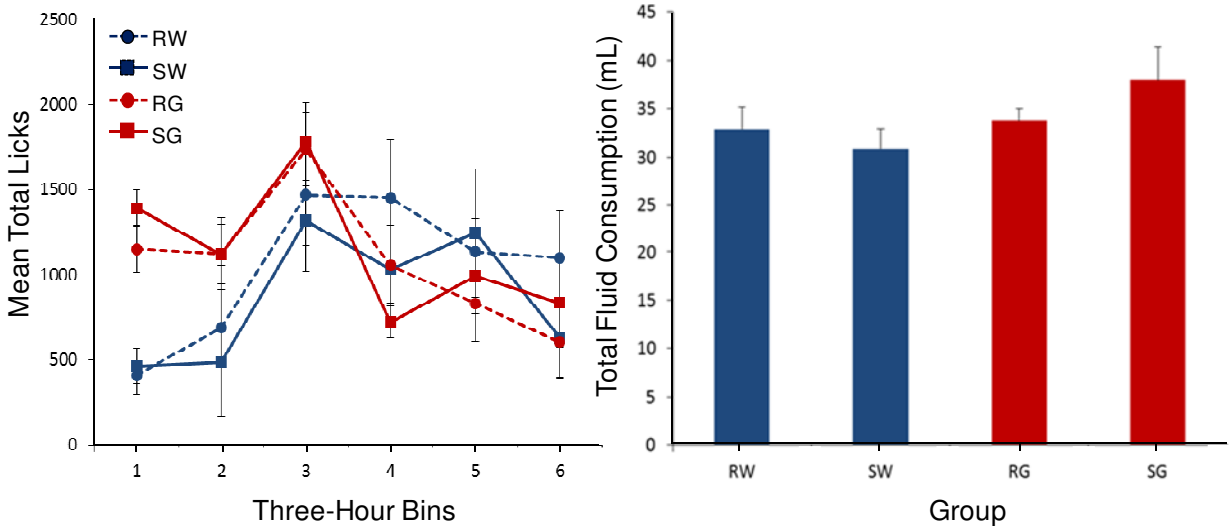

Figure 5. (Left Panel) Mean lick totals were assessed in 3-hours bins immediately following traumatic shock or restraint over a period of 18 hours. (Right Panel) Total fluid consumption in milliliters over an 18-hour period immediately following trauma.

A mixed-design analysis of variance (ANOVA: Stress condition x Fluid condition x Time Bin) yielded a significant main effect of Bin, $F(5, 24) = 13.02$, $p < .000$, and a significant interaction between Fluid condition and Bin, $F(5, 24) = 10.26$, $p < .000$. No other main effects or interactions were statistically significant. Post-hoc comparisons of grand mean lick totals across Bins indicated that drinking behavior was significantly greater during Bin 3 (onset of darkness) that at all other time points. No other contrasts were statistically significant. Newman-Keuls post-hoc comparisons ($\alpha = .05$) of group means at each time point indicated that

groups with access to glucose (RG & SG), which did not differ from one another, engaged in significantly greater drinking behavior during Bin 1 than did the water groups (RW and SW), which did not differ from one another. No other contrasts were statistically significant.

The right panel of Figure 5 shows mean total post-stress fluid consumption in each group. Differences in the pattern of drinking among groups did not affect total fluid consumption. Although Group SG consumed slightly more fluid that did other groups, a two-factor ANOVA (Stress condition x Fluid condition) yielded no statistically significant main effects or interactions.

Test data are shown in Figure 6. The left panel in Figure 6 shows post-trial freezing in each group. Group SW showed excessive levels of freezing from the outset of training. Consuming glucose following the pretreatment shock session eliminated this exaggerated fearfulness such the Group SG performed similarly to restrained controls (Groups RG & RW).

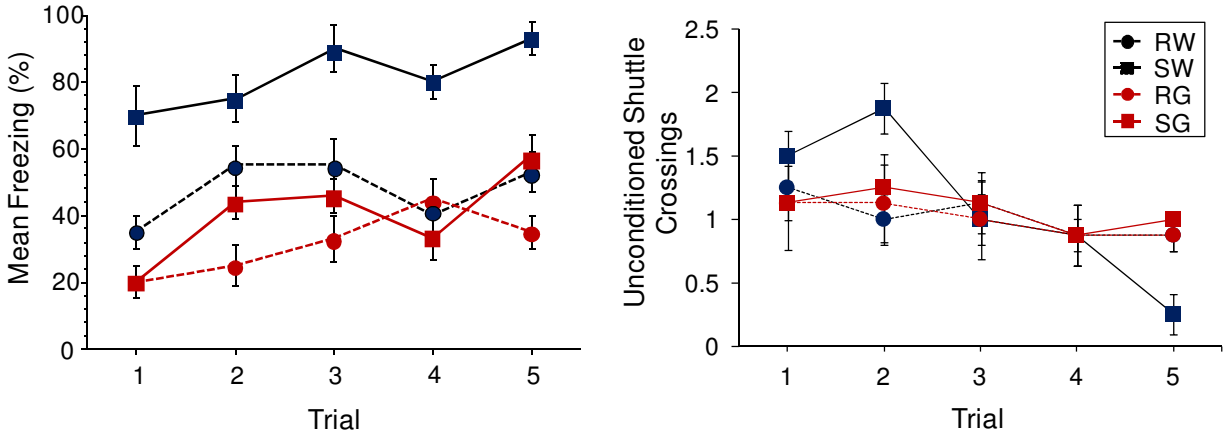

Figure 6. Percent freezing (left panel) and unconditioned shuttle escape crossings (right panel) for 4 groups in Experiment 1. Two groups received 18-hr access to 100mL of glucose following traumatic shock or restraint (Groups SG and RG, respectively). Two additional groups received 100mL of tap water following traumatic shock or restraint (SW and RW, respectively). Shuttle-box testing occurred 24 hours later. Animals were exposed to 5 trials of foot-shock. The number of unconditioned shuttle crossings was measured during each trial and freezing behavior was recorded during the inter-trial interval.

A mixed-design ANOVA (Stress x Fluid x Trial) yielded significant main effects of Stress, $F(1,28) = 7.112$, $p < .02$, Fluid, $F(1,28) = 26.346$, $p < .001$, and Trial, $F(4,112) = 11.96$, $p < .01$, and a significant interaction of Stress x Fluid, $F(1,28) = 19.755$, $p < .001$. Newman-Keuls post-hoc contrasts ($\alpha = 0.05$) on grand mean freezing suggested the following ordered relation among group means: SW > RW = RG = SG.

The right panel of Figure 6 shows unconditioned shuttle crossings in each group. Shocked rats given water post stress (Group SW) showed a pattern of responding suggesting that they were hyper responsive to shock at the start of the test session, but hypo responsive by the end. Consuming glucose following exposure in inescapable shock completely eliminated this pattern of responding: Group SG showed a pattern of unconditioned responses similar to Groups RG and RW.

A mixed-design ANOVA (Group x Trial) yielded a significant main effect of Trial, $F(4,112) = 5.33$, $p < .001$, indicating that number of crossings generally decreased across trials. The Group x Trial interaction was marginally significant, $F(12,112) = 1.84$, $p = .051$. The interaction in these data clearly is not robust, but is nonetheless theoretically important. Thus we conducted a liberal analysis of performance on trials 2 and 5. A single-factor ANOVA (Group) on unconditioned shuttle crossed on trial 2 was not statistically significant, $F(3, 31) = 2.6$, $P < .07$. A similar analysis of data on trial 5, however, yielded a significant main effect of group, $F(3, 31) = 7.9$, $p < .001$. Newman-Keuls post-hoc comparisons ($\alpha = .05$) indicated that Group SW made significantly few crossings that did all other groups, which did not differ from one another.

Experiment 2

The left panel of Figure 7 shows mean percent post-trial freezing in each group. Glucose consumption during hormetic training resulted in reduced fear conditioning relative to those with access to water only (Groups sw-sw-sw-S and swswswS). Giving glucose during hormetic training with interpolated days of rest (Group sg-sg-sg-S) afforded more protection than giving glucose during training with no rest (Group sgsgsgS).

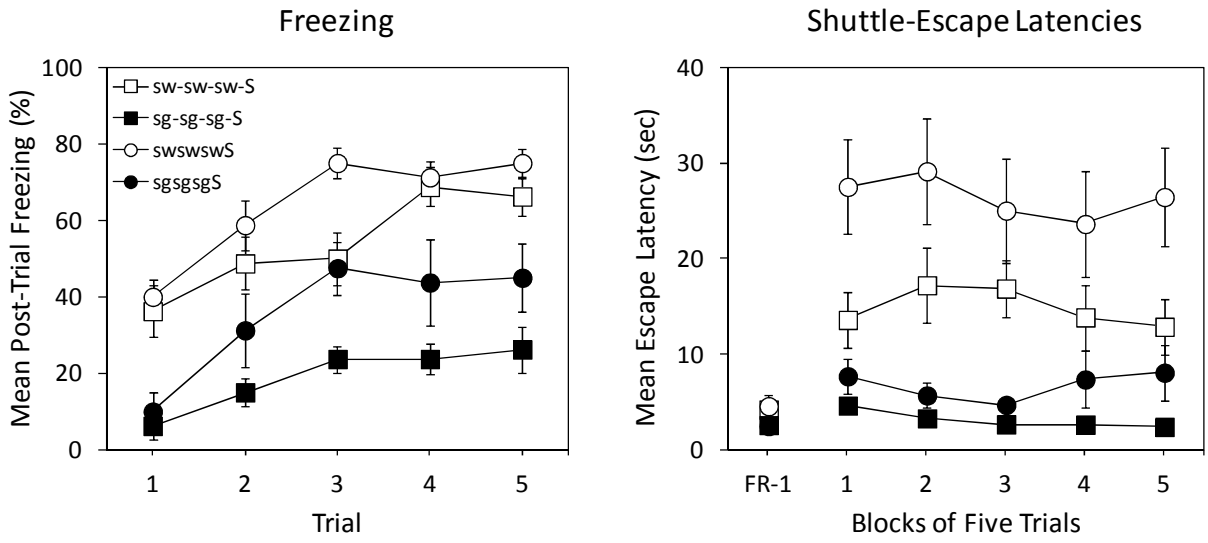

Figure 7. Percent freezing (left panel) and shuttle escape latencies (right panel) for 4 groups in Experiment 2. All groups received hormetic training in the form of 3, 30-minute sessions of 25 inescapable tail shocks (1 session per day) prior to traumatic stress. Group sw-sw-sw-S and Group sg-sg-sg-S received 100mL of water or glucose, respectively, following each hormetic session with interpolated days of rest. Group swswswS and Group sgsgsgS received 100mL of water or glucose, respectively, after each hormetic session with no days of rest. Shuttle-escape testing occurred 24 hours later. Freezing was measured over 5 trials (FR-1) at the start of the shuttle-escape test. Impaired escape performance was assessed over the next 25 trials (FR-2).

A mixed-design analysis of variance (ANOVA: Group x Trial) yielded significant main effects Trial, $F(4, 112) = 26.648$, $p < 0.001$, and Group, $F(3,28) = 27.537$, $p < 0.001$. There was no statistically significant interaction of Group x Trial. Newman-Keuls post-hoc contrasts ($\alpha =$

0.05) on grand mean freezing suggested the following ordered relation among group means: swswswS = sw-sw-sw-S > sgsgsgS > sg-sg-sg-S.

The right panel of Figure 7 shows mean escape latencies across blocks of five trials in each group. FR-1 escape latencies did not differ among groups, $F < 1$. Group swswswS exhibited the standard helplessness effect, as indicated by large escape deficits. Water consumption following hormetic training with interpolated rest afforded a moderate amount of protection (sw-sw-sw-S). Glucose consumption during hormetic training eliminated helplessness, regardless of pattern of rest.

A mixed-design ANOVA (Group x Trial Block) on FR-2 escape latencies yielded a significant main effect of Group, $F(3, 28) = 14.16$, $p < .001$. The main effect of Trial Block and the interaction between Group and Trial Block were not statistically significant. Newman-Keuls post-hoc contrasts ($\alpha = 0.05$) on grand mean FR-2 escape latencies suggested the following ordered relation among group means: swswswS > sw-sw-sw-S > sgsgsgS = sg-sg-sg-S.

Experiment 3

The left panel of Figure 8 shows mean percent post-trial freezing in each of the four groups in Experiment 3. Group SwSw showed exaggerated fear conditioning throughout the five FR-1 trials compared to all other groups.

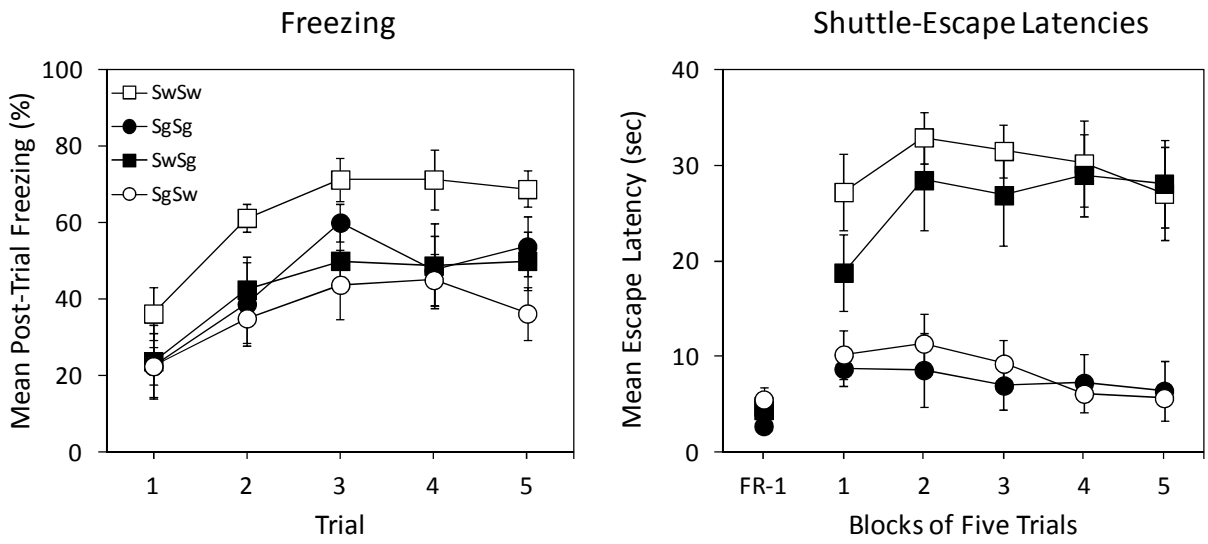

Figure 8. Percent freezing (left panel) and shuttle escape latencies (right panel) for 4 groups in Experiment 3. All groups were exposed to two traumatic stress sessions 24 hours apart. Two groups received either 100mL of water or 100mL of a concentrated 40% (wt/vol) aqueous glucose solution following both trauma sessions (Group SwSw and SgSg, respectively). One group received water following the first trauma session and glucose following the second trauma session (Group SwSg). The remaining group received glucose following the first trauma session and water following the second session (Group SgSw). Shuttle-escape testing occurred 24 hours later. Freezing was measured over 5 trials (FR-1) at the start of the shuttle-escape test. Impaired escape performance was assessed over the next 25 trials (FR-2).

A mixed-design ANOVA (Group x Trial) yielded significant main effects of Group, $F(3, 28) = 4.163$, $p < 0.02$, and Trial, $F(4, 112) = 20.047$, $p < 0.001$, but no significant Group x Trial interaction. Newman-Keuls post-hoc contrasts ($\alpha = 0.05$) on grand mean freezing suggested the following ordered relation among group means: SwSw > SgSg = SwSg = SgSw.

The right panel of Figure 8 shows mean escape latencies across blocks of five trials in each group. FR-1 escape latencies did not differ, $F < 1$. Glucose consumption following the first traumatic stress session dramatically improved escape performance, regardless of the type of liquid consumed after the second stress session. Consuming glucose following only the second traumatic stress session did not improve performance.

A mixed-design ANOVA (Group x Trial Block) yielded a significant main effect of Group, $F(3, 28) = 15.513$, $p < 0.001$, and Trial, $F(4,112) = 3.606$, $p < .01$, and a significant Group x Trial Block interaction, $F(12, 112) = 2.492$, $p < 0.01$. Newman-Keuls post-hoc contrasts ($\alpha = 0.05$) on grand mean FR-2 escape latencies suggested the following ordered relation among group means: SwSw = SwSg > SgSg = SgSw.

Discussion

The experiments described above provide evidence that post-stress glucose consumption is a simple and effective method of building stress resilience. Traumatic stress exposure is followed by a conservation-withdrawal state in which behavioral depression is a prominent symptom (Plumb et al. 2013). Allowing rats to drink a glucose solution following traumatic stress prevented the shift to conservation-withdrawal that is normally observed during subsequent shuttle-escape testing. Post-stress glucose also facilitates hormetic stress training and renders previously ineffective training procedures now effective in preventing behavioral depression. Lastly, it was discovered that glucose consumption can build resilience to back-to-back traumatic stress sessions. Interestingly, glucose consumption following the first trauma was the critical factor in building resilience to the second trauma. The fluid consumed following the second trauma was irrelevant.

Experiment 1 showed that rats exposed to inescapable shock and given water during training show a different pattern of responding during 5, fixed-duration shocks at test than restraint control groups, and even inescapably-shocked rats given glucose. These rats made a greater number of responses during the first two trials than all other groups. They then rapidly

decreased their responding over the remaining trials, resulting in a lower number of responses at Trial 5, relatively. We have argued that this signals a shift in state from anxiousness and agitation to one of conservation-withdrawal, and consuming glucose following inescapable shock eliminates this shift. An alternative explanation for these data may be that because they start out making a larger number of responses, the rats learned more rapidly during the first few test trials that their actions had no consequences (a zero response-outcome contingency), and simply gave up on the task. This explanation is consistent with the original learned helplessness hypothesis that argues for a combination of motivation, emotional and cognitive processes that result from a perceived lack of control over the environment (Maier and Seligman, 1976; Maier, Seligman and Solomon, 1969).

This alternative explanation is plausible for this particular experiment, but falls apart when you consider the larger literature. Standard shuttle-box testing consists of 5 FR-1 trials followed by 25 FR-2 trials. Three minutes separate the end of FR-1 and the beginning of FR-2. Inescapably-shocked (IS) rats perform similarly to restraint controls during all FR-1 trials; however, a large discrepancy is observed on the first trial of FR-2, with IS rats showing high escape latencies relative to the controls. In this case, all groups are exposed to the same positive response-outcome contingency during the FR-1 trials, yet they show this rapid drop in performance at the start of FR-2 trials.

The data from standard shuttle-box testing supports the theory that IS rats enter the test phase highly anxious and agitated, and rapidly transition in state to one of conservation-withdrawal during the 3-min interval between FR-1 and FR-2 trials. Support for this comes from work by Minor and his colleagues on adenosine-mediated conservation-withdrawal (Minor,

Huang & Witt 2006; Plumb, Sterlace, Cavanaugh & Minor 2013; Minor, Chang & Winslow 1994; Minor, Winslow & Chang 1994; Hanff, Furst & Minor 2010; Minor, Rowe, Cullen & Furst 2008). In one experiment, rats exposed to inescapable shock were given the anxiogen caffeine before test. This was predicted to facilitate the transition to conservation-withdrawal, but instead, rats given caffeine showed enhanced escape performance (Minor, Chang & Winslow, 1994). This led to an examination of adenosine as a mediator of conservation-withdrawal, as caffeine is a well-known adenosine receptor antagonist (Snyder, Katims, Annau, Bruns & Daly, 1981). They went on to show that blocking adenosine before test prevented the escape deficits normally observed in IS rats (Minor, Rowe, Cullen & Furst, 2008). Likewise, providing restraint controls with adenosine agonists induced shuttle-escape deficits (Minor, Winslow & Chang, 1994). Together, these data indicate that the transition in state during test to conservation-withdrawal is mediated by adenosine signaling.

There are a number of potential mechanisms through which post-stress glucose consumption can build resilience to traumatic stress. Chapter One highlighted the importance of rest between stressor exposure if the stressor was severe. This provided support for McEwen and colleagues (McEwen & Stellar 1993; McEwen & Gianaros 2011) who argue that rest is necessary to repair the damage inflicted by traumatic stress and allows for adequate recovery of anabolic hormones. Post-stress glucose consumption may significantly reduce the amount of rest time needed between stressors. As evidenced by Experiment 2, no rest day was needed between stress sessions as long as the stress was immediately followed by glucose consumption. Perhaps post-stress glucose allows for a quicker recovery to baseline before the next stress occurs, reducing the functional severity of the stress. In Experiment 3, the first trauma now becomes hormetic for the second trauma.

Another possible explanation for the effects of post-stress glucose on helplessness is increased context discrimination. Minor and LoLordo (1984) provided evidence that if the animal can discriminate the training context from the testing context, the helplessness effect is eliminated. Glucose may be allowing veridical encoding of the context, resulting in less generalization between the two contexts.

Glucose may also alter orthogonal mechanisms like neuropeptide Y (NPY). NPY is an anxiolytic peptide that inhibits both the hypothalamic pituitary adrenal cortical axis (HPAC) and sympathetic adrenal medullary (SAM) axis (Heilig 2004). Wang et al. (1999) provided evidence that consumption of a glucose solution elevated NPY mRNA and peptide immunoreactivity in the paraventricular nucleus (PVN) of the hypothalamus as well as the arcuate nucleus, which has projections to the PVN. Post-stress glucose consumption may be reducing the functional severity of the stressor through the release of NPY.

Lastly, perhaps the benefit of post-stress glucose consumption comes from its ability to prevent metabolic exhaustion. Fear is an intensely catabolic state, and results in exaggerated neuronal activity. Under these circumstances, adenosine is released to inhibit further activity in an effort to prevent cell death. Minor and colleagues (Minor, Huang & Witt 2006; Plumb, Sterlace, Cavanaugh & Minor 2013; Minor, Chang & Winslow 1994; Minor, Winslow & Chang 1994; Hanff, Furst & Minor 2010; Minor, Rowe, Cullen & Furst 2008) have shown that adenosine A_{2A} receptors are involved in the conservation-withdrawal symptoms normally observed following traumatic stress. Glucose consumption following trauma might restore metabolic homeostasis, eliminating the necessity for the compensatory adenosine response.

Chapter Three attempts to narrow down the locus of action of adenosine in the central nervous system.

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CHAPTER THREE

Adenosine A_{2A} Receptors in the Nucleus Accumbens Mediate Conservation-Withdrawal Following Traumatic Stress

Abstract

Traumatic inescapable shock in the learned helplessness paradigm results in exaggerated fear responding and deficits in escape performance when rats are tested 24 hours later. Previous research has indicated that brain adenosine signaling is a critical mediator of these deficits. Activation of A_{2A} receptors appears to be particularly critical to this process. A_{2A} receptors have a limited distribution in the brain, being primarily located in the striatum. Rats were implanted with bilateral cannulae into either the core (bregma: AP +1.2, ML $\pm$1.5, DV -6.5) or shell (bregma: AP +1.2, ML $\pm$1.5, DV -7.5) of the accumbens during stereotaxic surgery. Rats were then exposed to either traumatic inescapable tail shock or simple restraint following recovery from surgery. Shuttle-box testing occurred 24 hours later. Ten minutes prior to test, rats were infused with the adenosine A_{2A} receptor antagonist CSC (8-(3-chloro-styrl) caffeine) or vehicle. Blockade of A_{2A} receptors in both the core and shell of the accumbens after inescapable shock failed to reverse the exaggerated fear conditioning but completely reversed the deficits in escape performance. These data may have important implications for the treatment of Post-traumatic Stress Disorder (PTSD) with comorbid depression as the learned helplessness effect has long been thought of as an animal model of these disorders. The evidence provided that adenosine signaling in the nucleus accumbens mediates helplessness through shifts in motivational states implies a critical role in stress-induced disorders.

Introduction

The learned helplessness procedure is a traditional method for analyzing the effects of acute, traumatic stress and modeling related symptoms of post-traumatic stress disorder and comorbid major depression in rats (Minor, Plumb, Schell & Pham, 2011). The experiment consists of two phases in which rats are exposed to a series of escapable electric shocks, yoked inescapable shocks, or simple apparatus restraint in the pretreatment phase (Minor & LoLordo, 1984). The restrained group provides a baseline from which any effect of stressor controllability can be assessed during later testing. Although the nature of the test varies with the interests of the experimenter, the traditional measure of helplessness has been performance in a shuttle-escape task conducted 24 hr after stress pretreatment.

Rats pre-exposed to escapable shock in the pretreatment phase perform as efficiently as restrained controls during escape testing 24 hours later. By contrast, rats pre-exposed to yoked inescapable shock show severe impairment, with near-maximum escape latencies during shuttle-escape testing. This general pattern among groups holds for a wide variety of behavioral and biological stress indexes. Moreover, because escapably and inescapably shocked rats receive the same pattern, intensity, and durations of shock during pretreatment, the differential performance of these two groups in the test phase provides unequivocal evidence that some psychological variable related to behavioral control, or lack thereof, modulates the impact of the shock stressor (Overmier & Seligman, 1967).

Recent analyses of the psychological aspects of helplessness clearly implicate that animals exposed to inescapable shock suffer a prolonged, intense fear state while those receiving escapable shock suffer from dramatically lower levels of fear (Minor, Dess & Overmier, 1991).

The inter-trial intervals are highly variable with no signal conditions that allow the rats to predict when the shocks will begin or end. In the absence of clear signals for safety, rats are biased to remain chronically afraid. By contrast, stimuli generated during the act of escaping signal the termination of shock and a shock-free period. The consequence is that the overall aversiveness of the shock and the fear experienced during the intertrial interval are reduced (Weiss & Simson, 1985; Balleine & Curthoys, 1991; Minor, Pelleymounter & Maier, 1988; Hunter, Balleine & Minor, 2003). Fear is an intensely catabolic state and the consequences of maintaining this state appear to result in the loss of a variety of regulator mechanisms that render the rat highly fearful and vulnerable to subsequent stressors.

Animals receiving inescapable shock enter the test phase in a highly anxious/agitated state (Maier, 1990; Minor, 1990). Re-exposure to mild stress during testing provokes an excessive neural and behavioral fear response. Helpless rats rapidly transition from this catabolic state to one of *conservation- withdrawal*, characterized by sensory unresponsiveness, cognitive dullness, and behavioral depression. We have linked this transition in behavioral and emotional state to an increase in brain adenosine signaling.

Adenosine Neuromodulation

Figure 9 shows a generic synaptic cleft in the CNS as a means of summarizing main features of adenosine neuromodulation. The nucleoside adenosine is derived from the nucleotide adenosine-5'-triphosphate (ATP) under intense neural activation. ATP is the energy source of all cells, driving metabolism. Under sustained activation, ATP is broken down into adenosine both intracellularly and extracellularly. Adenosine is derived from the dephosphorylation of ATP intracellularly and is then extruded into extracellular space by bidirectional transporters.

Extracellular ATP is broken down into adenosine by enzyme families of ecto-nucleotidases (Burnstock, 2006; Vorhoff, Zimmermann, Pelletier, Sevigny and Braun, 2005). Activation of these enzymes is dependent on changes in pH of the surrounding space. As the level of neural activation increases, pCO2 concentrations rise and result in the acidification of extracellular space. This drop in pH disinhibits the ecto-nucleotidases, allowing for the breakdown of ATP and an increase in extracellular adenosine concentrations (Cunha, Sebastioa and Ribeiro, 1998; Dulla, Dobelis, Pearson, Frenguelli, Staley, and Masino, 2005; Dunwiddie, Diao and Proctor, 1997; Langer, Hammer, Kosalka, Schrader, Robson and Zimmermann, 2008; Vorhoff et al., 2005). Adenosine then acts to inhibit neurons by binding to receptors pre- and post-synaptically in an effort to prevent the utilization of all available ATP and ultimately, cell death (Dunwiddie, 1985).

Adenosine exerts its action at four G-protein coupled receptors: A_1, A_{2A}, A_{2B}, and A_3 (see Haas and Selbach, 2000 for a review). A_1 receptors are widely distributed throughout the brain and mediate adenosine's inhibitory actions (i.e., presynaptic inhibition of neurotransmitter release or postsynaptic hyperpolarization). A_2 receptors mediate the excitatory action of adenosine (i.e., exciting inhibitory GABAergic neurons). The A_{2A} receptor subtype is a high-affinity receptor that is localized primarily in the indirect pathway of the striatum (Svenningsson, Le Moine, Fisone, and Fredholm, 1999; Rosin et al., 1998). Limited concentrations are also found in the thalamus (Mishina et al., 2007), nucleus tractus solitarius (Scislo and O'Leary, 2006), and reticular formation (Coleman, Baghdoyan, and Lydic, 2006). The A_{2B} subtype is low-affinity and is distributed throughout the brain. A_3 receptors are found mainly in the peripheral nervous system and play an important role in regulating inflammatory processes (Gessi et al., 2008). The receptor-mediated actions of adenosine are mimicked by a number of synthetic

adenosine analogs that vary in their affinity for subtypes of the adenosine receptor. Moreover, methylxanthine stimulants (caffeine; theophylline) are nonselective high-affinity antagonists of the adenosine receptor, and derive their immediate stimulant properties by disinhibiting neurons from adenosine regulation during states of fatigue or depressed mood (Minor, Dess & Overmier, 1991; Mohta, Sethi & Tyagi, 2003; Svenningsson, Lindskog & Rognoni, 1998; Seligman, 1975).

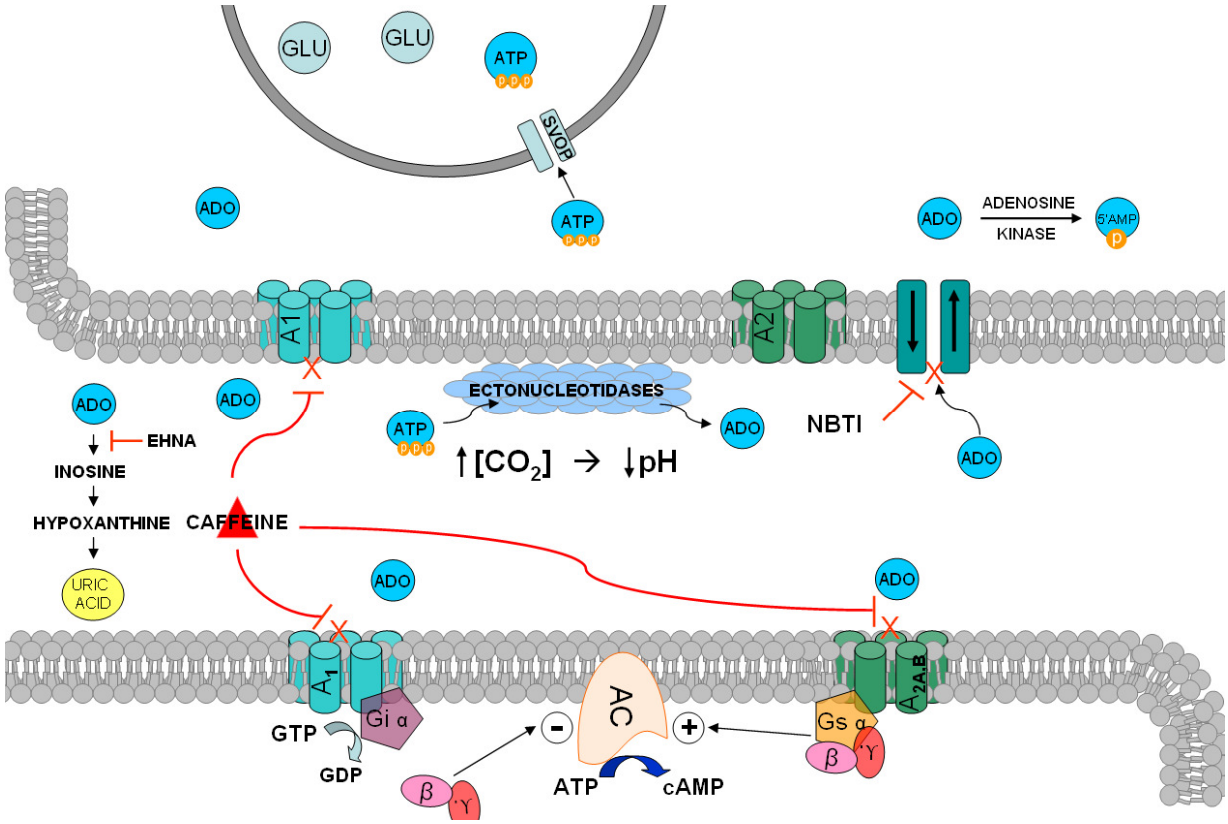

Figure 9. Regulation of adenosine in the synaptic cleft. Build up of adenosine in the synaptic cleft occurs by 1) conversion of ATP to ADO by ectonucleotidases when triggered by increased levels of CO2 or 2) through a bidirectional transporter after release from synaptic vesicles and dephosphorylation from ATP. Synaptic levels of ADO can be decreased by 1) Conversion of ADO into Inosine, hypoxanthine, and Uric Acid, which can be blocked by Inosine Deaminase. 2) ADO binding to Adenosine receptors A1, A_{2A}, A_{2B}. Caffeine acts as ADO receptor antagonist to increase ADO levels. 3) The bidirectional transporter also decreases ADO levels; this pathway can be blocked by EHNA. ADO receptor binding results in release of G protein ϒ and β units; this regulates levels of cAMP in postsynaptic space through binding to Adenylyl Cyclase.

Adenosine Signaling and Conservation-Withdrawal

Engel and Schmale (1972) argued that periods of intense activation, stress or anxiety are automatically and unconditionally followed by a transition to a state termed *conservation-withdrawal*. The sensory unresponsiveness, behavioral depression, and cognitive dullness that characterize the state were considered to be adaptive mechanisms for husbanding limited resources and facilitating the eventual recovery of energy homeostasis.

This proposition was presented, in part, as an explanation for the learned helplessness effect. Sensory unresponsiveness, cognitive dullness, and behavioral depression are reasonably good descriptors of the depression-like component of helplessness. Moreover, the symptoms of conservation-withdrawal are a good description of the fatigue component of major depression (Minor, Huang & Witt, 2006). Finally, the idea that conservation-withdrawal follows a period of intense catabolic output is consistent with the shift in behavior and emotional state that occurs in inescapably shock rats at the time of testing.

Despite these positive features of the construct, conservation-withdrawal has had little impact of theorizing in the helplessness paradigm. Part of the problem is that there was no know neural mechanism to produce such an instinctive, unconditional outcome. Nonetheless, the characterization of adenosine's function in the nervous system corresponds closely with Engel and Schmale's (1972) concept of conservation-withdrawal; indeed, adenosine's function appears to be the cellular equivalent of the behavioral state. The nucleoside appears to be capable of producing the global characteristics of conservation-withdrawal. The ability of adenosine and its analogs to inhibit spontaneous motor activity (behavioral depression) is well established (Mohta, Sethi & Tyagi, 2003; Weiner, 1983; Huang, Jiang, Hao & Minor, 2004; El Yacoubi, Costentin &

Vaugeois, 2003). Adenosine also is known to mediate the spinal effects of morphine (Huang & Minor, 2003) and participate in antinociception (Kent, Rodriguez, Kelly & Dantzer, 1994), certainly a form of sensory unresponsiveness that is relevant to helplessness (Milusheva et al, 1990). Finally, the inhibitory action of the nucleoside is implicated in cortical spreading depression (Burns, 1991; Ikemoto, 2002; Fuxe & Agnati, 2003; Overmier & Hellhammer, 1988) and tonically regulates hippocampus (Selye, 1946; Kern, Lamb & Reed, 1988). An increase in adenosine's receptor-mediated action could be responsible for the impaired learning or cognitive dullness that characterizes a state of conservation-withdrawal.

Role of Striatum in Behavioral Depression

A systematic analysis of the adenosine receptor subtype mediating escape deficits in the helplessness paradigm clearly implicates the A_{2A} receptor. Minor et al (2008) mimicked the effects of inescapable shock using NBTI, a nucleoside transport blocker. They then attempted to reverse the effects using adenosine A_1 and A_{2A} receptor antagonists, DPCPX (8-Cyclopentyl-1,3-Dipropylxanthine) and CSC (8-(3-chloro-styrl)caffeine) respectively. They determined that NBTI and shock-induced deficits in escape performance are reversed by pretest administration of the highly selective A_{2A} antagonist but not the adenosine A_1 antagonist.

The A_{2A} receptor subtype has a very limited distribution in brain, being expressed primarily on the dendritic spines of enkephalin-containing GABAergic neurons in the nucleus accumbens. Adenosine signaling in this region is linked to dopamine signaling in a manner that appears to functionally regulate the integration of motivation with ongoing behavior.

Function of the Striatum

The striatum (caudate, putamen, nucleus accumbens, and olfactory tubercle) is the most prominent structure of the basal ganglia and plays a key role in motor movement. This structure is highly complex in that it receives concurrent input from a number of brain regions, including the cortex, thalamus, and limbic system, and integrates these incoming signals to form a wide array of complex behavior.

Functionally distinct regions of the striatum are observed. Originally it was thought that the dorsal and ventral aspects where responsible for sensorimotor behaviors and emotionally-mediated behaviors, respectively, based upon their diverse connections to different regions (Heimer and Wilson, 1975). Recent findings have molded this view and indicate a much more complex system than originally thought. The functions of the dorsal and ventral striatum can be further broken down into medial and lateral components (Yin and Knowlton, 2004; Yin, Knowlton, and Balleine, 2004; Yin et al., 2008; Ena, de Kerchove d'Exaerde and Schiffmann, 2011). The dorsal medial striatum (DMS) is involved in the associative aspects of behavior and is necessary for goal-directed action. Recurring response-outcome associations formed by the DMS come under the control of the dorsal lateral striatum (DLS) where habitual behavior is produced (Balleine and O'Doherty, 2010; Corbit, Nie and Janak, 2012). The DLS performs the more traditional sensorimotor aspects associated with the striatum.

The ventral striatum, or nucleus accumbens as it is more often referred to, can be functionally divided into the core (ventral lateral striatum) and shell (ventral medial striatum). Neither region of the accumbens is necessary to form instrumental associations (Corbit, Muir and Balleine, 2001; de Borchgrave, Rawlins, Dickinson and Balleine, 2002), but are differentially necessary for performance of the instrumental association, as well as the formation of

associations between stimuli (Corbit and Balleine, 2011; Shiflett and Balleine, 2010). Furthermore, both the shell and core encode different hedonic aspects of rewards or outcomes (Yin et al., 2008; Laurent, Leung, Maidment and Balleine, 2012). The core encodes the general emotional qualities of outcomes while the specific sensory qualities are mediated by the shell.

The core and shell of the nucleus accumbens are integrated into the limbic network through a number of connections with different limbic structures. The emotional and motivational information received from this network is combined with ongoing behavior in these regions (Mogenson, Jones and Yim, 1980; Ferré et al., 2007). As such, the accumbens sub-regions have been greatly studied for their individual roles in addiction and reward-mediated behavior (Ostlund, Wassum, Murphy, Balleine and Maidment, 2011; Di Chiara, 2002; Parkinson, Olmstead, Burns, Robbins and Everitt, 1999).

Striatal Adenosine, Dopamine, and Metabotropic Heteromers

The striatum's role in complex behavior is a point of great interest to scientists across disciplines. This structure exerts its function by the use of medium-sized spiny GABAergic neurons, which make up more than 90% of the striatal neuronal population. These neurons exert behavioral control by two distinct efferent pathways, the direct and indirect pathways (Herrero, Barcia and Navarro, 2002).

The dopaminergic input to the striatum is of critical importance when discussing striatal control of behavior. Dopamine from the substantia nigra binds to either D_1 or D_2 receptors in the dorsal striatum, exciting opposing pathways. These pathways have been labeled the striatonigral and striatopallidal pathways, respectively. Tonic activation of the striatonigral pathway (also known as the direct pathway) facilitates behavior by sending excitatory signals from the

thalamus to the cortex. The striatopallidal pathway (also known as the indirect pathway) is responsible for the inhibition of behavior by inhibiting thalamic output. The ventral tegmental area (VTA) sends dopaminergic input to the nucleus accumbens through the mesoaccumbens pathway. Here it exerts the same influence over the direct and indirect pathways as the substantia nigra input.

Interestingly, the action of dopamine on these two pathways is opposite; dopamine binding at D_1 receptors excites the striatonigral pathway while binding at D_2 receptors inhibits the striatopallidal pathway. These two distinct pathways of the striatum facilitate movement when dopamine is introduced by excitation of the direct pathway and disinhibition of the indirect pathway (for reviews see Gerfen and Surmeier, 2011; Ferré et al., 2008; Haber, 2008). Recent evidence suggests that the dopaminergic signal from the substantia nigra and the ventral tegmental area may be serving different functions due to the projections to the functionally distinct areas of the striatum (Yin et al., 2008). The nigrostriatal pathway may reflect the value of performing an instrumental action as it mainly serves the dorsal striatum. The mesoaccumbens pathway, as it serves mainly the nucleus accumbens, may reflect a different function where it encodes the value of motivational states. The function of dopamine in the striatum is exceedingly complex and is still under much investigation.

Dopamine is a key component in the striatum's contribution to the learning and expression of behavior. However, the expression of dopamine is often modulated by several other factors. One key factor is adenosine, which is found coupled with dopamine throughout the striatum. Adenosine A_{2A} receptors are found mainly on the dendritic spines of enkephalin-containing GABAergic neurons in receptor complexes known as trimeric heteromers. These heteromers contain receptors for adenosine (A_{2A}), dopamine (D_2), and metabotropic glutamate

($mGLU_5$) and are mainly found postsynaptically at glutamatergic synapses (Ferré et al., 1997; Ferré et al., 2007). Adenosine and dopamine are involved in an antagonistic relationship within these heteromers where bound adenosine decreases the binding affinity of dopamine (Schultz, 2002). Moreover, glutamate may play a complex role in this relationship where it acts synergistically with adenosine to modulate dopamine binding (Ferré et al., 2002; Ferré et al., 2005; Anisman and Sklar, 1979). Adenosine A_{2A} receptors are also found to be colocalized with adenosine A_1 receptors presynaptically, but their influence on behavior is still unclear.

Adenosine and dopamine heteromers are distinguished between the two pathways of the striatum; A_1 and D_1 receptor complexes are found mainly throughout the direct pathway, while A_{2A} and D_2 receptor complexes are distributed in the indirect pathway. Adenosine exerts its influence on behavior by blocking the disinhibition of the indirect pathway provided by the dopamine signal. This results in the excitation of the indirect pathway and an overall inhibition of behavior. In conservation withdrawal, there appears to be potent activation of the indirect pathway. Given the evidence that the A_{2A} receptor is contributing to conservation withdrawal, the trimeric heteromer containing A_{2A}, D_2 and $mGLU_5$ receptors in the indirect pathway is of particular interest in the learned helplessness effect.

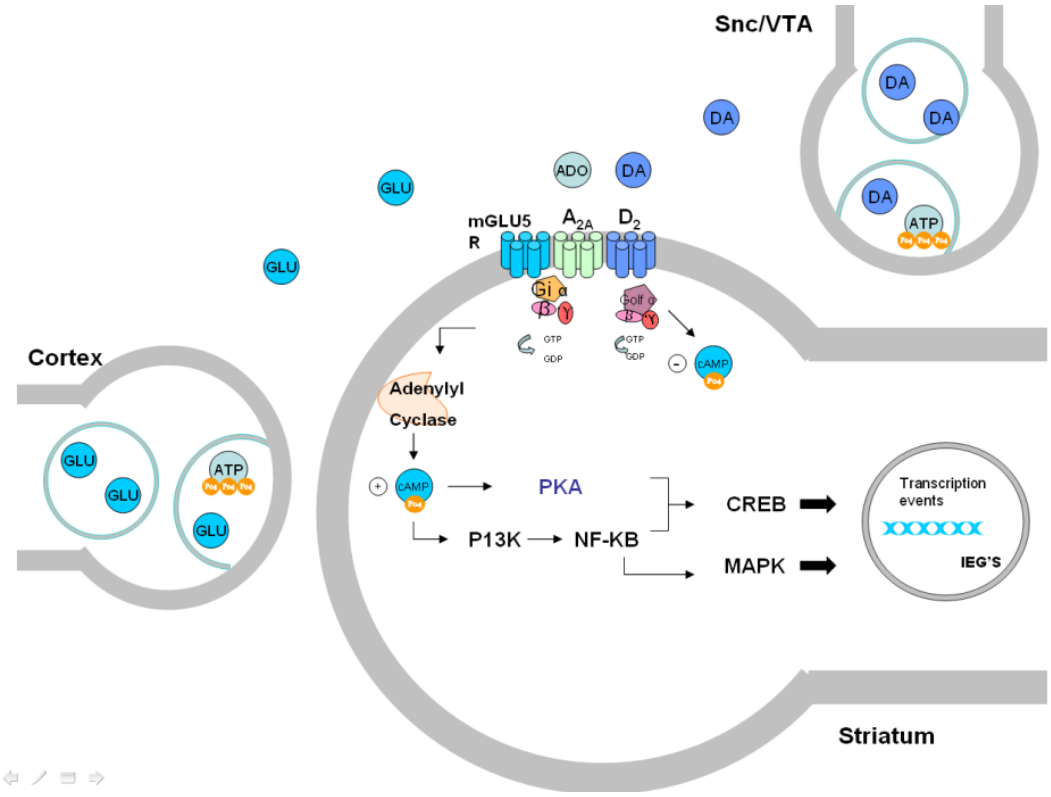

Figure 10. The striatum receives glutamatergic inputs from the cortex and dopaminergic inputs from the ventral tegmental area (VTA) and substantia nigra pars compacta (Snc). Glutamate $mGLU_5$ and dopamine D_2 receptors bond with adenosine receptors A_{2A} in the ventral striatum to form a metabotropic heteromer. Activation of the A_{2A} receptor by adenosine decreases the affinity for dopamine binding on the D_2 receptor. GLU – glutamate; DA – dopamine; ADO – adenosine; ATP – adenosine triphosphate.

Figure 10 reviews the relationship between adenosine and dopamine in the striatum. The antagonistic interaction of A_{2A} and D_2 receptors in the nucleus accumbens is likely to be responsible for the uncoupling of motivation from ongoing behavior often seen in behavioral depression (Minor, Huang & Witt, 2006). Therefore, blocking adenosine's action in this area should allow the motivational signal to be transmitted to performance, thereby eliminating the learned helplessness effect. The following experiments tested this hypothesis by injecting rats exposed to inescapable shock with an adenosine A_{2A} antagonist into the core (Experiment 1) or shell (Experiment 2) of the nucleus accumbens.

Method

Experiment 1

Subjects. Thirty-two Sprague-Dawley albino rats (290-320g) from Harlan Laboratories were housed in individual cages with free access to food and water in a room maintained on a 12:12-hour light/dark cycle for one week prior to experimental treatment. Experimentation occurred during the light portion of the cycle. All protocols in this paper were pre-approved by the UCLA IACUC.

Apparatus. Traumatic stress occurs in clear Plexiglas restraining tubes, measuring 23 cm in length and 6 cm in diameter. Adjustable front walls prevent the rats from moving forward in the tubes. A rat's tail extends through the rear door of each tube and is taped to a plastic rod. Unscrambled electric shocks are delivered from one of four constant-current shock generators (Lafayette Instrument Co., Model 82400) through electrodes attached to the rat's tail with electrode paste and tape. Each tube is housed in a sound-attenuating enclosure containing an exhaust fan that masks extraneous noises. A 7-W house light located in the center of the rear wall of the attenuating enclosure's rear wall provides constant illumination.

Escape testing occurs in a (45 cm x 20 cm x 20 cm) shuttle box (BRS-LVE model 146-40). The shuttle box is divided into two equal compartments by a metal barrier that has an 8 x 7 cm center opening flush with the grid floor. The floor consists of 2-mm diameter stainless-steel rods spaced 1.1 cm apart center to center. Scrambled shock is delivered to the grid floor from a Grason-Stadler (Series 700) shock generator. The floor pivots in the center and a response is recorded when a 300-g rat's front paws touches the center grid in a compartment. Two 6-W

lamps located in the center of each end wall provides constant illumination. The shuttle box is housed in a sound-attenuating chest, containing an exhaust fan that masks extraneous noise.

Procedure. Rats underwent stereotaxic surgery where they were anesthetized with sodium pentobarbital (50 mg/kg) and treated with a local numbing agent (Bupivocaine; 15mg/kg) on the skull. They were then implanted with bilateral 28-gauge cannulae in the nucleus accumbens core (bregma: AP +1.2, ML $\pm$1.5, DV -6.5). Dental cement and skull screws were used to keep the cannulae in place following surgery. Recovery time consisted of seven to ten days following surgery.

Rats were randomly assigned to one of four groups of 8 rats each following recovery from surgery. Two groups received traumatic restraint stress which consisted of restraint in a tube for 1.83 hours. The remaining two groups received traumatic shock stress, which consisted of 100, 1.0 mA variable-duration tailshocks (mean = 8.0 s; range: 3 to 15 s) on a 60 second variable time schedule (range: 20 to 150s) in restraining tubes. Shuttle-box testing occurred 24 hours later. Fifteen minutes before test, all rats were injected with either 0.3µL of a 30nM solution of the adenosine A_{2A} antagonist 8-(3-chloro-styrl)caffeine (CSC) or vehicle dimethyl sulfoxide (DMSO) in a 2x2 factorial design.

Shuttle-box testing consisted of five trials during which a rat had to cross from one side of the central barrier to the other in order to terminate shock (FR-1 trials). These trials occurred on a fixed-time 60-second schedule. Defensive freezing, defined as the absence of all bodily and vibrissae movement except for that related to respiration, was measured during each intertrial interval using a time-sampling procedure every 5 seconds (Fanselow, 1980). FR-1 trials were followed by 25 FR-2 trials during which a rat had to cross from one side of the central barrier

and then return to terminate shock. Shock terminated automatically if the appropriate response contingency was not met within 40 seconds of shock onset on a given trial. Shock intensity was set at 0.6 mA with FR-2 trials occurring on a variable time 60-second schedule (range: 20 to 230 seconds); however, three minutes intervened between FR-1 and FR-2 trials.

Experiment 2

Subjects and Apparatus. Thirty-two Sprague-Dawley albino rats (290-320g) were housed as described in Experiment 1. The apparatus was the same as described above.

Procedure. Rats underwent stereotaxic surgery as described in Experiment 1. For Experiment 2, bilateral cannula were placed in the nucleus accumbens shell (bregma: AP +1.2, ML $\pm$1.5, DV -7.5). All other procedures were the same as described above.

Results

Experiment 1

The left panel of Figure 11 shows mean percent post-trial freezing for each of the five FR-1 trials. Rats exposed to inescapable shock and treated with DMSO vehicle shortly before testing showed a substantial and excessive increase in freezing over five FR-1 trials relative to the restraint controls. Those exposed to inescapable shock and the A_{2A} adenosine antagonist (CSC) performed similarly to shocked controls. Thus, blockade of adenosine A_{2A} receptors in the accumbens core had no effect on fear behavior.

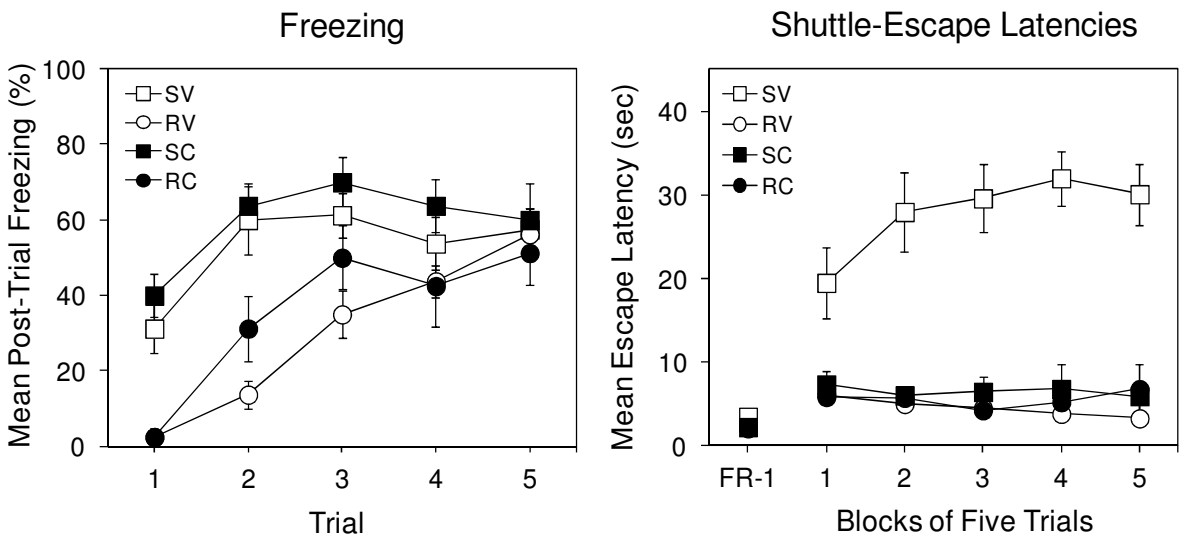

Figure 11. Percent freezing (left panel) and shuttle escape latencies (right panel) for 4 groups in Experiment 1. Rats underwent stereotaxic surgery where bilateral cannula were implanted into the nucleus accumbens core. Following recovery, rats were exposed to inescapable shock (S) or restraint (R) as a measure of traumatic stress and mild stress respectively. Shuttle-box testing occurred 24 hours later. Fifteen minutes before testing, they were injected with CSC (C), a highly selective adenosine A_{2A} antagonist, or a vehicle (V). Freezing was measured over 5 trials (FR-1) at the start of the shuttle-escape test. Impaired escape performance was assessed over the next 25 trials (FR-2).

A mixed-design analysis of variance (ANOVA: Stress Condition x Drug Condition x Trial) yielded significant main effects of Stress, $F(1, 28) = 29.72$, $p < .000$, and Trial, $F(4, 112) = 35.62$, $p < .000$. The Stress x Trial interaction also was significant, $F(4, 112) = 9.69$, $p < .000$. The main effect of Drug Condition was not significant. Newman-Keuls post-hoc contrasts ($\alpha = 0.05$) on grand mean freezing suggested the following ordered relation among group means: SV = SC > RV = RC.

The right panel of Figure 11 shows mean escape latencies across blocks of five trials. FR-1 escape latencies did not differ among groups, $F < 1$. Rats exposed to inescapable shock with the vehicle showed substantial impairment of escape performance over 25 FR-2 trials relative to

the restraint control. Those exposed to inescapable shock and the adenosine antagonist performed similarly to the restraint controls.

A mixed-design analysis of variance (ANOVA: Stress Condition x Drug Condition x Trial) yielded significant main effects of Stress, $F(1, 28) = 53.71$, $p < .000$, and Drug, $F(1, 28) = 28.49$, $p < .000$, and significant interactions of Stress x Drug, $F(1, 28) = 27.25$, $p < .000$, Drug x Trial, $F(4, 112) = 2.94$, $p = .02$, Stress x Trial, $F(4, 112) = 2.79$, $p = .03$, and Stress x Drug x Trial, $F(4,112) = 5.06$, $p = .001$. The main effect of Trial was not significant. Newman-Keuls post-hoc contrasts ($\alpha = 0.05$) on grand mean FR-2 escape latencies suggested the following ordered relation among group means: SV > SC = RV = RC.

Experiment 2

The left panel of Figure 12 shows mean percent post-trial freezing for each of the five FR-1 trials. Rats exposed to inescapable shock and treated with DMSO vehicle shortly before testing showed a substantial and excessive increase in freezing over five FR-1 trials relative to the restraint controls. Those exposed to inescapable shock and the A_{2A} adenosine antagonist (CSC) performed similarly to shocked controls. Thus, blockade of adenosine A_{2A} receptors in the accumbens shell had no effect on this measure of helplessness.

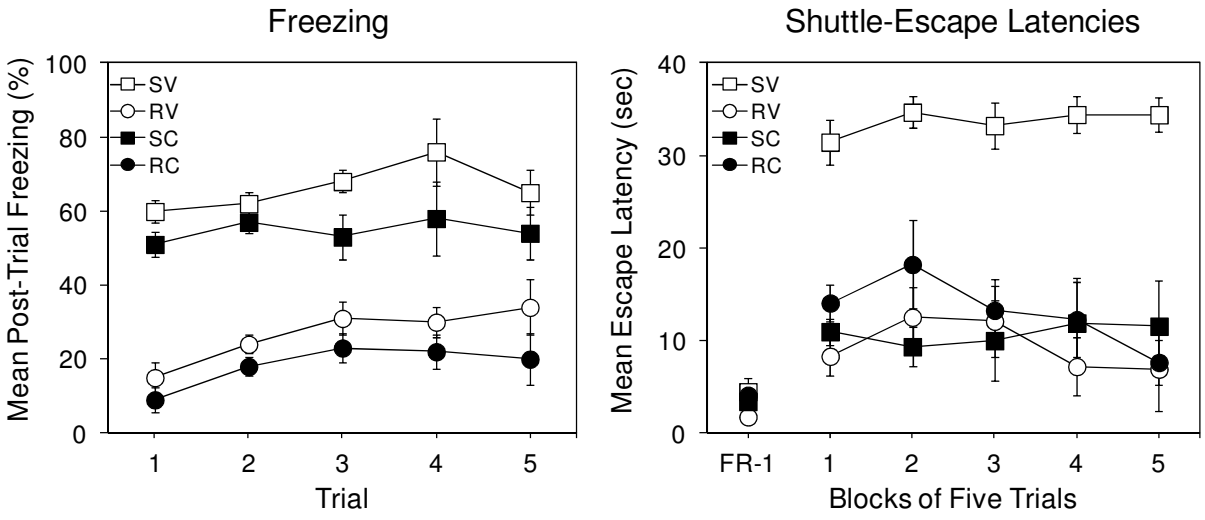

Figure 12. Percent freezing (left panel) and shuttle escape latencies (right panel) for 4 groups in Experiment 2. Rats underwent stereotaxic surgery where bilateral cannula were implanted into the nucleus accumbens shell. Following recovery, rats were exposed to inescapable shock (S) or restraint (R) as a measure of traumatic stress and mild stress respectively. Shuttle-box testing occurred 24 hours later. Fifteen minutes before testing, they were injected with CSC (C), a highly selective adenosine A_{2A} antagonist, or a vehicle (V). Freezing was measured over 5 trials (FR-1) at the start of the shuttle-escape test. Impaired escape performance was assessed over the next 25 trials (FR-2).

A mixed-design analysis of variance (ANOVA: Stress Condition x Drug Condition x Trial) yielded significant main effects of Stress, $F(1, 28) = 49.62$, $p < .001$, and Trial, $F(4, 112) = 3.86$, $p < .01$. The main effect of Drug and the Stress x Trial interaction were not significant. Newman-Keuls post-hoc contrasts ($\alpha = 0.05$) on grand mean freezing suggested the following ordered relation among group means: SV = SC > RV = RC.

The right panel of Figure 12 shows mean escape latencies across blocks of five trials. FR-1 escape latencies did not differ among groups, $F < 1$. Rats exposed to inescapable shock with the vehicle showed substantial impairment of escape performance over 25 FR-2 trials relative to the restraint control. Those exposed to inescapable shock and the adenosine antagonist performed similarly to the restraint controls.

A mixed-design analysis of variance (ANOVA: Stress Condition x Drug Condition x Trial) yielded significant main effects of Stress, $F(1, 28) = 18.11$, $p < .000$, and Drug, $F(1, 28) = 13.94$, $p = .001$, and significant interactions of Stress x Drug, $F(1, 28) = 26.69$, $p < .000$, and Stress x Trial, $F(4, 112) = 2.50$, $p < .05$. The main effect of Trial and the interactions of Trial x Drug and Trial x Stress x Drug were not significant. Newman-Keuls post-hoc contrasts ($\alpha = 0.05$) on grand mean FR-2 escape latencies suggested the following ordered relation among group means: SV > SC = RV = RC.

Discussion

Experience with unsignaled, inescapable shock in the learned helplessness paradigm represents a profound challenge to brain metabolic function and physiology. It is argued that behavioral impairment following this traumatic stress is a consequence of a transition in motivational state to one of conservation-withdrawal. This transition is mediated by enhanced brain adenosine signaling, which promotes metabolic recovery by profoundly inhibiting neural activation. Previous research linked the onset of a conservation-withdrawal response to the activation of highly selective adenosine A_{2A} receptors in the brain (see Minor, Rowe, Cullen, and Furst, 2008). Adenosine A_{2A} receptors have a highly selective regional distribution in the brain and are predominately located on the enkephalin-containing spiny GABAergic neurons of the striatum.

The present experiments showed that pharmacological blockade of A_{2A} receptors in either the shell or core of the accumbens after inescapable shock failed to reverse exaggerated fear

conditioning, but completely reversed the deficits in escape performance. As such, the nucleus accumbens appears to be a major locus of adenosine's influence on helplessness.

The nucleus accumbens functions as the site of integration of emotional and motivational input with behavior. Adenosine A_{2A} receptors are colocalized with dopamine D_2 receptors on the indirect pathway of the striatum where they modulate the binding affinity of dopamine to its receptor. Minor and colleagues (Minor, Huang, and Witt, 2006; Plumb, et al., 2013) have postulated that activation of A_{2A} receptors functionally uncouples the dopamine signal from the ventral tegmental area, thus uncoupling motivation from ongoing behavior. Performance deficits ensue. The evidence provided that adenosine signaling in the core and shell of the nucleus accumbens mediates helplessness implies a potential role in other stress-induced disorders. These data may have important implications for the treatment of Post-traumatic Stress Disorder (PTSD) with comorbid depression as the learned helplessness effect has long been thought of as an animal model of these disorders.

Striatal adenosine's involvement in ongoing behavior is complex. It is clear that A_{2A} receptors of the indirect pathway affect escape performance following traumatic stress; however, it is unclear how they are exerting their influence. Striatal adenosine signaling could be eliminating the motivation to respond as thought by Minor and colleagues; or, due to its inhibitory properties, could be effectively preventing a response from occurring by way of behavioral paralysis. Perhaps a combination of these two effects is necessary for induction of helplessness.

Some evidence for the motivational effects of adenosine comes from work on mouse models of behavioral despair. The tail suspension test is used to screen the effectiveness of

antidepressant drugs, indicated by a reduction in the amount of time the mouse remains immobile (Stéru et al., 1987). Immobility is often interpreted as a lack of motivation to respond; however, there is a locomotor component to the test as well. Therefore, simple locomotor tests should be run to distinguish motor effects from motivation effects (El Yacoubi, Costentin & Vaugeois, 2003; Perrault, Morel, Zivkovic & Sanger, 1992). El Yacoubi et al. (2003) studied the effect of adenosine antagonists on immobility in the tail suspension test. A non-selective adenosine antagonist (i.e. caffeine) or a selective adenosine A_{2A} receptor antagonist (SCH 58261) was given to mice 30 minutes before the tail suspension test. Both drugs were found to reduce immobility in a dose-dependent manner. To test for possible locomotor effects, both antagonists were given concomitantly with the dopamine D_2 receptor antagonist haloperidol, a drug known to reduce motor activity, in an open field task. The stimulant effects of caffeine were reduced with increasing doses of haloperidol, indicating that caffeine may be simply increasing locomotion in the tail suspension test. However, haloperidol had no effect on the increased locomotor activity resulting from the A_{2A} antagonist SCH 58261. Taken together, these data indicate that perhaps adenosine A_{2A} receptors are affecting motivational processes. Further experimentation is needed to accurately reflect the exact nature of adenosine's influence on stress-induced performance deficits.

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DISCUSSION

Traumatic stress exposure results in myriad psychological and physiological changes that are often deleterious in nature and can last a lifetime (see Minor, Huang & Witt 2006). The extreme state of fear that often accompanies traumatic stress can lead to the development of anxiety disorders, such as Posttraumatic Stress Disorder (PTSD) (Rosen & Schulkin, 1988; Fanselow & Lester, 1988). Common features of PTSD include an exaggerated and inappropriate stress response upon reminders of the trauma (Dykman, Ackerman & Newton 1997; Friedman 1994; Bremner, Krystal, Southwick & Charney 1995), drug and alcohol abuse (Dutten et al. 2014; van Dam, Ehring, Vedel & Emmelkamp 2013), and comorbity with other psychiatric disorders, such as depression (Stander, Thomsen & Highfill-McRoy 2014). Due to the potential severity of this disorder, methods that can prevent the development of PTSD or treat the symptomology are essential.

The three chapters of this dissertation examined methods to build stress resilience utilizing the learned helplessness procedure, an animal model of PTSD and comorbid depression. Multiple resilience-building techniques were assessed on their effectiveness of eliminating two components of the helplessness effect. Firstly, we observed if these techniques could eliminate the sensitization of the fear response in subsequent stressful situations by examining freezing behavior, a measure of fear in rats (Bouton and Bolles, 1980). Secondly, we recorded shuttle-escape latencies to determine if these techniques could prevent the transition in motivational state from anxiousness and agitation to one of conservation-withdrawal normally observed upon stress re-exposure. This shift in motivational state to one of conservation-withdrawal occurs unconditionally after periods of intense neural activation and is critical for conserving limited

resources. This state is characterized by sensory unresponsiveness, cognitive dullness, and behavioral depression, which are seen as adaptive mechanisms for recovering metabolic homeostasis (Engel & Schmale 1972). Unfortunately, the components of this state also prevent proper coping in subsequent stressful situations.

Chapter One provided one method to prevent PTSD by building stress resilience using hormetic stress. Hormesis is the process by which exposure to small stresses builds resilience to subsequent traumatic stress. We found that as few as three pre-exposure sessions were sufficient to eliminate the deleterious effects normally observed following trauma. The pre-exposure sessions could be mild or moderate in severity; however, when using a more moderate stress, rest between stress sessions became critical to building resilience to traumatic stress. There was also some evidence that increasing the number of pre-exposure sessions affords even greater protection from the effects of trauma, but this needs to be explored in greater detail.

Chapter Two examined a second resilience-building technique utilizing post-stress glucose consumption. Exposure to inescapable, traumatic stress results in an exaggerated fear state. This state is intensely catabolic and metabolic homeostasis is rapidly compromised. Glucose consumption following traumatic stress has been shown to eliminate the exaggerated fear responding and escape deficits normally observed after trauma (Minor & Saade 1997). We further explored the versatility of post-stress glucose in a number of experiments. Experiment 1 provided evidence that glucose prevents the transition into the conservation-withdrawal state commonly observed during testing. Experiment 2 showed that glucose consumption facilitates the effectiveness of other resilience-building techniques, such as hormetic stress. Consuming glucose following the pre-exposure sessions facilitated an already-effective hormetic stress

procedure, as well as made a previously ineffective procedure now effective. Lastly, Experiment 3 pushed the boundaries of the stress response and examined if glucose could build resilience when exposed to two back-to-back trauma sessions. Here, it was discovered that glucose consumption following the first trauma was critical in building resilience to the second trauma. Glucose consumed only after the second trauma provided no benefit, indicating that damage was already done.

Chapter Three provided further evidence that brain metabolic homeostasis is compromised following traumatic stress, and highlighted a potential treatment for the unresponsive state that is often seen in PTSD. Adenosine is released under intense neuronal activation and is a potent inhibitor. Under these conditions, adenosine is acting as a neural protectant in an effort to prevent the utilization of available ATP and possible excitotoxicity. Adenosine signaling has been linked to the conservation-withdrawal state, specifically at the A_{2A} receptor subtype (Minor, Huang & Witt 2006; Plumb, Sterlace, Cavanaugh & Minor 2013; Minor, Chang & Winslow 1994; Minor, Winslow & Chang 1994; Hanff, Furst & Minor 2010; Minor, Rowe, Cullen & Furst 2008). Adenosine A_{2A} receptors are found mainly on enkephalin-containing GABAergic neurons in the indirect pathway of the striatum. This pathway has an overall inhibitory effect on behavior. Here, A_{2A} receptors are colocalized with dopamine D_2 receptors where they act in an antagonistic fashion (Ferré et al., 1997; Ferré et al., 2007; Schultz, 2002). Dopamine D2 receptor binding disinhibits the indirect pathway, resulting in facilitation of movement (Gerfen and Surmeier, 2011; Ferré et al., 2008; Haber, 2008). It appears in conservation-withdrawal that there is potent activation of the indirect pathway. Two experiments attempted to further narrow down the locus of action of adenosine in conservation-withdrawal. We discovered that blocking adenosine A_{2A} receptors in both the core and shell of the nucleus

accumbens did not affect fear responding but did eliminate shuttle-escape deficits. This indicates that adenosine does not affect fear, but does mediate an important downstream mechanism of fear – conservation-withdrawal.

A larger question remains as to why this A_{2A}-D_2 heteromer would evolve to control behavior. A plausible explanation revolves around a system of checks and balances, where excitation and inhibition of behavior are regulated. One example of this can be found in sickness behavior (Kent, Bluthé, Kelley, & Dantzer, 1992). Following an infection, behavior changes dramatically. Common symptoms include fatigue, anhedonia, decreased motor activity, lack of motivation to eat, and disruptions in sleep (Kelley et al., 2003; Minor, Huang & Witt, 2006). Sickness behavior is adaptive in that it reduces unnecessary energy expenditures in order to fight infection and promote recovery. Conservation-withdrawal is a critical component of sickness behavior (Minor, Huang & Witt, 2006). As discussed in Chapter Three, adenosine A_{2A} receptors mediate the state of conservation-withdrawal. Dopamine from the ventral tegmental area (VTA) provides a motivational signal to respond in the presence of a reward. The A_{2A}-D_2 heteromer may have evolved to allow for regulation of conflicting signals from adenosine and dopamine during illness. Simply put, energy shouldn't be wasted seeking rewards when it is needed to fight an infection.

A second possible explanation for the development of this heteromer relates to recent work on effort. Effort-based decision making is the match between motivation and the costs of responding (Nunes et al., 2014; Salamone & Correa, 2012; Salamone et al., 2002). This reflects a more general class of behaviors than sickness behavior, affecting everyday decisions like food seeking and predator avoidance. Commonly, animals will actively choose a task that requires

more effort if the reward is of high value (Salamone et al., 1991). Both dopamine and adenosine have been implicated in effort-related decision making on appetitive choice tasks. Dopamine depletion or antagonism of either D_1 or D_2 receptors in the nucleus accumbens all alter choice behavior such that a rat will more readily choose a less valuable reward with low effort costs rather than a high-value reward requiring high effort (Nunes et al., 2010; Salamone et al., 1991; Salamone, 1986; Salamone, 1988). Conversely, administration of an A_{2A} antagonist fully reverses the effects of a D_2 antagonist and partially reverses the effects of a D_1 antagonist; A_1 antagonists had no effect (Font et al., 2008; Nunes et al., 2010). Clearly, adenosine A_{2A} and dopamine D_2 receptors interact to regulate effort-based decision making. Perhaps the A_{2A}-D_2 heteromer exists for this very purpose.

This theory would also relate to work on finickiness. Finickiness is seen in feeding and foraging behavior, and refers to the high degree of selectivity that occurs following traumatic stress (Dess, Chapman & Minor, 1988). For example, rats will avidly consume a sucrose solution following inescapable shock, but refuse to drink a more bitter-tasting saccharin or quinine solution (Dess, 1992; Dess, Chapman & Minor, 1988). This indicates that stress may enhance sensitivity to tastes that have aversive qualities, perhaps through the interaction of adenosine A_{2A} and dopamine D_2 receptors.

The interaction of adenosine and dopamine is less clear in risk-based decision making. Here, an animal can choose between two tasks that will result in either a certain, low-value reward or an uncertain, high-value reward. Stopper, Khayambashi, and Floresco (2013) found that accumbal D1 and D3 receptors mediate risk-based decision making. Interestingly, they found that antagonism or stimulation of D_2 receptors had no effect on risky behavior. Others

have found that dopamine D_1 and D_2 receptors have functionally distinct roles in risky behavior depending on their location with the brain (St. Onge, Ahn, Phillips, Floresco, 2012). Clearly, the role of dopamine in risk-based decision making is complex, and the interaction between dopamine and adenosine in these tasks still needs to be examined.

In conclusion, this dissertation provided three peritraumatic interventions for building stress resilience and eliminating PTSD-like symptoms. Our work on hormetic stress highlights the importance of life experiences prior to trauma. People that are exposed to intermittent stresses throughout their life may be more resilient to the development of PTSD. However, frequent exposure to stress may be harmful if the stressor is too severe, or adequate recovery time was not allowed between stresses. Ethnic minorities and those in low socioeconomic statuses are at higher risk of developing PTSD, perhaps due to an increased frequency of stressor exposure that often accompanies these groups (Feske, 2001; Pole, Gone & Kulkarni, 2008; Roberts et al., 2011). The incidence and pattern of rest surrounding stressor exposure may be critical factors in determining who is at risk for developing PTSD.

Post-stress glucose consumption is a simple and effective method that may help repair the damaging effects of trauma exposure and allow for a quicker recovery back to baseline. This could be particularly useful in a military population as a glucose solution could easily be taken onto the battlefield and consumed immediately after a traumatic event. Likewise, glucose could be given to sexual assault victims or trauma patients upon admittance to the emergency room as part of a medical intervention to help prevent the development of PTSD.

And lastly, adenosine antagonists that target A_{2A} receptors in the striatum may be an effective tool for eliminating the conservation-withdrawal state that is commonly observed after

trauma. Conservation-withdrawal is the initial phase of withdrawal following traumatic stress or injury. Patients in this state show profound detachment (Barach, 1991; Schore, 2002). They have little interest in family and friends and tend to avoid social situations (Mohta, Sethi, Tyagi & Mohta, 2003; Schore, 1994). Perhaps an adenosine A_{2A} antagonist given while a patient is experiencing these symptoms will bring them out of this conservation-withdrawal state and allow for active commerce with the environment. Together, the three interventions outlined in this dissertation may provide prevention of or partial recovery from PTSD.

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