Title

The parametric determinants of heterogeneity in the behavioral and neurobiological impact of stress.

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Publication Date

2020

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

The parametric determinants of heterogeneity in the behavioral and neurobiological impact of stress.

A dissertation submitted in partial satisfaction of the
Requirements for the degree of Doctor of Philosophy
in Psychology
by

Michael Anthony Conoscenti

2020

ABSTRACT OF THE DISSERTATION

The parametric determinants of heterogeneity in the behavioral and neurobiological impact of stress

by

Michael Anthony Conoscenti
Doctor of Philosophy in Psychology
University of California, Los Angeles, 2020
Distinguished Professor Michael S. Fanselow, Chair

Exposure to traumatic stress can lead to a wide range of persistent, deleterious biological and behavioral effects. Despite a significant national investment of resources directed toward both basic and clinical stress research, the field has come up short in the development of effective treatments for stress disorders. One potential explanation for this discrepancy is a lack of field-wide procedural and theoretical cohesion. There are a great number of different stress procedures used by basic research scientists and yet there has been no attempt to comprehensively consolidate findings across stressors. For example, we have previously shown that giving a rat access to a glucose solution following stress exposure alleviates the deleterious effects of stress. However, the effects of post-stress glucose have yet to be tested outside of this specific stress procedure. We have hypothesized that differences in the dimensions of stress exposure (such as quality, volume, and chronicity of the stressor) may in part account for the biological and behavioral heterogeneity of stress disease. Here, we test this hypothesis.

In the second chapter of this dissertation, we assess the physiological impacts of post-stress glucose in order to better understand its potential mode of action. In a series of experiments, we found that glucose may be alleviating the negative sequelae of stress exposure by helping the organism maintain energetic homeostasis. The study also rules out a corticosterone-mediating mechanism of action for glucose's prophylactic effects. In the third chapter of this dissertation, we examine the behavioral and biological effects of stress procedures with very different stress volumes (as defined by shock length x intensity x number). We found that stress volume counter-intuitively impacts the resultant behavior and neurobiology. Namely, we found that high-volume stress does not produce stress-enhanced fear learning (SEFL), a quintessential effect of moderate-volume stress. However, when rats exposed to high-volume stress were given glucose, SEFL behavior appeared. We identify a few behavioral and biological differences that provide a potential mechanism of the effect. These studies suggest that the volume of the stressor has a clear impact on the resultant disease phenotype and intervention efficacy. In the fourth chapter, we examine the effects of a different stress dimension: chronicity. We found that stress chronicity impacts the resultant behavior and neurobiology in an additive way. Namely, we found that chronic stress appears to produce the same non-associative enhancements of fear quintessential to the acute stressor, plus a unique associative component. We provide evidence for this conclusion through several behavioral and neurobiological means. These studies suggest that the chronicity of the stressor can impact the mechanism and severity of disease. Finally, all of the above findings are discussed in terms of their implications in the stress field.

The dissertation of Michael Anthony Conoscenti is approved.

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2020

This dissertation is dedicated to those who have had a great hand in shaping my mind, body, and heart, but are not here to celebrate the occasion- Dr. Thomas Minor, Coach Lazslo Tabori, and my grandfather "Mickey".

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ACKNOWLEDGMENTS

At twenty-one, I would never have guessed in my wildest dreams that in a decade's time I would be defending my doctoral thesis. At the time, I was still fairly certain that I wanted to pursue a career as an actor. As you can perhaps imagine, it has been a long and winding road to get here. I wouldn't have made it to this point without an incredible amount of support from family, friends, and mentors along the way.

First, I would like to thank the teachers and coaches that sparked and shaped the inquisitiveness and scholarship which has led me to this point. My high school track and cross-country coach, Marty Maciel, was the first non-familial adult to make me feel truly needed and appreciated. As cliché as it sounds, he also instilled in me a true love of the daily effort that leads to a goal. I'd like to thank my high school teachers, Steve Johnson and Christine Thompson, for making learning a joyous experience; I always felt that my thoughts and ideas were heard and appreciated in your classrooms. I'd also like to thank my high school counselor, Richard Intlekofer. I would not have reached my full potential without your support and encouragement. I'd like to especially thank Dr. Lorraine Turcotte, who first introduced me to laboratory research at USC, and has continued to provide valuable mentorship throughout grad school. I would not be here without you. This dissertation is in part dedicated to the memory of my college track and cross-country coach, Lazlo Tabori, who taught me to "put the guts to it" and the joy of a lifetime pursuit of your passion.

I had no true career pursuit or sense of self in my early twenties. Therefore, I would be remiss to not mention my closest friends who not only avoided pointing out this fact to me, but agreed to live with me during this tumultuous time. Scott, thank you for putting up with all of my nonsense and for holding back your typical skepticism when I announced that I was going to pursue a doctorate in a field of which I had no formal education. Brooks, thank you for being my eternal

wingman. Thomas, I am very thankful, among many other things, to have shared the uncertainties of post-bacc education with you. Bobby, your constant optimism and subtle encouragement did not go unseen. Shawn, we too shared in the uncertainty of those formative years; look at how far we've come! I'd like to thank our imaginary butler Jaeyves for his superior imaginary service and aptitude in beezle. I also want to thank Dan for being my workout buddy and Rahad, among other things, for providing much of my musical backdrop while writing this dissertation.

As those who have pursued a higher degree know, the uncertainty doesn't end when you are accepted into a graduate program- it has only begun. I enjoyed a massive case of "imposter syndrome" during my early graduate career. It was through the encouragement of my mentors and peers that I was able to persevere. First, I would like to thank my committee, Dr. Aaron Blaisdell, Dr. Pamela Kennedy, and Dr. Nigel Maidment, for their guidance and support. Thank you to my research assistants, Nina, Jazmine, Nicole, Alex, Kayla, Tracy, J.P., and Jenny. You not only followed orders, but were intellectually engaged in the work and showed a vested interest in the result. Thank you to the entirety of the Fanselow lab. Nancy, we've gone through this crazy ride together. Thanks for always being there. Sonya, thank you for being my western blot partner in crime. I'd like to thank Lisa Lee for her guidance through the graduate program. I'd like to thank Cheryl Polfus for her support regarding TA appointments, and discussions on our shared love of the theatre and a good cocktail. Dr. Evan Hart, you were my go-to person. Anytime I was feeling uncertain, insufficient, uneducated, unintelligent, or simply had a question I was too embarrassed to ask anyone else, I would come to you. And you were always there. Thank you. This dissertation is in part dedicated to the memory of Dr. Thomas Minor, who gave me a perhaps unwarranted shot when no one else would. I owe this all to you. You are greatly missed. Finally, to Dr. Michael

Fanselow, who took over the reins without hesitation and has not only made me feel welcome, but also wanted.

I would like to also thank my family for their constant support. First, I'd like to thank my father for his support and encouragement. My "halflings", Julia, Dominic, and Olivia: it has been a delight to watch you grow-up. My grandparents "Millie" and "Mickey", aunts, cousins, and the rest of my crazy Italian family. I love you all. Grandpa Mickey, you are deeply missed. This one's for you. I would like to thank my grandparents "Frenchie" and "Jerpa", as well as my aunts and uncles for all helping raise me. I'll never forget the countless boardgames and rollercoasters with you, Grandma. Grandpa, I'll always cherish our afternoon t.v. time, juxtaposed with episodes of "M.A.S.H." and "Catdog". Aunt Mary Alice, I am so thankful to have gotten to grow-up with such a badass, loving, and kind "big sister". Garrett, I couldn't ask for a better brother-cousin. I could write a dissertation length thank you to my mother and my wife, who I lump together in this with a bit of intentional irony. Mom, I could not ask for a better role model, a better mentor, or a better friend. You worked so unbelievably hard and sacrificed so much to give me the opportunities that led me where I am today, without showing me a hint of the struggle. Leanna, I love you so much. Thank you for being the most amazing wife I could have asked for. Thank you for your undaunting support and encouragement. Thank you for listening intently to all of the half-baked versions of my presentations. Thank you for being the best self-isolation partner a man could have.

Research support. This work was supported by the National Institute of Mental Health, grant R01 MH115678 (Fanselow), The Staglin Center for Brain and Behavioral Health (Fanselow), Office of Naval Research Grant #N00014-16-2799/N00014-15-1-0038, UCLA Graduate Division Dissertation Year Fellowship (Conoscenti), Graduate Research Mentorship (Conoscenti), and Graduate Summer Research Mentorship (Conoscenti).

Permissions. Portions of this dissertation are adapted from previously published articles. These data are published in:

Conoscenti, MA & Fanselow, MS (2019). Dissociation in effective treatment and behavioral phenotype between stress-enhanced fear learning and learned helplessness. *Frontiers in behavioral neuroscience*, 13, 104.

Conoscenti, MA, Williams, NM, Turcotte, LP, Minor, TR, Fanselow, MS (2019). Post-stress fructose and glucose ingestion exhibit dissociable behavioral and physiological effects. *Nutrients*, 11(2), 361.

Contributions of co-authors. For work that has been previously published or is in preparation, I would like to thank my co-authors. Nicole Williams assisted with data collection and manuscript preparation. Dr. Lorraine Turcotte assisted with conceptualization, methodology, and manuscript preparation. Daniel Weatherill assisted with data collection, experimental design, and manuscript preparation. Sonya Watson assisted with data collection and manuscript preparation. Dr. Michael Fanselow and Dr. Thomas Minor assisted with experimental design, manuscript preparation, and were principal investigators on these projects.

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Smith, N.J., **Conoscenti, MA**, Minor, TR, Fanselow, MS (*In prep*). Effectiveness of post-stress glucose in the prevention of dissociative amnesia and post-traumatic stress disorder.

Traci N. Plumb, TN*, **Conoscenti, MA***, Minor, TR, Fanselow, MS (*In prep*). Post-stress glucose consumption facilitates hormesis and resilience to severe stress.

Conoscenti, MA & Fanselow, MS (2019). Dissociation in effective treatment and behavioral phenotype between stress-enhanced fear learning and learned helplessness. *Frontiers in behavioral neuroscience*, 13, 104.

Conoscenti, MA, Williams, NM, Turcotte, LP, Minor, TR, Fanselow, MS (2019). Post-stress fructose and glucose ingestion exhibit dissociable behavioral and physiological effects. *Nutrients*, 11(2), 361.

Conoscenti, MA, Hart, EE, Smith, NJ, Minor, TR. (2017). Temporal Parameters of Post-Stress Prophylactic Glucose Treatment in Rats. *Stress*, 1-37.

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Hart, EE, **Conoscenti, MA,** & Minor, TR (2014). Animal Models of Depression: A Focus on Adenosine Signaling at A2A Receptors. *Ann Depress Anxiety*, *1*(3), 1011.

Chapter 1: Introduction

Portions adapted from publication in *Frontiers of Behavioral Neuroscience*

Background and significance

Exposure to traumatic stress results in a number of physiological and psychological changes in both human and non-human species [1,2]. These changes are often deleterious in nature and can endure throughout a lifetime. Clinical impacts of exposure to traumatic stress include a wide array of anxiety disorders, major depressive disorder, and post-traumatic stress disorder (PTSD). In fact, approximately 20% of those that experience a trauma will go on to develop PTSD [3].

Post-traumatic stress disorder is a debilitating disease marked by high symptom heterogeneity among patients. It is estimated that approximately 7% of United States citizens, and up to 20% of military personnel, will develop PTSD within their lifetimes [4,5]. PTSD patients show a wide array of symptoms such as anhedonia, avoidance behaviors, dissociative amnesia, exaggerated fear startle, hypervigilance, and insomnia [6]. Patients diagnosed with PTSD also exhibit high comorbidity with several other anxiety, depression, and substance abuse disorders [7-10]. Over the past decade, there has been a large ongoing research effort focused on identifying the neurobiological mediators of stress-induced disease. Yet despite great headway made in understanding the neurobiology of stress, there has been little-to-no improvement in effective clinical intervention. This has led to widespread critique regarding the reliability of animal models of stress disorder.

A review by Richter-Levin, Stork, and Schmidt provides several suggestions on how we might best improve basic research on stress [11]. In the review, they suggest that the translational relevance of animal models can be improved if modified to adequately capture the heterogeneity

of stress disorders. They suggest that stress models should be modified to more accurately represent genetic factors that may lead to greater susceptibility or resilience. They also suggest that different stress procedures likely lead to dissociable behavioral and biological impacts. Therefore, researchers should carefully select study parameters to reflect their patient population of interest. If the type of stress exposure does indeed account for a portion of the symptom variability, parametric study of stress and its impact may help us better understand where stress-induced disease biologically converges, and where it diverges.

Modeling Post-Traumatic Stress Disorder

A wide host of stress procedures and behavioral assays have been utilized to model PTSD in rodents. Stressors include physical restraint, electric shock, social defeat, social isolation, maternal separation, tail suspension, forced swim and underwater submersion, exposure to ether vapor, exposure to predators and/or predator-related stimuli, and a range of chronic, variable stressors (see [11-13] for review). Not surprisingly, this wide variety of stressors seemingly induces a variable array of behavioral consequences. However, due to confounds across several dimensions, side-by-side comparison across models that vary in stress type is unlikely to garner appreciable and informative progress towards understanding stress-induced psychiatric disease. Therefore, it is pertinent to select a stressor for comparison that is both capable of parametric manipulation and has historically displayed variable behavioral outcomes when modulating these parameters. Electric shock affords us this opportunity. It is an aversive, discrete, and highly manipulatable stimulus capable of producing a wide array of persistent behavioral effects. Two commonly utilized models of PTSD that use electric shock as the instigating stressor are *learned helplessness* and *stress-enhanced fear learning*. Both models utilize exposure to a single session of shock as the stress pretreatment, but vary greatly in the volume of shock exposure (shock

number x intensity x length) within the session. Importantly, there are key differences in the reported behavioral effects and mediating neurobiology of these two stress procedures.

A Brief History of Learned Helplessness

The learned helplessness procedure is a traditional method for analyzing the effects of acute, traumatic stress and modeling related symptoms of PTSD and comorbid major depression in rats [14-18]. Seligman and colleagues first discovered in 1967 that exposure to inescapable shock, but not escapable shock, results in failure to perform future escape responding in a novel apparatus [19,20]. The classic experiments utilized dogs and a triadic design. In this design there are three groups. One group is able to perform a response to escape the shock. Another group is able to perform the same response non-contingently, as their exposure to shock is yoked to that of the escapable group. A final group is exposed to the same apparatus, but no shock is administered. This design allows for dissociable assessment of the effects of escapable and inescapable shock. The term "learned helplessness" was originally coined as it was initially believed that the escape latency deficits were due to the animals learning that they had no control over the environment [20,21]. However, others have provided subsequent evidence which has suggested that it instead may be the unpredictability of shock that is the root of the subsequent maladaptive behavior [16,22,23]. The model has since transitioned to rats and LH has been used extensively as an animal model of human disorders, such as PTSD and MDD [24,25]. Though the learned helplessness model has been used extensively as a model of depression and PTSD, it does have a scientifically contentious history. The relatively short 24 to 72-hour lifespan of many of the observed behavioral and cognitive deficits, which can be moderately extended using a reinstatement procedure [26], has been a point of which its opponents cite when discussing its inefficacy as a model of psychiatric

disease [27-31]. However, face, construct, and predictive validity maintain its place as one of the leading models of PTSD and MDD.

A Brief History of Stress-Enhanced Fear Learning

Our first indication of enhanced fear learning following stress was suggested by two papers published in 1979 [32,33]. In these experiments rats that received an identical single shock in the same novel context froze at very different rates depending on whether or not they received prior experience with a robust fear conditioning protocol in a completely different context. Interestingly, while both 15 forward (tone-shock) and backward (shock-tone) trials enhanced subsequent contextual fear conditioning, predictive signaling of the shock reduced the magnitude of this enhancement. Importantly, the lack of freezing observed prior to the single shock indicated that this enhancement was not caused by generalization of fear from the 15 shock to the 1 shock contexts.

This ability of stress to enhance fear learning was then used as a tool to explore two deficits in contextual fear conditioning [34]. One was the deficit seen when only a minimal period of exploration was allowed prior to delivery of a single shock. Prior stress facilitated conditioning with this procedure that typically supports little to no conditioning. Another deficit in contextual fear conditioning occurs when shocks are closely spaced rather than given in a more distributed manner. In this case, prior stress eliminated the difference between massed and spaced trials. These studies also revealed an important boundary conditioning to SEFL; when multiple conditioning shocks were well spaced prior stress caused no enhancement in fear learning. These findings indicate that stress enhances the rate but not the asymptote of the learning curve.

Glucose as a potential stress intervention

The brain consumes a large amount of energy, relative to its size. While it constitutes only about 2% of total body weight, the brain accounts for 20% of oxygen consumption and 25% of glucose consumption [35]. Unlike many other organs in the body, energy expenditure remains relatively constant in the brain across wake and sleep cycles [36] and degree of mental effort [37]. However, energy expenditure dramatically increases on a total-brain scale when the animal is in a state of fear [38-40], and dramatic regional changes occur in areas such as the hippocampus [38], and amygdala [41]. In general, the brain does not store metabolic substrates and needs a constant supply of oxygen for normal functioning [42], though there is evidence suggesting that some glycogen and lactate are stored in glia, but not in neurons [43-46]. The brain primarily utilizes glucose for the anaerobic phase of respiration, and uses its carbon backbone for neurotransmitter synthesis [47-49]. Therefore, neurons are highly vulnerable to even transient changes in energy homeostasis [50-54]. It should be noted that under prolonged conditions in which glucose is not available (weeks to months), the brain can shift to utilizing ketone bodies to fulfill its energy demands [55,56]. Glucocorticoids inhibit glucose uptake in regions such as the hippocampus when in high concentrations, further exacerbating the homeostatic challenge of stress [51,57]. Fortunately, the body is equipped with mechanisms to increase energy availability during an energy exhaustive event.

When met with a challenge to energy homeostasis, there are three primary routes to increase blood and brain glucose concentrations. The simplest, and perhaps quickest, way to increase circulating glucose concentrations is to consume it. Blood glucose levels rapidly rise, and peak within thirty minutes of glucose consumption [58]. The sympathetic-adrenal-medullary (SAM) response is another mechanism that increases glucose availability. Epinephrine and norepinephrine are released during the SAM response and target alpha cells in the pancreas to

increase secretion of glucagon and inhibit secretion of insulin in beta cells [59]. Glucagon, in turn, upregulates glycogenolysis (breakdown of glycogen to glucose) in the liver. The net result is increased glucose availability in the brain, because unlike other tissues in the body such as muscle, transport of glucose to brain tissue is not dependently-mediated by insulin [60]. The other intrinsic modulator of blood glucose levels is the Hypothalamic-Pituitary-Adrenal (HPA) axis. Activation of the HPA axis results in the release of cortisol. Cortisol-activated glucocorticoid receptors bind to a glucocorticoid response element on the phosphoenolpyruvate carboxykinase (PEPCK) gene, which results in transcription upregulation of PEPCK- an enzyme with an essential role in the gluconeogenesis protein cascade [61]. At rest, the HPA axis is responsible for upregulation of gluconeogenesis to maintain liver glycogen and blood glucose concentrations during sleep [62].

As can be surmised, removal of the adrenal gland leads to major metabolic consequences. Adrenalectomy results in a reduction in food consumption, body weight, concentrations of insulin and leptin, as well as salt and blood volume loss and increased metabolism via thermogenesis [63,64]. These widespread deleterious effects can be reversed by corticosterone replacement. Adrenalectomized rats given access to a sucrose (50% glucose, 50% fructose) solution, but not corticosterone replacement, also exhibit normalized metabolism, caloric intake, and hormone concentrations [64]. Voluntary sucrose ingestion restores concentrations of metabolic hormones such as insulin, CRF, and leptin, while recovering fat deposition and caloric efficiency to levels similar to sham controls and groups given corticosteroid replacement therapy [64,65]. Furthermore, evidence suggests that these rats will preferentially consume a sucrose solution over a saccharine solution when given the choice [65]. Interestingly, intracerebroventricular infusions of corticosterone do not show the same metabolic benefits of peripheral injection, and in fact eliminate the beneficial effects of sucrose ingestion [63,66]. These data taken together suggest a

complex interaction between adrenal steroids and intrinsically- and extrinsically-produced saccharides, while also suggesting differential behavioral and physiological roles of the central and peripheral glucocorticoid systems.

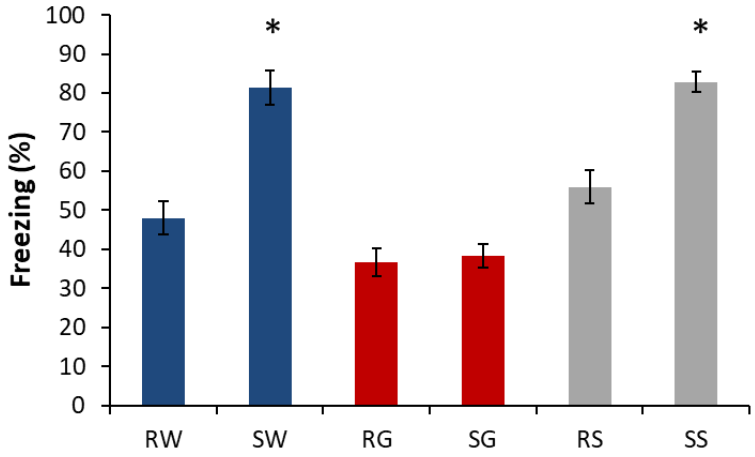

Figure 1.1. Post-stress saccharine consumption does not reduce fear responding in shuttle box. Average percent freezing between FR-1 trials is depicted among groups. Rats given access to glucose (_G) following inescapable shock (S_) exhibited a freezing frequency similar to restraint (R_) controls. However, rats given saccharine (_S) following inescapable shock exhibited heightened freezing similar to the group that received inescapable shock and access to only water (_W). Error bars denote mean ± SEM. * $p < .05$ (compared to RW)

A large number of interventions and preventions have been introduced that eliminate the deleterious effects of inescapable and unpredictable tail shock. Minor and Saade (1997) hypothesized that simply treating rats with glucose following traumatic stress would restore energy homeostasis and eliminate the helplessness effect [67]. They found that rats given 18-hour access to 100 mL of a 40% (wt/vol) aqueous glucose solution immediately following traumatic shock stress showed reduced freezing behavior and reduced escape latencies during shuttle-box testing equal to that of restraint controls. A recent parametric study suggests that glucose may be working in a dose dependent manner, and that rats must have free access to the glucose solution within

three hours of acute stress session termination [68]. Interestingly, the same behavioral benefits are not observed when the rats are given free access to the artificial sweetener, saccharine, matched for taste (unpublished; see Figure 1), or another monosaccharide, fructose, matched for caloric density [69]. Furthermore, artificial glucose depletion via peripheral injection of 2-deoxy-d-glucose (2DG) mimics the behavioral effects of inescapable shock [70]. It has been shown that these 2DG-induced escape latency deficits are reversed by central administration of the non-specific adenosine antagonist, caffeine. Recent data suggests that activity of adenosine 2a receptors in the nucleus accumbens shell modulate shuttle escape, but not fear sensitization [71]. Additionally, both running-wheel exercise and hormetic stress sessions, prior to the traumatic shock session, eliminate learned helplessness behavior at the time of testing [72,73]. Interestingly, all of these interventions share a common thread: they all either submaximally tax energy homeostasis (preventions) or rapidly induce/reverse the physiological effects of a challenge to energy homeostasis (interventions). It is therefore possible that a traumatic episode severely taxes energy homeostasis, leading to future behavioral and psychological consequences. However, the underlying processes of this hypothesized mechanism have yet to be identified. Furthermore, the benefits of glucose appear only after exposure to high-volume stressors, as it does not appear to be an effective intervention for the smaller-volume SEFL stress procedure (unpublished; see Figure 2). This suggests stress procedures that differ on volume may not only exhibit dissociable behavioral phenotypes, but also may differ in effective interventions.

Stress volume as a disease prediction factor

Based on the literature, exposure to inescapable, unpredictable shock appears to incorporate some homogenous peripheral and central mechanisms and induce a series of consistent trans-situational behaviors, regardless of volume (see Table 1 for review). It appears that stress-induced anxiety phenotypes are first to arise during exposure to a stressor, as anxiety-related

behaviors are conserved across high (LH) and moderate-volume (SEFL) stress models. The HPA axis appears to play a critical, permissive role in the development of both LH and SEFL-induced behavior. It also appears that the immune response, specifically IL-1, plays a critical role in the development of stress-induced psychopathology. Regarding neurocircuitry, converging evidence suggests that the amygdalar complex is involved in the neurocircuitry of shock stress regardless of volume. The vmPFC has also been implicated in both behavioral models, though it appears to have opposing effects.

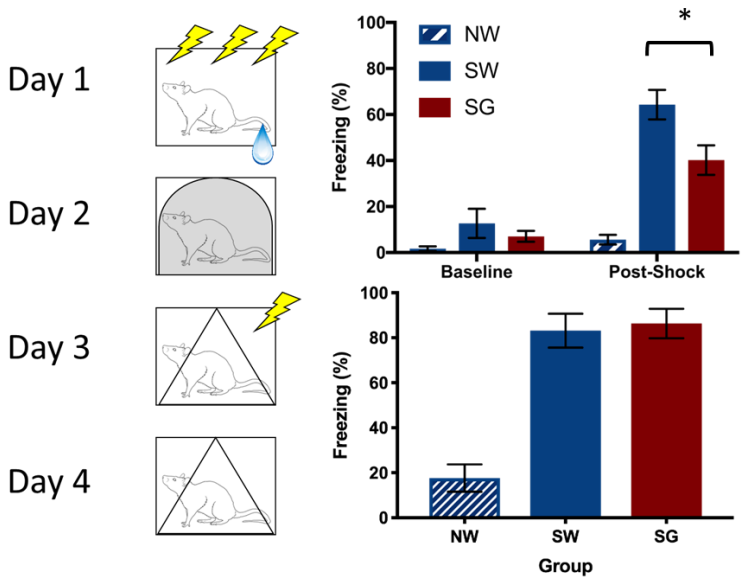

Figure 1.2. Post-stress glucose consumption does not impair stress-enhanced fear learning. Stress and testing procedure (left), pre- and post-shock freezing during 1-shock exposure (top right), and freezing during contextual fear learning test day (bottom right) are depicted. Rats given glucose (G) following stress pretreatment (S) did exhibit a modest decline in post-shock freezing compared to the stress group given water (W). However, glucose had no effect on fear expression during the context test. "N" indicates no-stress controls. Error bars denote mean ± SEM, * $p < .05$.

Several dissociable behavioral and neurobiological aspects of the two procedures stand out. The most obvious division is the induction of a depression-like phenotype in LH-stressed animals that appears absent in SEFL-stressed animals. Another interesting difference is the apparent generalization necessary for LH's characteristic deficits in shuttle-escape performance, which does

not appear necessary for the SEFL phenotype. Perhaps the most perplexing difference is that of symptom persistence. The LH-stressor produces many behavioral changes that appear to persist for only a few days. Meanwhile, SEFL produces a set of behaviors which persist for at least several months. Given that there is a much greater volume of stress in the LH procedure it is surprising that many of its effects do not persevere. However, it should be noted that several of these short-lived changes are in behaviors that do not overlap with the behavioral effects of SEFL. Therefore, it may be a product of the behavioral phenotype assayed, and not an effect directly related to stress volume. It is important to note that there are several outstanding questions that have been left unanswered. For example, the role of 5-HT neurons in the DRN has been well characterized in LH, but has yet to be investigated in SEFL.

Use of the same stressor can produce dissociable behavioral and neural consequences by simply modulating stress volume. Notably, the degree of stress does not necessarily make the effects quantitatively greater, but rather there seems to be qualitative changes in the consequent behavioral reactions. Based on the literature reviewed, it appears that the SEFL procedure may produce several phenotypes specific to model PTSD without depression comorbidity, while LH may model a PTSD comorbid with depression. This notion sits perfectly in-line with the heterogeneity of PTSD described in the review by Richter-Levin [11]. Within that review, the authors describe an outstanding fundamental question about PTSD: is PTSD with depression a unique subtype, or do the diseases merely show a high comorbidity. Approximately half of patients diagnosed with PTSD also concurrently meet criteria for Major Depressive Disorder [10,102-105]. Perhaps even more staggering is the statistic that 95% of those with PTSD will be diagnosed with MDD within their lifetime [95]. Patients with MDD exhibit symptoms such as chronic depressed mood, anhedonia, anorexia or hyperphagia, insomnia or hypersomnia, fatigue, and cognitive

deficits [6]. These symptoms are consistent with several of the symptoms observed following LH, but not SEFL, stress exposure. It is possible that human PTSD development is influenced by similar factors. For example, stress volume may influence both the quality and quantity of symptoms. It is also possible, that disease persistence does not positively correlate with stress volume, but may be predicted by another variable of stress exposure. Only through careful, focused study examining the neurobiological effects of modulating stress volume may we begin to unravel the dissociable aspects of PTSD and PTSD with comorbid depression.

Phenotype	Present in LH?	Present in SEFL?	Source
Future Enhanced Fear Learning	Yes	Yes	[74-76]
Anxiety; Elevated Plus Maze	Yes	Yes	[77,78]
Anxiety; Open Field	Yes	Yes	[79,80]
Anxiety; Exaggerated Startle	Yes	Yes	[79,81]
Anxiety; Social Interaction	Yes	Not reported	[82]
Depression; Shuttle Escape Deficit	Yes	No	[20,83]
Depression; Forced Swim	Yes	Maybe	[79,84,85]
Depression; Sucrose Preference	Yes	Not reported	[86,87]
Anorexia	Yes	Not reported	[28,88]
Reinstatement of Drug Seeking	Yes	Yes	[89,90]
Neurobiology			
Amygdala	Yes	Yes	[79,91]
Ventromedial Prefrontal Cortex	Yes	Yes	[21,92]
Dorsal Raphe Nuclei	Yes	Not reported	[21]
Nucleus Accumbens	Yes	Not reported	[71]
Dorsal Striatum	Yes	Not Reported	[93]
BNST	Yes	Not reported	[94,95]
Habenula	Yes	Not Reported	[96]
Corticosterone	Yes	Yes	[78,79,97]
Serotonin	Yes	Not reported	[21]
Norepinephrine	Yes	Not reported	[29,98]
Interleukin-1	Yes	Yes	[99,100]
Glucose	Yes	Not reported	[67,68]
Adenosine	Yes	Not reported	[71,83,101]

Table 1: Summary of LH and SEFL-induced change. This table displays a summary of the behavioral, neural, and pharmacological effects of LH and SEFL stressors

Further precise exploration to assess the behavioral and neurobiological dissociation between the two procedures is necessary. By further understanding the mechanisms of each stressor we may be able to more accurately target investigation into neural mechanisms and effective treatment of specific disease phenotypes. This goal can best be reached by minimizing the lab-specific stress procedure permutations that are presently under use and focusing on stressors that can be parametrically titrated and objectively compared.

The issue of stress chronicity research

There is considerable experimental and clinical interest in the effects of stress chronicity on behavioral and biological outcomes. Despite a large chronic stress literature, the question of whether acute and chronic stress produce dissociable phenotypic profiles remains unanswered. The reason little-to-no headway has been made toward answering this question is a simple matter of inadequate experimental control groups in most studies. Indeed, the majority of studies claiming to provide information on the effects of chronic stress use a non-stressed group as their control. Therefore, any conclusions made in regards to the effects of chronic stress are invalid. The differences between groups may be attributed to stress exposure, but there is not a proper comparison to resolve if the observed effects can be attributed to the *chronicity* of the stressor. In those studies that provide an acute stress control, there tend to be issues of unmatched stress quality and/or volume between groups. These issues even appear in the highest-profile chronic stress literature. For example, we see blatant comparison confounds in the landmark paper by Firdaus Dhabar and Bruce McEwen, "Acute stress enhances while chronic stress suppresses cell-mediated immunity *in vivo*: A potential role for leukocyte trafficking" [106]. In this paper, the acute-chronic stress comparison is confounded by both volume and quality differences. Acute groups receive a single 2 or 5-hour stress exposure consisting of restraint, shaking, or both. Meanwhile chronic-

stressed groups receive daily 6-hour stress exposures for 3 to 5 weeks consisting of equal parts restraint, shaking, and both. This amounts to a comparison between acute and chronic-stressed groups that differ in total stress exposure time by up to 28 hours! Not only that, but chronically-stressed groups receive variable stress, while acute-stressed animals receive exposure to a single stress type. One may surmise, if these confounds are present in the most popular works of the field (this paper has been cited over 1,000 times) by leading researchers in the field (Bruce McEwen has been cited over 150 thousand times) then it is likely a widespread issue within the field. And it is. Similar issues show-up again and again throughout the chronic stress literature (for example, [107-112], and so on). It is therefore essential to develop a research strategy which allows us to test the effects of chronicity without volume and/or quality confounds. The SEFL procedure provides us with a unique opportunity to do just that.

The SEFL stress procedure can be easily manipulated to test the effects of stress chronicity, while controlling for other factors such as volume, quality, and severity. As previously described, the acute SEFL procedure consists of 15, 1 mA unpredictable footshocks that occur over a 90-minute session. We have designed a chronic stress procedure that controls for these dimensions by simply dividing this 90-minute procedure into 15 exposures, each consisting of a pre-shock interval and a single shock. Thus, time in the stress context, time to next shock, and shock volume are equated between the two conditions, with the only distinction being distribution of the experiences. This allows us to more accurately assess the behavioral and biological effects of chronicity.

We hypothesize that chronically-stressed rats will exhibit several key differences from the acutely-stressed rats. For one, we hypothesize that the SEFL behavior produced from chronic stress, unlike acute stress, will have an associative component. We suggest that chronic stress's

differential engagement of associative influences is an example of the ubiquitous rule that spaced experiences are more effective at promoting learning and memory than massed experiences. Obviously, the chronic condition administers stress in a more spaced manner. There is clear theoretical and empirical precedent for the premise that massed trials favor non-associative processes, and spaced trials favor associative processes in the conditioning and habituation literatures [113-116]. Additionally, we suspect that chronically-stressed rats will exhibit depression-like behavior commonly reported in the chronic stress literature, but not seen following our acute stress procedure [117,118]. Due to the potential depression-like effects of chronic stress, which mirror similar effects observed after high-volume stress exposure, we suspect that post-stress glucose will impact the behavioral effects of chronic stress exposure. Finally, we believe that we will see biological changes that reflect these behavioral differences.

Dissertation overview

Here I will answer three questions:

Q1: What are the peripheral impacts of post-stress glucose consumption, as they relate to its prophylactic effects within the learned helplessness model of PTSD?

Q2: What is the impact of stress volume on subsequent fear learning, depression-like behavior, and neurobiology?

Q3: What is the impact of stress chronicity on subsequent fear learning, depression-like behavior, and neurobiology?

The conclusion aims to apply findings from these three studies toward future study of the basic mechanisms of stress, with a specific focus on identifying effective intervention for stress-induced disorders.

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Chapter 2: Post-stress fructose and glucose ingestion exhibit dissociable behavioral and physiological effects

Published in *Nutrients*

Abstract: An acute traumatic event can lead to lifelong changes in stress susceptibility and result in psychiatric disease such as Post-Traumatic Stress Disorder (PTSD). We have previously shown that access to a concentrated glucose solution for 24 hours beginning immediately after trauma decreased stress-related pathology in the learned helplessness model of PTSD and comorbid major depression. The current study sought to investigate the peripheral physiological effects of post-stress glucose consumption. We exposed 128 male Sprague-Dawley rats to inescapable and unpredictable 1-milliamp electric tail shocks or simple restraint in the learned helplessness procedure. Rats in each stress condition had access to a 40% glucose solution, 40% fructose solution, or water. Blood and liver tissue were extracted and processed for assay. We assessed corticosterone, corticosteroid-binding globulin (CBG), glucose, and liver glycogen concentrations at various time points following stress. We found that rats given access to glucose following exposure to traumatic shock showed a transient rise in blood glucose and an increase in liver glycogen repletion compared to those that received water or fructose following exposure to electric shock. We also found that animals given glucose following shock exhibited reduced free corticosterone and increased CBG compared to their water-drinking counterparts. However, this difference was not apparent when glucose was compared to fructose. These data suggest that post-stress glucose prophylaxis is likely not working via modulation of the HPA axis, but rather may provide its benefit by mitigating the metabolic challenges of trauma exposure.

Keywords: glucose; fructose; liver glycogen; CBG; cortisol; learned helplessness; PTSD; rat

1. Introduction

Exposure to traumatic stress results in a number of physiological and psychological changes in both human and non-human species [1,2]. 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.

The learned helplessness procedure is a classic model used to analyze the behavioral symptoms of Post-Traumatic Stress Disorder (PTSD) and comorbid depression related to an acute, traumatic stressor in rats [3–7]. The procedure consists of two phases, which are an acute-traumatic shock phase and a testing phase that occurs 24 hours later. In the initial phase, rats are either exposed to 100 inescapable and unpredictable shocks over an extended period, or restrained in plexiglass tubes for that same interval. All rats are then tested 24 hours later for escape-performance in a shuttle box. Rats that receive inescapable shock show a profound, exaggerated fear response and shuttle-escape deficits during testing [8–10]. This transition to an unresponsive, depression-like state is referred to as conservation-withdrawal [11].

A number of findings suggest that metabolic homeostasis is challenged by exposure to uncontrollable, traumatic stress [8–10,12,13]. Minor and Saade (1997) hypothesized that simply treating rats with glucose following traumatic stress would restore energy homeostasis and eliminate the helplessness effect [14]. They found that shocked rats given 18-hour access to a 40% (wt/vol) aqueous glucose solution immediately following traumatic shock stress no longer exhibited exaggerated fear responding and escape latency deficits in the shuttle-box. However, the mechanism by which glucose exerts its prophylactic effects has yet to be investigated.

Several studies have indicated that corticosteroids (CORT, cortisol in humans and corticosterone in rodents) are necessary to develop learned helplessness [15,16]. Uncontrollable

stress causes elevation in CORT, which creates abnormalities in the hypothalamic-pituitary-adrenocortical axis [17]. Metyrapone blocks CORT synthesis and upregulates CORT catabolism. Injection of metyrapone before inescapable shock prevents learned helplessness [18,19], which illustrates that the stress-induced rise in CORT is necessary for the development of the learned helplessness phenotype.

The actions of corticosteroids are not only modulated by production and release of this hormone via the HPA axis. In fact, 95% of cortisol is bound under resting conditions [20,21]. Approximately 80% of CORT is bound to the high-affinity, low capacity corticosteroid-binding globulin (CBG), 15% bound to the low-affinity, high capacity albumin, with the remaining 5% consisting of its free (or "freed") form. Qian et al. (2011) showed that CBG regulated levels of free CORT in rats during a stressor [22]. CBG binds corticosterone to produce a functionally inactive form [23]. CBG is also released from the liver when blood glucose levels have risen [23]. Therefore, we hypothesize that post-trauma glucose ingestion may upregulate CBG protein synthesis, which allows for the increased binding of circulating glucocorticoids. This downregulates free CORT.

An alternative explanation is that the prophylactic effects of glucose are independent of glucocorticoid action and merely lie in its ability to prevent the negative metabolic sequelae of trauma. Rats receive inescapable, unpredictable shock transition from an initial anxious reaction to an inactive, depression-like state when exposed to test stimuli [8,9,24]. This state serves as an adaptive mechanism for husbanding limited resources and facilitating the recovery of metabolic homeostasis [11] and is likely mediated by brain adenosine signaling [6,8,9,25]. Given that energy expenditure dramatically increases on a total-brain scale when the animal is in a state of fear [26–

28] and glucose transport is impaired during a stress response [12], it is possible that glucose is simply mitigating the negative metabolic impacts of stress.

This study used the learned helplessness procedure to examine the physiological impacts of post-stress glucose consumption. This study aimed to examine the impact of glucose for reducing the circulating levels of free CORT, increasing CBG, and increasing liver glycogen following stress pre-treatment and time of testing.

2. Materials and Methods

2.1. Subjects

One hundred twenty-eight Sprague-Dawley albino male rats (290–320 g) from Envigo (Placentia, CA, USA) were housed in individual cages in a room maintained on a 12:12-hour light/dark cycle (6:00–17:59 lights on, 18:00–5:59 lights off). Animals were housed in the room for approximately two weeks prior to testing. During this time, all animals had free access to water and food. All experimentation took place during the early light cycle (7:00–10:00, approximately). A timeline of all procedures is presented in Figure 1. The protocols in this paper received pre-approval by the UCLA Institutional Care and Use Committee.

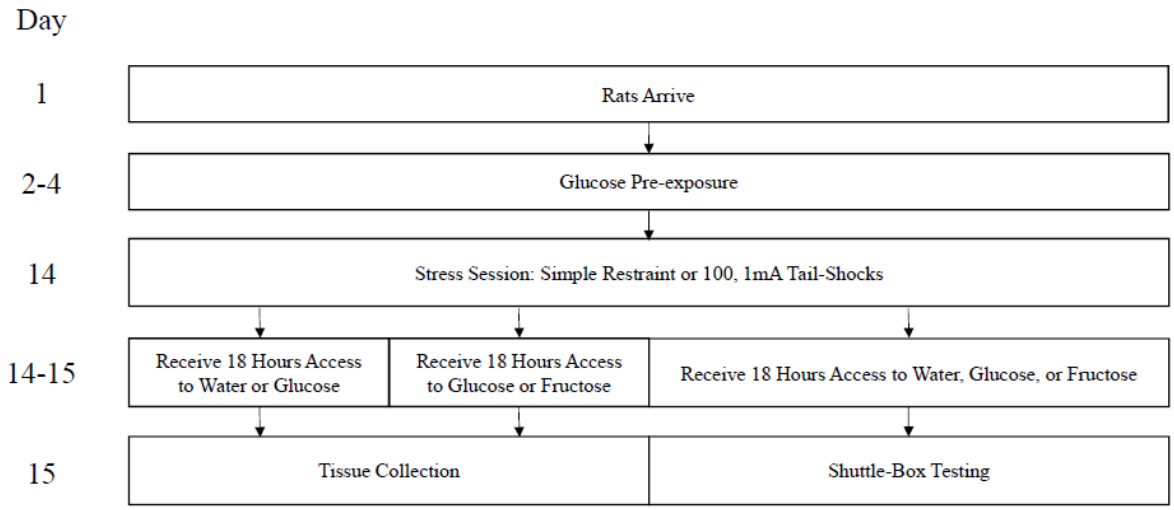

Figure 1. Timeline of events. Day 1 coincides with postnatal day (PND 50), approximately.

2.2. Apparatus

Rats were housed in metal hanging cages. Each cage was equipped with a standard glass (250 mL) water bottle with a rubber stopper and metal spout.

Rats were restrained in Plexiglass clear restraining tubes during stress pre-treatment, as previously described [24]. Unscrambled electric shock was administered via electrodes attached to a rat's extended tail. Each restraining tube was housed during the session in an illuminated, sound-attenuating chamber. Testing occurred in a shuttle box, as previously described [24]. A metal barrier divided the shuttle box into equal chambers. The chamber contained a center-pivoting grid floor that delivered scrambled shock.

2.3. Procedure

Rats were assigned randomly to groups of eight rats each. Every group was pre-exposed to a glucose cocktail and a fructose cocktail over four consecutive days [14]. The cocktails consisted of 40% glucose or fructose and 5% sucrose dissolved in tap water (weight/volume).

Rats were exposed to either inescapable shock or simple restraint, which was followed by free access to glucose, water, or fructose. Twenty-four hours following stress pre-treatment, one cohort of rats was sacrificed via rapid decapitation. Trunk blood and liver samples were collected for later analysis. It should be noted that, after evidence of a dissociable behavioral effect between post-stress glucose and fructose, a fructose group was later added to this analysis and all samples were compared to a glucose group using a new cohort of rats. Another group of rats was exposed to the same stress pre-treatment and fluid access as above, but underwent serial blood draw before and after stress pre-treatment. These same animals also underwent testing 24 hours later.

We exposed half of the groups (S: shocked) to 100, 1.0 mA variable-duration (mean = 8.0 s, range: 3 to 15 s), and inescapable tail shocks on a variable-time 60-s schedule (range: 20 to 150 s) in restraining tubes during a 110-min stress pre-treatment session. The other groups (R: restrained) were restrained in tubes for the same period and received no shock. Groups received free access to water (W: Groups SW and RW), glucose (G: Groups SG and RG), or fructose (F: Groups SF and RF) for 18 h beginning immediately following the pre-treatment stress session. We recorded total fluid consumption during this interval. All rats had free access to water over the final 6 h.

Testing or tissue collection occurred 24 h after the pre-treatment stress session in all groups. Testing began with five FR-1 trials on a 60-s fixed-time schedule. These trials required a rat to cross from one chamber to the other to terminate foot shock. During the inter-trial interval, freezing was assessed using a six-second time-sampling procedure. Freezing is defined as total immobility

of the animal [29]. Twenty-five FR-2 trials on a 6-second variable time schedule (range: 20–230 s) followed three minutes after FR-1 trial completion. These trials required a rat to cross from one chamber to the other and back to terminate foot shock. Shock was terminated on a given trial after 40 s if the animal did not meet the response contingency. Latency to terminate shock was recorded for each FR-2 trial. The intensity of shock was set at 0.6 mA.

2.4. Plasma Sample Analyses

Blood was collected from the tail prior to the acute stress session and 0, 3, and 6 h following the acute stress session in one group of animals. At the time of typical testing (24 h after stress pretreatment), another group of rats was sacrificed using a small rat guillotine. Blood was collected from the trunk of the rat and the right lateral lobe of the liver was extracted.

Assay of CBG, free corticosterone, and total corticosterone plasma concentrations were determined by using a commercially-available ELISA kit (Cat# E-EL-R1112, Elabscience, Bathesda, MD, USA; ADI-900-097, Enzo Life Sciences, Farmingdale, NY, USA). The assays were performed according to the manufacturer's instructions. Liver tissue was pulverized using an electric pestle [30]. To prepare liver tissue for the glycogen assay, we followed procedures for hydrolysis [31], standard preparation [32], and analysis of tissue [33]. The concentration of CBG is presented as ng/mL of plasma, free and total corticosterone as ug/dL, and glycogen as ug/g of tissue, which accounts for the dilution factor.

2.5. Statistical Analysis

Software package SPSS (SAS Institute, Inc., Version 16.0, Cary, NC, USA) was used for statistical analyses. A multivariate analysis of variance (MANOVA) with stress type and fluid type as the between-subjects factors was conducted for free and total corticosterone plasma

concentrations. A mixed-design ANOVA with stress type and fluid type as the between subjects factors was conducted for post-stress glucose consumption and CBG plasma concentrations. *A priori* planned comparisons were also made to determine whether inescapable tail-shock would reduce liver glycogen concentrations, and if post-stress glucose would replenish these depleted stores. Following significant interactions, Neuman-Keuls post-hoc analysis are reported. Statistical significance was noted when p values were less than 0.05. Data is presented as group means with error bars denoting group mean +/− SEM. No statistical outliers were removed from the data. Animals were excluded solely based on equipment malfunction.

3. Results

3.1. Effects of Post-Stress Glucose on Peripheral Physiology at the Time of the Test

Baseline glucose consumption for individual rats ranged between 21 and 45 ml. Mean intake was similar among groups and across pre-exposure days. A mixed-design analysis of variance (ANOVA: Group × Pre-exposure Day) yielded no statistically significant main effects or interactions, $F(3, 69) = 0.798$, $p = 0.499$. Post-stress fluid consumption ranged between 15 and 48 ml. A single-factor ANOVA showed no statistically significant effect of group, $F(3, 69) = 1.398$, $p = 0.251$.

Figure 2 shows free and total corticosterone, CBG, and liver glycogen concentrations among groups. Shock groups showed much higher concentrations of both free and total corticosterone compared to their restraint counterparts. Restraint groups showed no differences in free or total corticosterone levels regardless of the type of solution they consumed (Figure 2A). Shocked rats that received glucose following the stress session (SG) showed decreased concentrations of free corticosterone compared to shocked rats that received only water. Shocked rats showed no

differences in total corticosterone levels regardless of the solution consumed. The water groups (RW & SW) showed lower concentrations of CBG compared SG (Figure 2B). RG showed modest, but not significant elevations of CBG compared to both water groups. The group that received the traumatic shock condition followed by *ad libum* access to water (SW) showed much lower liver glycogen concentrations compared to all other groups (RW, RG, SG, Figure 2C). No other groups appear to differ in liver glycogen concentrations. Groups did not differ in blood glucose concentrations (Figure 2D), $F(3, 26) = 1.584$, $p = 0.217$.

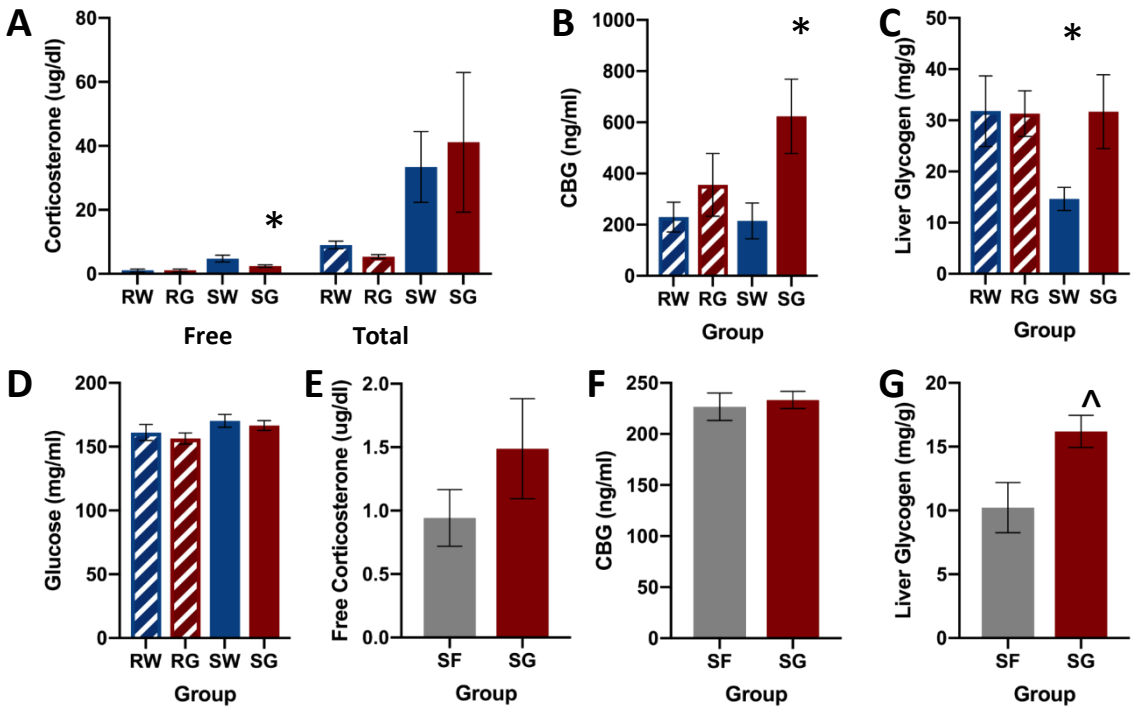

Figure 2. Corticosterone (panels **A** & **E**), CBG (panels **B** & **F**), liver glycogen (panels **C** & **G**), and glucose (panel **D**) concentrations among groups, following FR-1 shuttle-escape testing. Animals received either inescapable and unpredictable shock (S) or simple restraint (R). Following the stress session, animals were given 18-h free access to a 40% glucose cocktail (G), 40% fructose cocktail (F), or water (W). In shocked rats, glucose reduced free CORT, increased plasma CBG, and increased liver glycogen compared to water controls. However, CBG and corticosterone concentrations did not differ between shocked rats that received glucose or fructose. Liver glycogen concentrations were higher in shocked rats that received glucose compared to their fructose-drinking counterparts. Error bars denote mean ± SEM. * $p < 0.05$ (comparison: SG, SW), ^ $p < 0.05$ (comparison: SG, SF).

A multivariate ANOVA on corticosterone concentrations yielded a significant main effect of Group on Free CORT, $F(3, 28) = 20.039$, $p < 0.001$, as well as a significant main effect of the Group on Total CORT, $F(3, 28) = 5.032$, $p < 0.001$. Neuman-Keuls post-hoc comparisons ($\alpha = 0.05$) on group means indicated a relationship among groups for Free CORT, such that: RW = RG < SG < SW. Neuman-Keuls post-hoc comparisons ($\alpha = 0.05$) on group means indicated a relationship among groups for Total CORT, such that: RW = RG < SW = SG.

A one-way ANOVA on CBG concentrations yielded a significant main effect of Group, $F(3, 28) = 3.384$, $p = 0.034$. Neuman-Keuls post-hoc comparisons ($\alpha = 0.05$) on means indicated a relationship among groups such that: RW = RG = SW < SG.

A priori planned comparisons using two-tailed t-tests were conducted to compare restraint and shock conditions (RW, SW), and glucose and water groups within the shock condition (SW & SG). Unpaired, two-tailed t-tests showed a significant difference in liver glycogen between RW and SW groups, $t(14) = 2.31$, $p = 0.036$, and between SW and SG groups, $t(14) = 2.52$, $p = 0.025$.

Also pictured in Figure 2 are identical measures assayed in a new cohort of rats that received either glucose or fructose following shock. Baseline glucose and fructose consumption for individual rats ranged between 20 and 31 ml. Mean intake was similar among groups and across pre-exposure days. A single-factor analysis of variance (ANOVA: Group) yielded no statistically significant main effects for glucose, $F(1, 13) = 0.394$, $p = 0.541$, or fructose, $F(1,10) = 3.954$, $p = 0.075$. Post-stress fluid consumption ranged between 20 and 47 mL. A single-factor ANOVA showed no statistically significant effect in the group, $F(1, 14) = 3.384$, $p = 0.087$.

No group differences were observed for free corticosterone or CBG plasma concentrations (Figures 2E and 2F). However, the SG group showed a significantly higher concentration of glycogen in the liver compared to SF (Figure 2G).

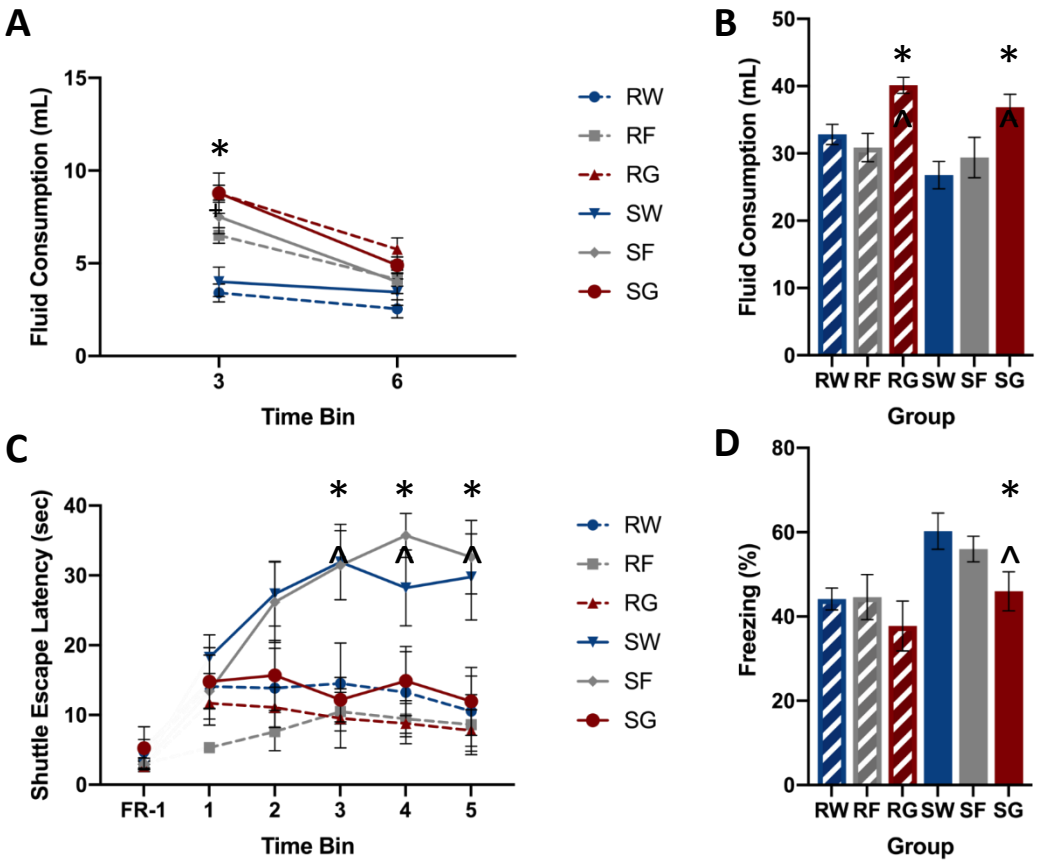

Figure 3. Mean fluid consumption at 3 and 6 h post-stress (panel **A**) and 18 h post-stress (panel **B**), escape latencies (panel **C**), and percent freezing for FR-1 trials (panel **D**) among groups. Rats were exposed to inescapable shock (S) or restraint (R) over a 110-min period. Rats from each stress condition had free access to water (W), a concentrated glucose solution (G), or a concentrated fructose solution (F) for 18 h, beginning immediately following stress. Shuttle-box testing occurred 24 h later. Rats were exposed to five FR-1 trials of the foot-shock. These trials were run from one side to the other shut-off shock. The amount of time spent freezing between trials was measured. Twenty-five FR-2 trials, which were broken into five groups of five, required two shuttle-crossings to shutoff shock. The time it took for required shuttle crossings was measured during each trial. Shocked animals that received glucose performed similarly to restraint controls, while animals that received water or fructose following shock exhibited increased escape latencies and freezing during testing. Rats that received glucose or fructose consumed more during the first three hours after trauma compared to their water-drinking counterparts. Rats that received post-stress glucose consumed more fluid over the 18-hour period compared to rats that received water or fructose. Error bars denote mean ± SEM. * $p < 0.05$ (comparison: SG, SW), ^ $p < 0.05$ (comparison: SG, SF), + $p < 0.05$ (comparison: SF, SW).

Single-factor ANOVA yielded no statistically significant effects of the group on free corticosterone, $F(1,14) = 2.292$, $p = 0.152$, or CBG, $F(1,14) = 0.174$, $p = 0.683$. A single-factor ANOVA analysis yielded a significant effect of the Group on liver glycogen concentrations, $F(1,12) = 5.917$, $p = 0.032$.

3.2. Effects of Post-Stress Glucose on Peripheral Physiology Following Stress Pre-Treatment

Baseline glucose and fructose consumption for individual rats ranged between 16 and 37 mL. Mean intake was similar among groups and across pre-exposure days. A mixed-design analysis of variance (ANOVA: Stressor × Fluid Type × Pre-exposure Day) yielded no statistically significant main effects or interactions for glucose, $F(2, 50) = 0.516$, $p = 0.600$, or fructose $F(2, 42) = 0.928$, $p = 0.403$. Post-stress fluid consumption ranged between 1 and 4 mL per hour. A mixed-design ANOVA (Stressor × Fluid Type × Time Bin) yielded statistically significant interactions of Time Bin by Stressor, $F(2, 92) = 6.689$, $p = 0.002$, and Time Bin by Fluid Type, $F(4, 92) = 10.313$, $p < 0.001$. Newman–Keuls post-hoc comparisons ($\alpha = 0.05$) indicated the following order of relationship among group means: W = F < G.

Figure 3 shows post-stress fluid consumption, shuttle-escape latencies, and freezing among groups. Shocked groups that received water or fructose following trauma showed significantly higher escape latencies compared to the restraint controls (Figure 3A). However, the shocked group that received glucose following trauma did not show this increase in escape latency. Shocked groups that received water or fructose following trauma showed exaggerated fear responding with respect to the restraint controls (Figure 3B). However, the shocked group that received glucose following trauma did not show this increase in freezing.

A mixed-design ANOVA on FR-2 shuttle-escape latencies (Stressor × Fluid Type × Time Bin) yielded a significant interaction for Time Bin by Stressor, $F(4, 156) = 3.890$, $p = 0.005$, and

Time Bin by Fluid Type, F(8, 156) = 3.914, $p < 0.001$. Newman–Keuls post-hoc comparisons ($\alpha = 0.05$) indicated the following order of relationship among group means: RW = RG = RF = SG < SW = SF. A single-factor ANOVA (Stressor × Fluid Type) on FR-1 shuttle-escape latencies showed no significant main effects or interactions, F(2, 50) = 1.508, $p = 0.231$.

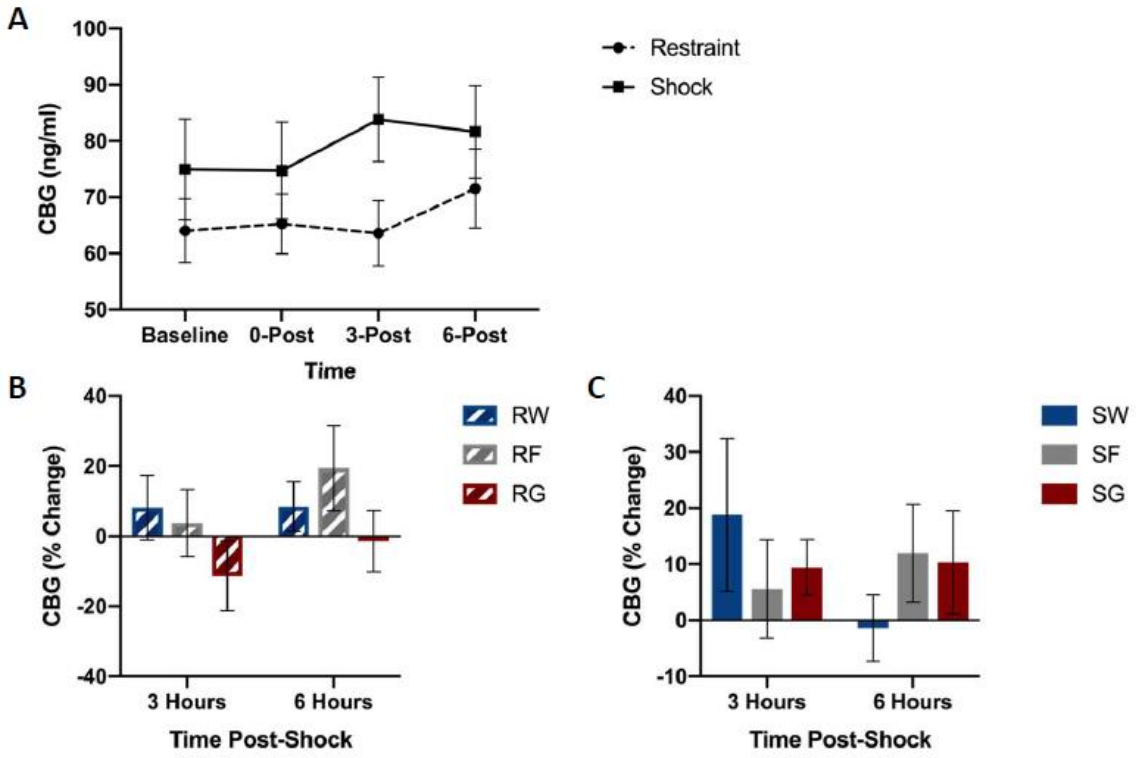

Figure 4. CBG concentrations between stress treatments (panel **A**), and percent change from 0 h post-stress among fluid conditions in restraint (panel **B**) or shock (panel **C**) stress treatments. Blood was collected for analysis immediately before the acute stress session, and 0, 3, and 6 h following the acute stress session. Animals received either inescapable and unpredictable shock (S) or simple restraint (R). Following the stress session, animals were given 18-h free access to a 40% glucose cocktail (G), 40% fructose cocktail (F), or water (W). CBG concentrations were not influenced by stress or fluid type.

A single-factor ANOVA on freezing (Stressor × Fluid Type) yielded the significant main effects of the Stressor, F(1,52) = 10.021, $p = 0.003$, and Fluid Type, F(2, 52) = 4.612, $p = 0.014$. Newman–Keuls post-hoc comparisons ($\alpha = 0.05$) indicated the following order of relationship among group means: RW = RG = RF = SG < SW = SF.

Figure 4 shows CBG concentrations among groups. No differences were observed in CBG levels based on the fluid or the stressor type. A mixed-design ANOVA on CBG (Stressor × Fluid Type × Time Bin) yielded no significant main effects or interactions, $F(2, 39) = 0.309$, $p = 0.736$.

Figure 5 shows free corticosterone concentrations among groups. Shock groups showed much higher concentrations of free corticosterone compared to their restraint counterparts immediately following the termination of stress pre-treatment (Figure 5A). However, no differences were observed in free corticosterone levels based on the type of solution consumed.

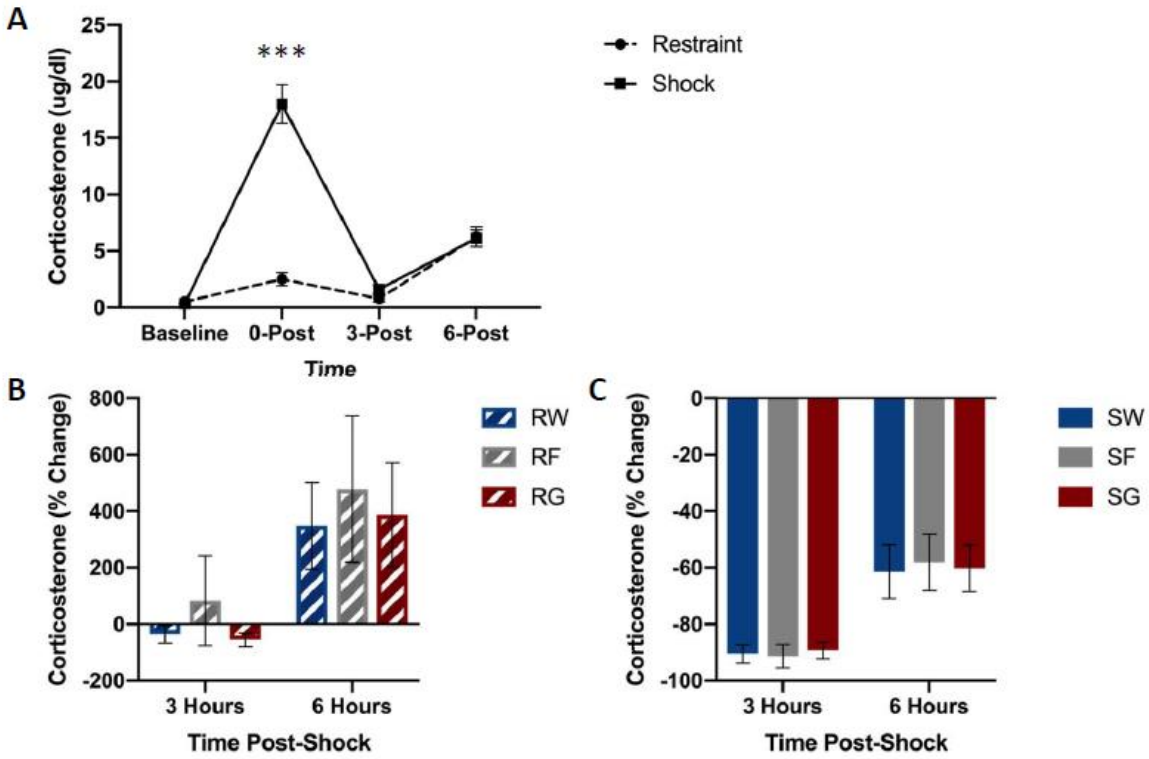

Figure 5. Free corticosterone concentrations between stress treatments (panel **A**), and percent change from 0 h post-stress among fluid conditions in restraint (panel **B**) or shock (panel **C**) stress treatments. Blood was collected for analysis immediately before the acute stress session, and 0, 3, and 6 h following the acute stress session. Animals received either inescapable and unpredictable shock (S) or simple restraint (R). Following the stress session, animals were given 18-h free access to a 40% glucose cocktail (G), 40% fructose cocktail (F), or water (W). Shocked animals exhibited higher concentrations of free corticosterone immediately after the stress session (0-Post). Error bars denote mean ± SEM. *** $p < 0.001$ (comparison: Restraint, Shock).

A mixed-design ANOVA on free corticosterone concentrations (Stressor × Fluid Type × Time Bin) yielded a significant Time Bin by Stressor interaction, $F(1, 42) = 14.618$, $p < 0.001$. Post hoc analysis indicated that corticosterone concentrations 0 hours after stress pre-treatment were significantly higher in rats that received shock compared to the restraint, $t(47) = 7.197$, $p < 0.001$.

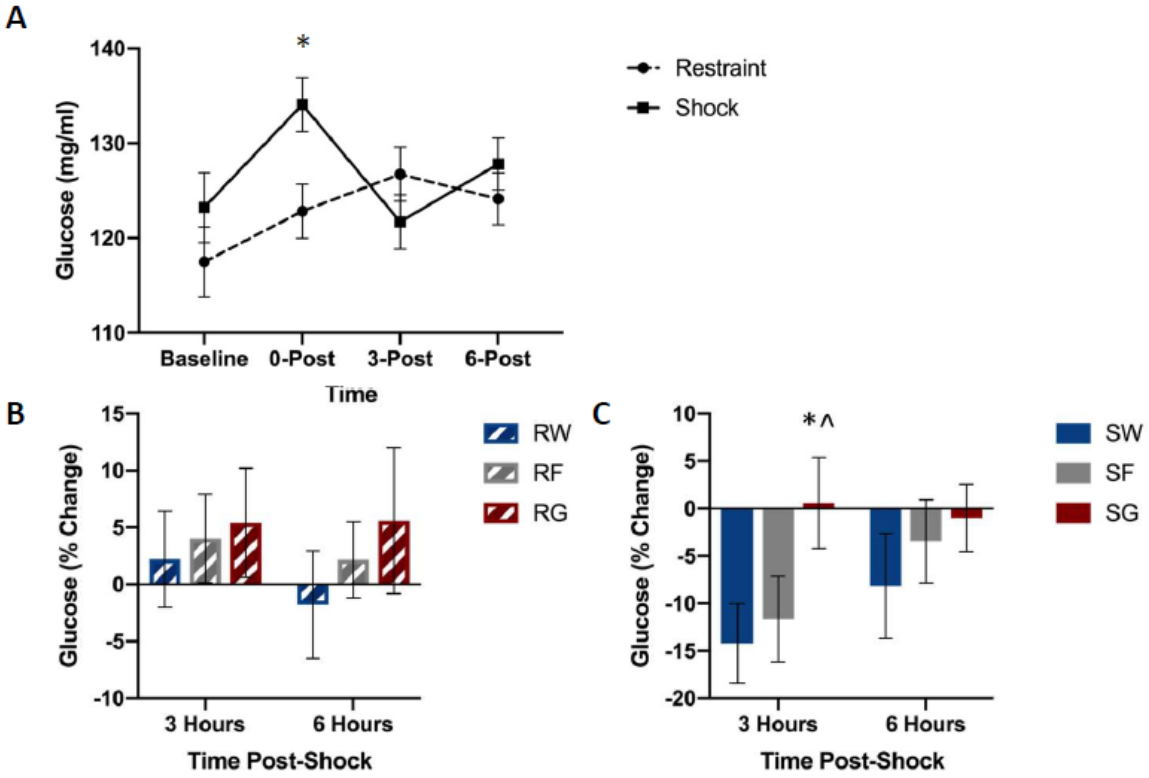

Figure 6. Blood glucose levels between stress treatments (panel **A**), and percent change from 0 h post-stress among solution conditions in restraint (panel **B**) or shock (panel **C**) stress treatments. Blood was collected for analysis before the acute stress session, and 0, 3, and 6 h following the acute stress session. Animals received either inescapable and unpredictable shock (S) or simple restraint (R). Following the stress session, animals were given 18-h free access to a 40% glucose cocktail (G), 40% fructose cocktail (F), or water (W). Glucose mitigated the post-stress decline in blood glucose concentrations in shocked animals. Error bars denote mean ± SEM. * $p < 0.05$ (comparison for top figure: Restraint, Shock, comparison for bottom figures: SG, SW), ^ $p < 0.05$ (comparison: SG, SF).

Figure 6 shows blood glucose concentrations among groups. Shocked groups exhibited a transient rise in blood glucose concentrations immediately following shock, followed by a dip in concentrations three hours later, which did not occur in restrained controls (Figure 6A). However, this drop did not occur in rats that received access to post-shock glucose (Figure 6C).

A mixed-design ANOVA on blood glucose concentrations (Stressor × Fluid Type × Time Bin) yielded a significant Stressor by Fluid Type by Time Bin interaction, $F(2,45) = 0.894$, $p = 0.038$. Post-hoc analysis indicated that glucose concentrations 3 h after stress pre-treatment were significantly higher in the SG group compared to groups SW, $t(16) = 2.583$, $p = 0.020$, and SF, $t(17) = 2.577$, $p = 0.020$.

4. Discussion

These experiments indicate that post-stress glucose consumption alleviates the energy homeostasis challenge of traumatic shock. It also suggests that the prophylactic effects of glucose are independent of HPA-axis activity. Furthermore, it appears that these effects are specific to glucose since fructose does not eliminate the negative behavioral consequences of stress nor does it impact blood glucose or liver glycogen concentrations in a similar way.

Figure 2 depicts corticosterone, CBG, glucose, and liver glycogen concentrations in rats that received free access to water, glucose, or fructose following traumatic stress or simple restraint. We found a large increase in both free and total corticosterone concentrations between groups that received traumatic shock compared to groups that received simple restraint. In groups that received simple restraint, there were no observed differences in free or total corticosterone concentrations between rats that received water or glucose. However, in groups that received traumatic shock, rats that received access to glucose following the acute stress session exhibited lower concentrations of free corticosterone compared to their water-drinking counterparts. No difference

in total corticosterone was observed between these two groups (SW & SG). When comparing glucose to fructose in shocked rats, we observed an effect of fluid type on liver glycogen concentrations, but not corticosterone or CBG. Figures 3–6 depict corticosterone, CBG, and blood glucose concentrations before and after stress pre-treatment. Rats in this study were also tested 24 hours following stress pre-treatment for the learned helplessness phenotype. We found that glucose, but neither water nor fructose, eliminated the negative behavioral consequences of traumatic shock. Shocked rats exhibited a transient rise in blood glucose concentrations immediately following termination of the stress session, which was followed by a decline in blood glucose three hours following stress pre-treatment. This rise in glucose concentrations is most likely due to epinephrine-induced glycogenolysis [34] even though the cause of the subsequent decline is less clear. Notably, glucose, exclusively, eliminated the transient decline of blood glucose concentrations 3 hours following shock. These findings show that the post-stress consumption of glucose, specifically, transiently raises blood glucose levels and mitigates liver glycogen depletion following stress exposure. Therefore, it may be this ability of glucose to reduce the metabolic challenges of stress that provide its prophylactic effects. However, how this effect works remains unclear. For example, the neural consequences of this post-stress glucose ingestion have yet to be investigated.

One potential neural pathway of glucose prophylaxis involves the hippocampus, which is a structure that is particularly vulnerable to the metabolic consequences of stress [35]. Following a stressor, CORT is one of many hormones and peptides upregulated. When, at high concentrations, CORT promotes mild insulin resistance to mobilize glucose for the brain [36]. However, not all brain regions benefit equally from this increase in circulating glucose. An increase in circulating CORT during stress impairs glucose uptake in the hippocampus and severely impairs contextual

processing [12,13,37–39]. Furthermore, studies have shown that high CORT levels cause high levels of hippocampal atrophy compared to moderate CORT levels [40,41]. Inescapable shock in rats creates a similar neuroplastic deficit in the hippocampus [42]. Such deficits are reversed by increasing hippocampal glucose concentrations by any number of means [43]. This suggests that increasing hippocampal glucose concentrations could decrease glutamate toxicity and potentially reduce some of the sequalae of depression. This indicates that learned helplessness and PTSD-like symptoms may be in part due to the mechanism in which traumatic stress elevates cortisol levels, and that consumption of a high concentration glucose solution may moderate the CORT-dependent stress effects.

Minor and LoLordo (1984) demonstrated that the helplessness effect is eliminated when rats can discriminate the training context, in which inescapable shock is delivered, from the shuttle-escape testing context [44]. Contextual learning critically depends on hippocampal processing [45]. Thus, post-stress glucose consumption may allow veridical encoding of the context in the hippocampus, which results in less generalization between the two contexts. This hypothesis is further supported by our previous finding that post-stress glucose only exhibits its prophylactic effects if given within the first three hours of stress pre-treatment [24]. We observed that the glucose-induced transient rise in blood glucose levels occurs three hours post-stress, which suggests that this may play a role in the beneficial effects of glucose consumption.

Perhaps the benefit of post-stress glucose is independent of hippocampal processing and is instead simply derived from its ability to prevent metabolic exhaustion. Fear is an intensely catabolic state and rapidly challenges brain metabolic homeostasis [2,8–10,19,46]. Under these circumstances, adenosine is released to inhibit further activity in an effort to prevent cell death. Minor and colleagues have shown that adenosine A_{2A} receptors are involved in the conservation-

withdrawal symptoms normally observed following traumatic stress [2,6,8–10,15,19,25,46,47]. Glucose consumption following trauma might restore metabolic homeostasis, as shown by the rise in liver glycogen concentrations. This could, thereby, eliminate the necessity for the compensatory adenosine response.

The data provide evidence supporting the role of glucose in diminishing the energetic challenges of traumatic shock. These findings illustrate that consumption of glucose directly following an acute traumatic stressor reduces the transient drop of blood glucose levels following stress pre-treatment and restores glycogen in the liver. The data also suggest that the behavioral effects of post-stress glucose consumption are independent of corticosterone's role in the induction of stress-induced behavioral effects. This indicates that glucose has a major role in mitigating the physiological and psychological challenges posed by stress, but the exact mechanism remains unclear. Lastly, it should be noted that there are several other animal models of PTSD that model many different aspects of the disease. It is possible that there are dissociable mechanisms by which different stressors induce their behavioral effects. It is, therefore, imperative to examine the behavioral and physiological effects of post-stress glucose in alternative models of PTSD in order to increase external validity and the potential for translational efficacy.

Author Contributions: Conceptualization, M.A.C., T.R.M., M.S.F., and L.P.T. Methodology, M.A.C., T.R.M., M.S.F., and L.P.T. Software, T.R.M. Validation, M.A.C. and M.S.F. Formal analysis, M.A.C. Investigation, M.A.C. and N.M.W. Resources, M.S.F. and T.R.M. Data curation, M.A.C.. Writing—original draft preparation, M.A.C. and N.M.W. Writing—review and editing, M.S.F., L.P.T., and M.A.C. Visualization, M.A.C. Supervision, M.S.F. and T.R.M. Project administration, M.A.C. and M.S.F. Funding acquisition, M.S.F.

Funding: Supported by NIMH Grant R01 MH115678

Acknowledgments: This is a posthumous authorship for Dr. Thomas Minor who made major contributions to the study design and data collection.

Conflicts of Interest: The authors declare no conflict of interest.

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Chapter 3: Modeling Stress Disorders: Behavioral and neurobiological consequences of stress volume in the rat

In preparation for submission

Abstract

Exposure to traumatic stress leads to persistent, deleterious behavioral and biological changes in both human and non-human species. Despite great headway made in understanding the biological basis of stress, effective clinical application of these findings has been scant. It has been hypothesized that this may be in part due to widespread procedural differences between basic research laboratories. For example, rats given access to glucose following stress has been shown to eliminate a subset of behaviors quintessential to the learned helplessness phenotype. However, the prophylactic effects of glucose have not been replicated following other stress procedures. The current study sought to test the hypothesis that procedural differences in stress volume (presentation length x intensity x number) may account for a portion of the symptom heterogeneity exhibited in both the clinic and the laboratory. We exposed 208 sprague-dawley male rats to inescapable and unpredictable 1-milliamp electric tail shocks or simple restraint. Rats that received tail shock received either 15 or 100 shocks in a procedure that mirrored the common stress procedures used to induce stress-enhanced fear learning or learned helplessness, respectively. To test the hypothesis that glucose has stressor- and phenotype-specific effects, a subset of animals received 18-hours free access to a 40% glucose solution following stress pretreatment or intraperitoneal injection of 2-deoxy-D-glucose. Rats underwent behavioral testing, or sacrifice for tissue analysis, one to seven days later. We found a double-dissociation, such that moderate-volume stress produced the SEFL phenotype but had no effect on weight maintenance, while high-volume stress produced the opposite behavioral effects. Interestingly, the SEFL behavior was

rescued when rats were given glucose following high-volume stress. However, glucoprivation did not inhibit the formation of SEFL in rats exposed to moderate stress. These effects seem specific to contextual fear conditioning, as cued fear conditioning was enhanced following both moderate and high-volume stress. Finally, we show that rats exposed to high-volume stress show elevations of GluA1 in the basolateral amygdala (BLA) similar to the moderate-volume group. However, high-volume stress also increased BLA GluA2 and decreased hippocampal NR1 when compared to controls. These data suggest that differences in the volume of stress exposure differentially impact the behavioral and biological effects of stress. However, this relationship cannot simply be summarized as more stress results in more negative effects.

Introduction

Acute, intense stressors can lead to a wide variety of physiological and psychological conditions in both human and non-human species [1,2]. One such disease, Post-Traumatic Stress Disorder (PTSD), develops in up to 20% of those that experience a traumatic stressor [3]. PTSD is a debilitating and heterogenous disease marked by a wide array of potential symptoms such as amnesia, anhedonia, avoidance behaviors, exaggerated fear-potentiated startle, hypervigilance, and insomnia [4]. PTSD patients also exhibit a wide array of comorbidities [5-8]. Great strides have been made in understanding the neurobiological consequences of severe stress, yet there has been little headway made in identifying effective treatment of stress-induced psychiatric diseases such as PTSD.

One possible explanation is that the animal-model research has failed to accurately capture and account for the apparent heterogeneity of PTSD seen in the clinical population [9]. Stress models vary widely between research groups, which leads to divergent behavioral and biological findings [9,10]. Despite the apparent disparity among groups, there has been little to no attempt to

thoughtfully consolidate the stress literature. This can be, in part, attributed to the fact that many stressors used in the laboratory are qualitatively different, making it nearly impossible to responsibly compare findings. In a recent review, we looked at two stress procedures which appear somewhat comparable, due to their mutual use of inescapable and unpredictable electric shock as the stressor [10]. In this review, we compared the behavioral and biological impacts of the stressors used to induce *learned helplessness* and *stress-enhance fear learning*.

The *learned helplessness* stressor consists of 100, 1 mA tailshocks of variable length (mean: 8 seconds) that occur during a 2-hour session [11,12]. The hallmark behavior of this stressor is the subsequent deficit in escape performance within the shuttle-box apparatus [13,14]. However, rats exposed to 100 shocks also exhibit a wide array of behavioral characteristics that parallel several of the symptoms of PTSD and depression ([15,16] for review, see [10]). Furthermore, several neurobiological mediators of the shuttle-escape deficit have been identified. Specifically, a pathway involving serotonin release from the dorsal raphe nucleus has been well defined through decades of research [11]. The impacts of the energetically-demanding fear state caused by this extensive stress session have also been implicated in the deleterious behavioral consequences. The 100-shock session has been shown to transiently stress energy homeostasis [17]. Furthermore, access to a concentrated glucose solution or an adenosine antagonist reverse the shuttle-escape deficits produced by the stressor [18-21], while artificial glucoprivation using 2-deoxy-D-glucose and adenosine agonists promote shuttle-escape deficits in unstressed rats [22,23]. We have therefore hypothesized that the energetic challenge induced by the stressor is a key mediator for the observed deleterious behavioral effects.

The *stress-enhanced fear learning* stressor consists of 15, 1 mA footshocks of fixed length (1 second) that occur during a 1.5-hour session. This stress procedure and subsequent behavioral

phenomena initially became popular due to its ability to enhance subsequent fear learning under novel conditions [24,25]. This allowed for the manipulation and study of several deficits in contextual fear conditioning [26]. It was then later discovered that this shock procedure produced a robust array of anxiety-like behaviors [27,28]. Rats exposed to 15 shocks also exhibit a wide array of behavioral characteristics similar to the symptoms of PTSD, but do not exhibit depression-like behavior as reported following 100 shocks (for review, see [10,28]). Evidence of the neurobiological mediators for SEFL are relatively limited, but initial evidence points toward a rise in GluA1 in the basolateral amygdala as a mediator for the sensitization effect [28].

The stressors induce several similar behavioral characteristics that model anxiety in the rat. The research also suggests that they may diverge in the induction of depression-like behavior. However, no direct comparison has been performed. Here we test the theory that more stress equates to greater behavioral and biological consequences. The *learned helplessness* and *stress-enhanced fear learning* stressors are particularly useful in examining this question as they are qualitatively similar yet vary on one major dimension: shock volume (shock number x mA x length). To avoid confusion between the stressors and behavioral consequences which popularized them, we will subsequently refer to the 100-shock procedure as *high-volume shock (HVS)*, the 15-shock procedure as *moderate-volume shock (MVS)*, and the restraint controls as *no shock (NS)*.

Five experiments are reported that investigate the behavioral and neurobiological consequences of stress volume. Rats were restrained in tubes and exposed to either 0, 15, or 800 cumulative seconds of shock over a 1.5 to 1.83 hour interval. Rats were assessed for enhanced fear learning or sacrificed for tissue analysis one day or one week after stress pretreatment. Pharmacological and glucose manipulation (if any) occurred immediately after the termination of

the stress session. All rats were weighed throughout the study and weight gain was compared across groups.

2. Materials and Method

2.1. Subjects

Two hundred and eight Sprague-Dawley albino male rats (290–320 grams) from Envigo (Placentia, CA, USA) were housed in individual cages in a room maintained on a 12:12-hour light/dark cycle (6:00–17:59 lights on, 18:00–5:59 lights off). Animals were housed in the room for approximately two weeks prior to testing. During this time, all animals had free access to food and water. All experimentation took place during the early light cycle (7:00–10:00, approximately). The protocols in this paper received pre-approval by the UCLA Institutional Care and Use Committee.

2.2. Apparatus

Rats were housed in metal hanging cages. Each cage was equipped with a standard glass (250 mL) water bottle with a rubber stopper and metal spout.

Rats were restrained in clear Plexiglass restraining tubes during stress pre-treatment, as previously described [18]. Unscrambled electric shock was administered via electrodes attached to a rat's extended tail. Each restraining tube was housed during the session in an illuminated, sound-attenuating chamber. Testing occurred in Med Associates (St Alban, Vt) behavioral testing chambers. Each chamber is equipped with an infrared camera, speaker for tone delivery, shock scrambler, and fluorescent and infrared light sources. The behavioral testing chambers in each testing room are controlled by a PC using Med Associates Video Freeze software that also automatically scores motion and freezing of the animal during the test session. To create distinct contexts between stress pretreatment and subsequent fear conditioning and testing, the chamber's

contextual features were modified using differential lighting and odors, and interchangeable grid floors and wall inserts.

2.3. Procedure

Rats were assigned randomly to groups of eight to ten rats each. Rats were exposed to restraint, fifteen (moderate-volume) or one hundred (high-volume) inescapable tailshocks. One day or one week later, rats underwent a fear conditioning procedure or sacrifice for tissue analysis.

Rats that received high-volume shock were exposed to 100, 1.0 mA variable-duration (mean = 8.0 s, range: 3 to 15 s) and inescapable tail shocks on a variable-time 60-s schedule (range: 20 to 150 s) in restraining tubes during a 113-min stress pre-treatment session. Rats that received moderate-volume shock were exposed to 15, 1.0 mA fixed-duration (1 second) and inescapable tail shocks on a variable-time 360-s schedule (range: 120 to 900 s) in restraining tubes during a 90-min stress pre-treatment session. The other groups were restrained in tubes for the same period (113 or 90 minutes) and received no shock. A homecage control was added for all experiments involving tissue analysis. These animals were handled the same as other groups, but were not exposed to stress pretreatment.

In the experiment involving the glucose intervention, every group was pre-exposed to a glucose cocktail over three consecutive days [19]. The cocktail consisted of 40% glucose and 5% sucrose dissolved in tap water (weight/volume). Rats received 18-hours of free access to glucose or water immediately following the termination of stress pre-treatment. In the experiment involving peripheral injection of 2DG, rats were injected intraperitoneally with either vehicle or 600 mg/kg of 2DG dissolved in distilled water immediately following the termination of stress pretreatment.

The fear conditioning procedure was as follows. On the first day of testing, rats were placed in a novel environment and received a single, 1-second and 1 mA footshock after three minutes of free exploration. Rats were retrieved thirty seconds after shock exposure and returned to their homecage. The following day, rats were placed back into this context for eight minutes. Time spent freezing was assessed during both days. In the cued fear learning experiment, a 30 second, 65 decibel, 2800 hz tone preceded and coterminated with shock. Rats were preexposed to a novel context one day following contextual fear conditioning testing. Following preexposure, all rats received a tone test, which consisted of three, 30-second tone presentations spaced one minute apart and following a three-minute baseline period.

2.4. Western Blot Analyses

Dorsal hippocampus, ventral hippocampus, and basolateral amygdala were dissected and flash frozen for western blot analysis. Tissue was homogenized and spun to separate crude and synapto-neurosome homogenate and diluted in a synaptic protein extraction reagent containing protease and phosphatase inhibitors (ThermoFisher, Cat #s 87793 & 78440). Protein concentrations of diluted homogenate were estimated using BCA assay (ThermoFisher, Cat # 23225). 15ug of protein was loaded into a 10% polyacrylamide gel for electrophoretic separation, and then transferred to a PVDF membrane (Bio-Rad, Cat #s 5671035 & 1704157). Lanes were assessed for total protein using Ruby protein blot staining (ThermoFisher, Cat # S11791). Primary antibody was then applied overnight and secondary antibody (fluorescent or chemiluminescent) was applied for one to two hours the following day. Tissue was analyzed for GluA1 (Millipore cat # ABN241, 1:5000), GluA2 (Millipore cat # MABN1189, 1:1000), NR1 (Millipore cat # AB9864, 1:5000), NR2a (Millipore cat # AB1555P, 1:10000), NR2b (Abcam cat # AB28373, 1:5000), and GAPDH (Abcam cat # AB8245, 1:5000). Secondary antibodies were applied at a 1:10000 to

1:5000 dilution depending on primary antibody specifications (Abcam cat # AB205719, Bio-Rad cat #s 12005867 & 12004162). Blots were imaged using a ChemiDoc MP imager and analyzed using Image Lab software (Bio-Rad, cat #s 17001402 & 1709690).

2.5. Statistical Analysis

Software package SPSS (SAS Institute, Inc., Version 16.0, Cary, NC, USA) was used for statistical analyses. One-way, two-way, three-way, and mixed-design ANOVAs were used when appropriate. Following significant interactions, Neuman-Keuls post-hoc analyses are reported. Statistical significance was noted when p values were less than 0.05. Data is presented as group means with error bars denoting group mean +/− SEM. No statistical outliers were removed from the data. Animals were excluded solely based on equipment malfunction.

Results

Moderate and high-volume stressors result in dissociable behavioral effects

Here we test the hypothesis that high and moderate-volume stressors will exhibit distinct behavioral phenotypes. Specifically, we hypothesized that rats exposed to high-volume stress will not express the SEFL phenotype characteristic of moderately-stressed rats. Conversely, we hypothesized that moderate-volume stress will not induce a pronounced challenge to energy homeostasis, as exemplified by the long-lasting suppression of weight gain seen in rats exposed to a high-volume stressor.

Figure 1 shows percent freezing to the conditioned context and weight change following stress pretreatment. Baseline and post-shock freezing are also shown. The moderate-volume shock group showed higher levels of freezing compared to the restraint and high-volume shock groups immediately following the single shock as well as 24 hours later. Conversely, the high-volume shock group showed greater weight loss compared to the restraint group on the days following

stress exposure. No differences in baseline freezing (prior to the single shock exposure) were observed ($F < 1$). Identical behavioral effects were found when the latency between stress pretreatment and 1-shock conditioning was one day, instead of one week, apart (see Supplemental Figure 1).

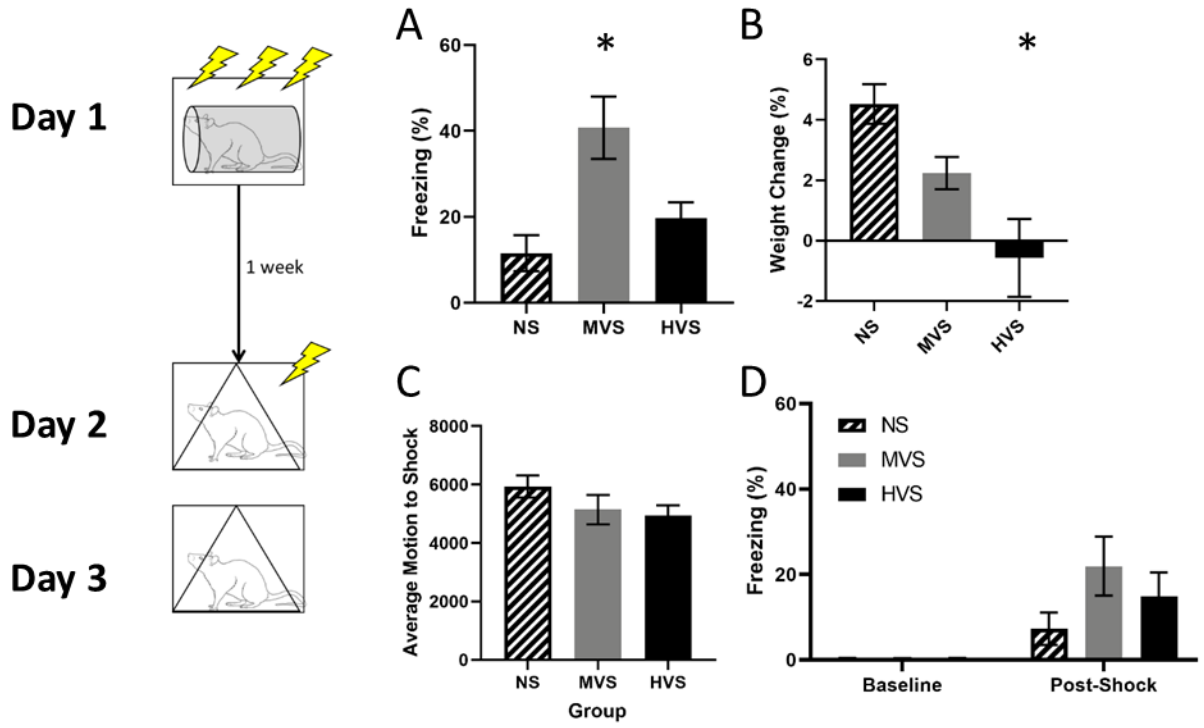


Figure 1. Effects of stress volume on fear learning and weight maintenance. Depicted: Percent freezing prior to and following exposure to one shock (panel D) and during context fear test the following day (panel A), weight change (panel B), and average motion to shock (panel C). Rats were exposed to 0 (NS), 15 (MVS), or 100 (HVS) tailshocks one week prior to fear conditioning testing. Testing consisted of exposure to a single, 1 mA shock in a novel context. Rats were then returned to this same context 24-hours later. Rats were weighed prior to stress exposure and prior to fear conditioning testing. The MVS group spent more time freezing during the context group when compared to NS and HVS groups. The HVS group showed significantly less weight gain when compared to MVS and NS groups. There were no observed differences in shock reactivity or baseline freezing among groups. As is typically observed, post-shock freezing behavior exhibited a similar trend as that seen during the context test, but no statistically significant differences were found. Error bars denote mean ± SEM. * $p < .05$ (compared to NS).

A one-way analysis of variance (ANOVA) on post-shock freezing yielded a significant main effect of Group on Freezing (%), $F(2, 21) = 3.903$, $p = .036$. Neuman-Keuls post-hoc comparisons on groups means indication a relationship among groups, such that: MVS < NS. A one-way ANOVA on freezing during the context test yielded a significant main effect of Group on Freezing (%), $F(2, 23) = 7.095$, $p = .005$. Neuman-Keuls post-hoc comparisons on groups means indication a relationship among groups, such that: MVS > HVS = NS. A one-way ANOVA on Weight yielded a significant main effect of Group, $F (2, 20) = 8.860$, $p = .0018$. Neuman-Keuls post-hoc comparisons on groups means indication a relationship among groups, such that: MVS = NS > HVS. A one-way ANOVA showed no statistically significant effect of group on shock reactivity, $F(2, 21) = .751$, $p = .484$, or baseline freezing to the 1-shock context, $F(2, 21) = .846$, $p = .443$.

Post-stress glucose rescues SEFL behavior in rats exposed to high-volume stress

The previous experiment showed that differences in stress volume impact subsequent behavior and physiology of the animal. Specifically, we found a double-dissociation such that rats exposed to moderate-volume stress exhibited SEFL but did not have suppressed weight gain; rats exposed to high-volume stress showed the opposite effect. Prior studies have indicated that ingestion of glucose following high-volume stress reverses several of the stressor's behavioral impacts [17-19]. We therefore hypothesize that post-stress glucose may (somewhat counterintuitively) induce the SEFL phenotype not previously observed in rats exposed to high-volume stress. We also suggest that glucose may mitigate the suppression of weight gain observed following high-volume stress.

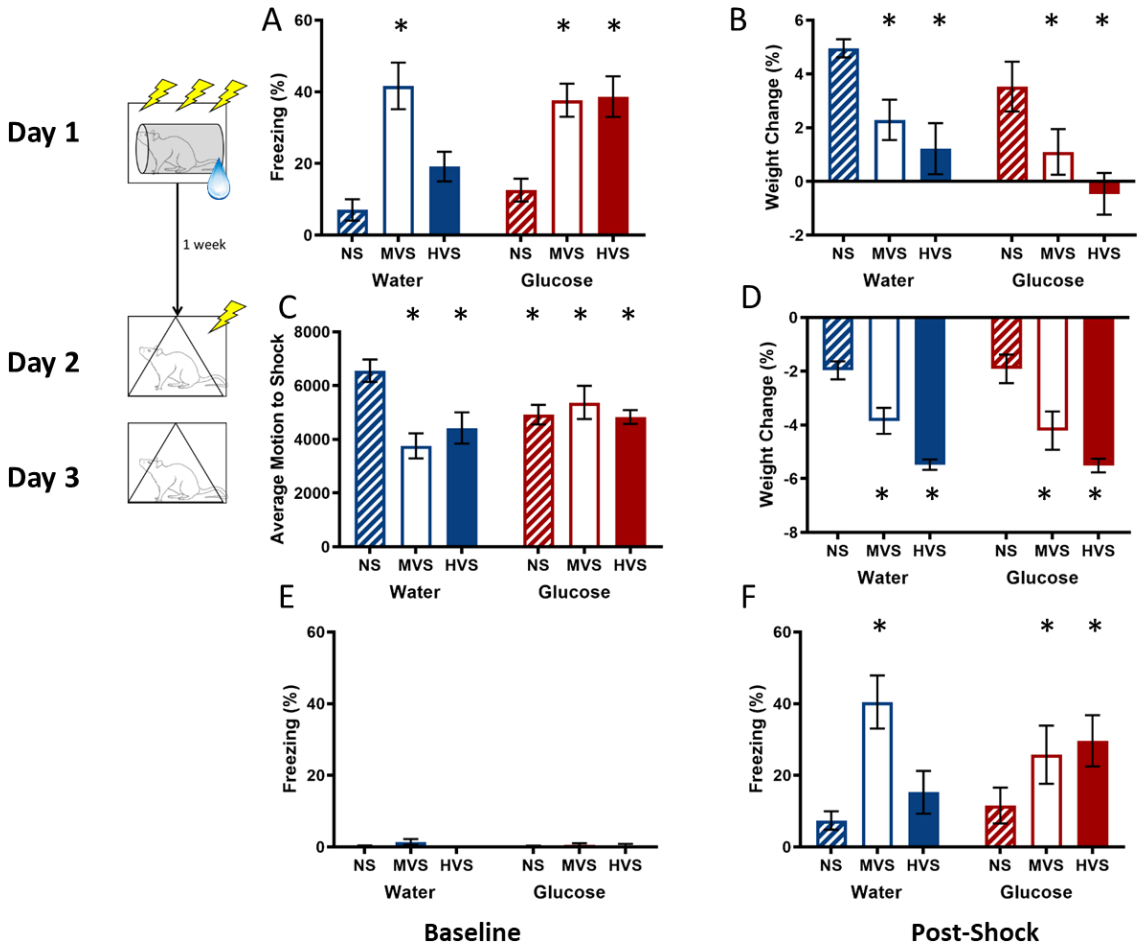


Figure 2. Impacts of glucose ingestion on the fear learning and weight maintenance stress volume effects. Depicted: Percent freezing prior to and following exposure to one shock (panel E/F) and during context fear test the following day (panel A), weight change (panel B & D), and average motion to shock (panel C). Rats were exposed to 0 (NS), 15 (MVS), or 100 (HVS) tailshocks one week prior to fear conditioning testing. Following stress exposure, all groups received 18-hour free access to a 40% glucose solution or tap water. All bottles were then switched back to tap water for the remainder of the experiment. Testing consisted of exposure to a single, 1 mA shock in a novel context. Rats were then returned to this same context 24-hours later. Rats were weighed prior to stress exposure and prior to fear conditioning testing. In groups that received water only, the MVS group spent more time freezing during the context group when compared to NS and HVS groups. However, in groups that received post-stress glucose, both MVS and HVS groups exhibited freezing levels higher than the NS group. Regardless of fluid condition, both the HVS and MVS groups showed significantly less weight gain when compared to the NS group. The group that received water-only following no shock exhibited greater shock reactivity than all other groups. There were no observed differences in baseline freezing among groups. As is typically observed, post-shock freezing behavior exhibited a similar trend as that seen during the context test, but no statistically significant differences were found. Error bars denote mean ± SEM. * $p < .05$ (compared to NS Water).

Figure 2 shows percent freezing to the conditioned context and weight change following stress pretreatment (one day and one week following). Baseline and post-shock freezing are also shown. In rats given water following shock, the moderate-volume shock group showed higher levels of freezing compared to the restraint and high-volume shock groups during the context test (as seen in previous experiment). However, rats given glucose following high-volume shock exhibited freezing levels higher than their water drinking counterparts and similar to rats given moderate volume shock. Interestingly, weight gain was depressed in both HVS and MVS groups when compared to NS. Furthermore, there appeared to be an overall depression of weight gain in groups that received access to post-stress glucose. A one-way ANOVA showed no statistically significant effect of group on shock reactivity, $F(2, 21) = 1.622$, $p = .221$, baseline, $F(2, 21) = .001$, $p = .999$, or post-shock freezing to the 1-shock context, $F(2, 21) = 1.889$, $p = .176$.

A two-way ANOVA on freezing during the context test yielded a significant Stress x Fluid interaction, $F(2, 42) = 3.499$, $p = .0393$. Neuman-Keuls post-hoc comparisons on groups means indicated a relationship among groups, such that: NS-W = NS-G = HVS-W < HVS-G = MVS-W = MVS-G. A two-way ANOVA on weight change (%) yielded significant main effects of Stress, $F(2, 40) = 12.34$, $p < .0001$, and Fluid , $F (1, 40) = 4.945$, $p = .0319$. Neuman-Keuls post-hoc comparisons on Stress indicated a relationship among groups, such that NS > MVS = HVS.

2DG-induce glucoprivation does not inhibit the formation of SEFL behavior in rats exposed to moderate-volume stress

The previous experiment showed that consumption of a glucose solution is enough to produce SEFL in rats exposed to high-volume stress, which otherwise do not exhibit the phenotype. Here we test the opposite: is artificial glucose deprivation sufficient to inhibit the

expression of SEFL in moderately-stressed animals? Previous research has shown that glucoprivation of unstressed controls, using the compound 2-deoxy-D-glucose (2DG), was enough to induce several of the behavioral phenotypes typically observed following high-volume stress [23]. Here we test the hypothesis that injection of 2DG following stress pretreatment or at the time of the single-shock exposure will suppress expression of the SEFL phenotype in rats exposed to moderate-volume stress.

Figure 3 shows percent freezing during the contextual fear test, and weight change among groups following stress pretreatment and drug or vehicle injection. Rats that received vehicle and moderate-volume shock showed higher levels of freezing compared to the vehicle-restraint group during the context test 24 hours after 1-shock exposure (as previously seen). Injection of 2-DG had no effect on contextual fear expression. Rats that received injection of 2-DG following moderate-volume shock exhibited a greater percent of body weight lost when compared to vehicle groups. A one-way ANOVA showed no statistically significant effect of group on shock reactivity, baseline, or post-shock freezing to the 1-shock context.

A one-way ANOVA on freezing during the context test yielded a significant main effect of Group, $F(2, 16) = 4.688$, $p = .0250$. Neuman-Keuls post-hoc comparisons on groups means indicated a relationship among groups, such that: NS-V < MVS-V = MVS-D. A one-way ANOVA on Weight Change (%) yielded a significant main effect of Group, $F(2,28) = 5.039$, $p = 0.0135$. Neuman-Keuls post-hoc comparisons on groups means indicated a relationship among groups, such that: NS-V = MVS-V > MVS-D.

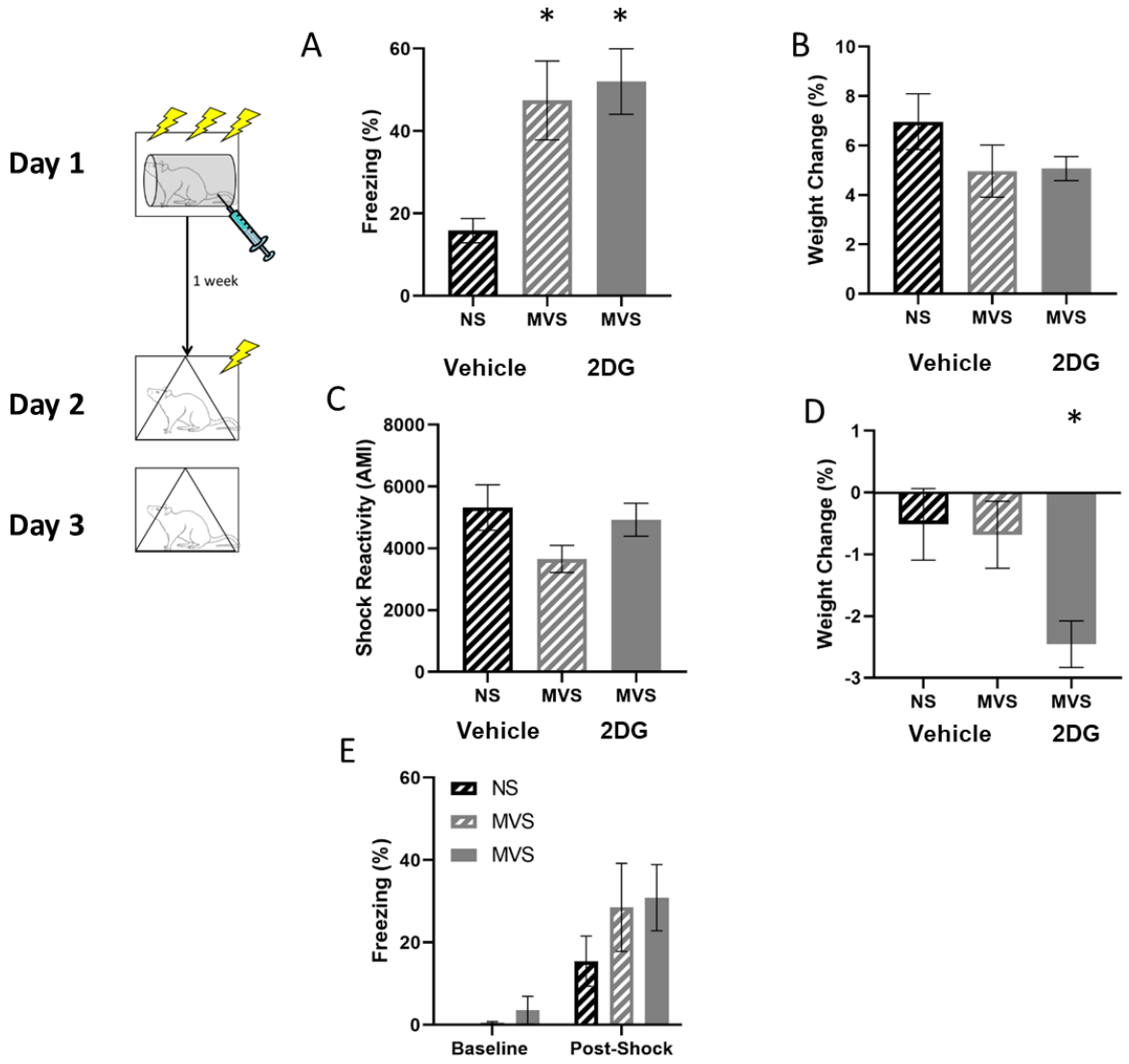


Figure 3. Post-stress injection of 2-deoxy-d-glucose does not inhibit SEFL in rats exposed to MVS. Depicted: Percent freezing prior to and following exposure to one shock (panel E) and during context fear test the following day (panel A), weight change (panel B & D), and average motion to shock (panel C). Rats were exposed to 0 (NS), or 15 (MVS) tailshocks one week prior to fear conditioning testing. Following stress exposure, all groups received intraperitoneal injection of 2-deoxy-D-glucose or vehicle. Testing consisted of exposure to a single, 1 mA shock in a novel context. Rats were then returned to this same context 24-hours later. Rats were weighed prior to stress exposure and prior to fear conditioning testing. Regardless of drug condition, the MVS groups spent more time freezing during the context group when compared to the NS group. There were no observed differences in weight gain, shock reactivity, or baseline freezing among groups. As is typically observed, post-shock freezing behavior exhibited a similar trend as that seen during the context test, but no statistically significant differences were found. Error bars denote mean ± SEM. * $p < .05$ (compared to NS Vehicle).

Moderate and high-volume stress exposure results in enhanced cued fear conditioning

The previous experiment showed that while consumption of glucose is enough to provoke the expression of SEFL in high-volume stressed animals, peripheral glucoprivation does not inhibit SEFL in moderately-stressed animals. This suggests that while adequate circulating glucose may be an important component of the formation of SEFL, artificial peripheral glucose deprivation is not sufficient to inhibit the phenotype. While high volume stress has great physiological impact on the hippocampus, evidence of the stressor's functional impact remains elusive [29]. While there is no evidence of stress' impact on function during an unstressed state, there is a small body of evidence which suggests that hippocampal processing may be impaired during subsequent testing that elicits the stress response [30,31]. Here, we test the hypothesis that high-volume stress impairs the enhancement of subsequent contextual fear conditioning by decreasing hippocampal function during a stressful event. Specifically, we hypothesize that while rats exposed to high-volume stress do not express SEFL to a context, they will express SEFL to a tone- an association that does not require the hippocampus [32].

Figure 4 shows percent freezing prior to and following exposure to a single shock, freezing to the 1-shock context 24-hours later, freezing during preexposure to a novel context, and freezing to the shock-associated tone in the preexposed context. The moderate-volume shock group showed higher levels of freezing compared to the restraint and high-volume shock groups during the contextual fear test and the first day of context preexposure. However, compared to the no-shock group, both moderate-volume and high-volume shock groups exhibited higher levels of freezing to the tone. No differences in baseline freezing (prior to the single shock exposure or tone presentation) were observed ($F < 1$).

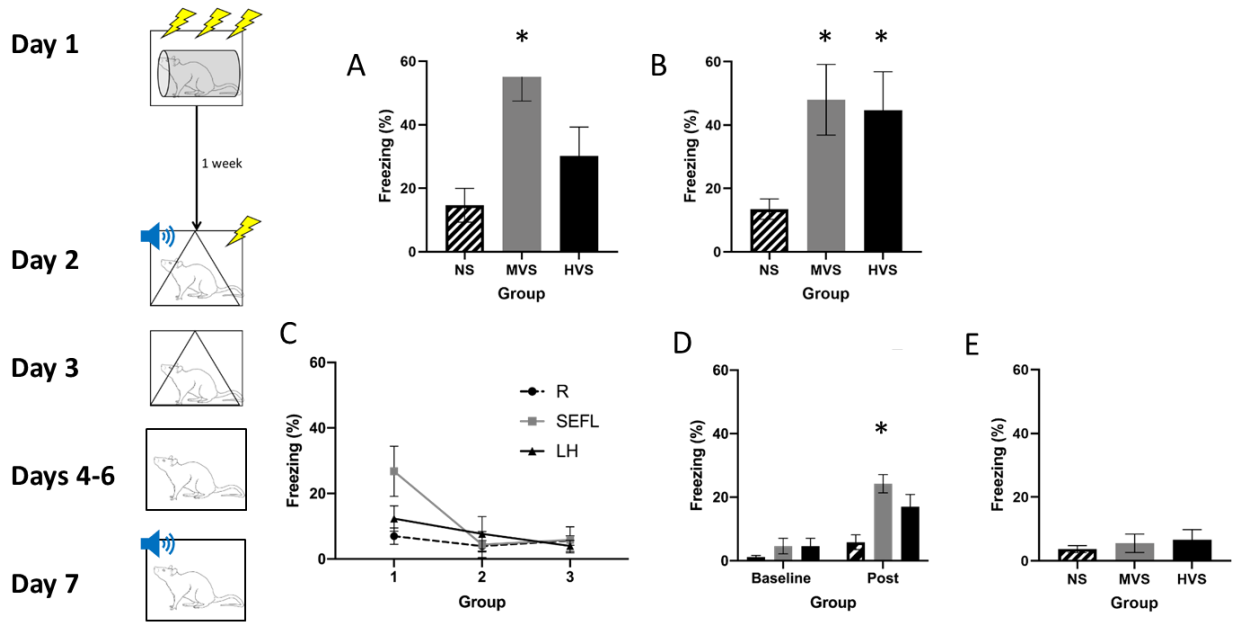


Figure 4. Moderate and high-volume stress exposure results in enhanced cued fear conditioning. Depicted: Percent freezing during context fear test (panel A) and during presentation of the conditioned tone (panel B). Percent freezing during context preexposure (panel C), prior to and following exposure to one shock (panel D), and prior to tone presentation during the tone test (panel E). Rats were exposed to 0 (NS), 15 (MVS), or 100 (HVS) tailshocks one week prior to fear conditioning testing. Testing consisted of exposure to a single, 1 mA shock in a novel context following presentation of a 30-second tone. Rats were then returned to this same context 24-hours later. Rats were then preexposed to a novel context. In this context, the previously-conditioned tone was presented and freezing was assessed. The MVS group spent more time freezing during the context group when compared to NS and HVS groups. Interestingly, both MVS and HVS groups showed higher levels of freezing to the tone compared to the NS group. There were no observed differences in shock reactivity or baseline freezing among groups. As is typically observed, post-shock freezing behavior exhibited a similar trend as that seen during the context test, but no statistically significant differences were found. Error bars denote mean ± SEM. * $p < .05$ (compared to NS).

A one-way ANOVA on freezing during the context test yielded a significant main effect of Group, $F(2, 24) = 7.944$, $p = .0023$. Neuman-Keuls post-hoc comparisons on groups means indicated a relationship among groups, such that: MVS > HVS = NS. A mixed-design ANOVA on freezing during context preexposure yielded a significant Group x Trial interaction, $F(4, 26) = 3.185$, $p = .0296$. Tukey's post-hoc comparisons on groups means indicated a relationship among

groups on Trial 1, such that: MVS > HVS = NS. A one-way ANOVA on freezing during tone presentation yielded a significant main effect of Group, $F(2, 22) = 4.327$, $p = 0.0260$. Neuman-Keuls post-hoc comparisons on groups means indicated a relationship among groups, such that: NS < MVS = HVS.

Moderate and high-volume stressors result in dissociable neurobiological effects

We have shown thus far that stress volume impacts the subsequent behavioral phenotype in a dissociable manner. Here we test the hypothesis that stress also produces dissociable neurobiological effects. Our lab has previously shown that moderate-volume stress increases the concentration of the GluA1 α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit in the basolateral amygdala [28]. Since we have shown that high-volume stress inhibits hippocampal-dependent (context) SEFL, we hypothesize that high-volume stress will produce a reduction in N-methyl-D-aspartate (NMDA) receptor concentrations in the hippocampus. We also hypothesize that high-volume stress will induce a similar increase of GluA1 in the BLA, since high-volume stress induced hippocampal-independent (tone) SEFL.

Figure 5 shows AMPA and NMDA receptor subunit protein quantification in the BLA and the DH one week after stress treatment. Rats exposed to high-volume stress exhibited greater weight loss seven days after stress exposure as previously seen. Rats exposed to moderate or high-volume stress exhibited greater levels of GluA1 in the BLA; rats exposed to high-volume stress also exhibited higher levels of GluA2. Rats exposed to high-volume stress exhibited decreased concentrations of NR1 in the DH compared to restraint controls. All stressed groups exhibited a decreased NR2a:2b ratio in the DH compared to homecage controls.

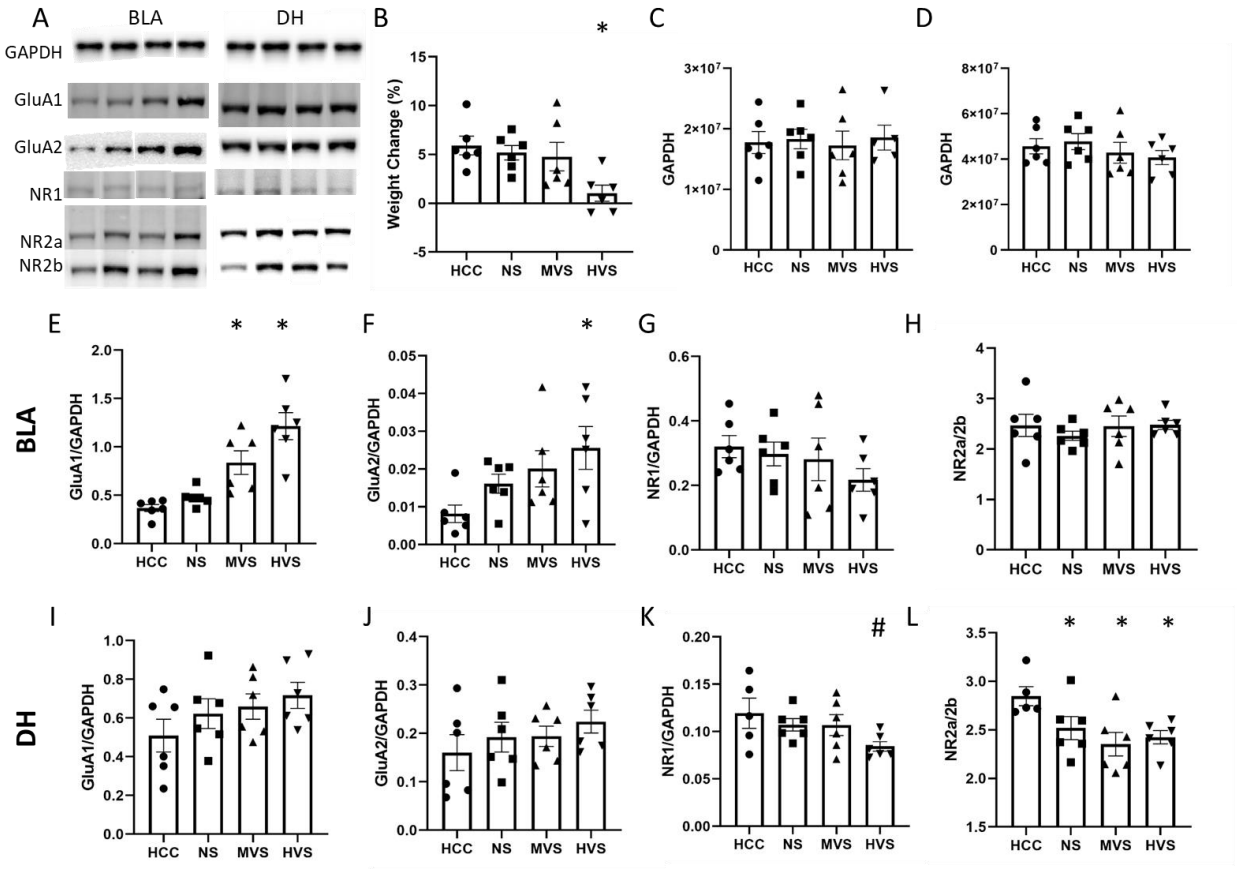


Figure 5. Neurobiological effects of stress volume. Depicted: Basolateral amygdalar (BLA) and dorsal hippocampal (DH) concentrations of GluA1 (panels E & I), GluA2 (panels F & J), NR1 (panels G & K), and NR2a/2b (panels H & L) as determined by western blot analysis. GluA1, GluA2, and NR1 are depicted as a ratio over GAPDH concentrations (panels C & D). Rats were exposed to 0 (NS), 15 (MVS), or 100 (HVS) tailshocks, or remained in their homecage (HCC), one week prior to sacrifice for tissue analysis. Rats were weighed prior to stress exposure and prior to sacrifice. MVS and HVS groups exhibited higher BLA concentrations of GluA1 compared to HCC and NS groups. The HVS exhibited higher concentrations of GluA2 compared to the HCC group. The HVS group had lower concentration of DH NR1 when compared to all other groups. All groups exhibited a lower NR2a/2b ration in the DH when compared to the HCC group. Error bars denote mean ± SEM. * $p < .05$ (compared to HCC).

One-way ANOVAs on BLA protein analysis yielded significant main effects of Group on GluA1/GAPDH, $F(3, 20) = 15.93$, $p < .0001$, and GluA2/GAPDH, $F(3, 20) = 3.214$, $p = .0449$. Neuman-Keuls posthoc comparisons ($\alpha = .05$) on GluA1/GAPDH indicated the following ordered

relationship among group means: HCC = NS < MVS < HVS. Neuman-Keuls posthoc comparisons ($\alpha = .05$) on GluA2/GAPDH indicated the following ordered relationship among group means: HCC < HVS. One-way ANOVAs on DH protein analysis yielded a significant main effect of Group on NR2a/2b, $F(3, 20) = 3.980$, $p = .0234$. Neuman-Keuls posthoc comparisons ($\alpha = .05$) on NR2a/2b indicated the following ordered relationship among group means: HCC > NS = MVS = HVS. Due to high variability in the homecage controls, any effect of group on DH NR1 was statistically washed-out. However, if HCC is removed from analysis, a one-way ANOVA on DH protein analysis yields a significant main effect of Group on NR1, $F(2, 14) = 4.651$, $p = .0283$. Neuman-Keuls posthoc comparisons on NR1 indicated the following ordered relationship among group means: NS = MVS > HVS. A one-way ANOVA on Weight Change (%) yielded a significant main effect of Group, $F(3,20) = 4.413$, $p < .0155$. Neuman-Keuls posthoc comparisons ($\alpha = .05$) on Weight Change indicated the following ordered relationship among means: HCC = NS = MVS > HVS. No significant main effects of Group were found during protein analysis of the VH.

Discussion

The experiments described above provide evidence that the volume of a stressor is a key factor in determining the behavioral and neurobiological consequences of stress and that this cannot simply be summarized as more stress leads to greater deleterious effects. Furthermore, we found evidence that further supports the notion that high volume stressors may model stress-induced conditions that have a depression component or comorbidity, while moderate volume stressors may better model anxiety-only disorders. We found evidence that suggests that glucose exerts its behavioral effects exclusively in high volume-stressed rats. The effects of glucose appear to not only to eliminate high-volume stress-induced phenotypy, but in the case of stress-enhanced fear learning, facilitate it. Finally, we provide evidence that stressors of different volumes produce

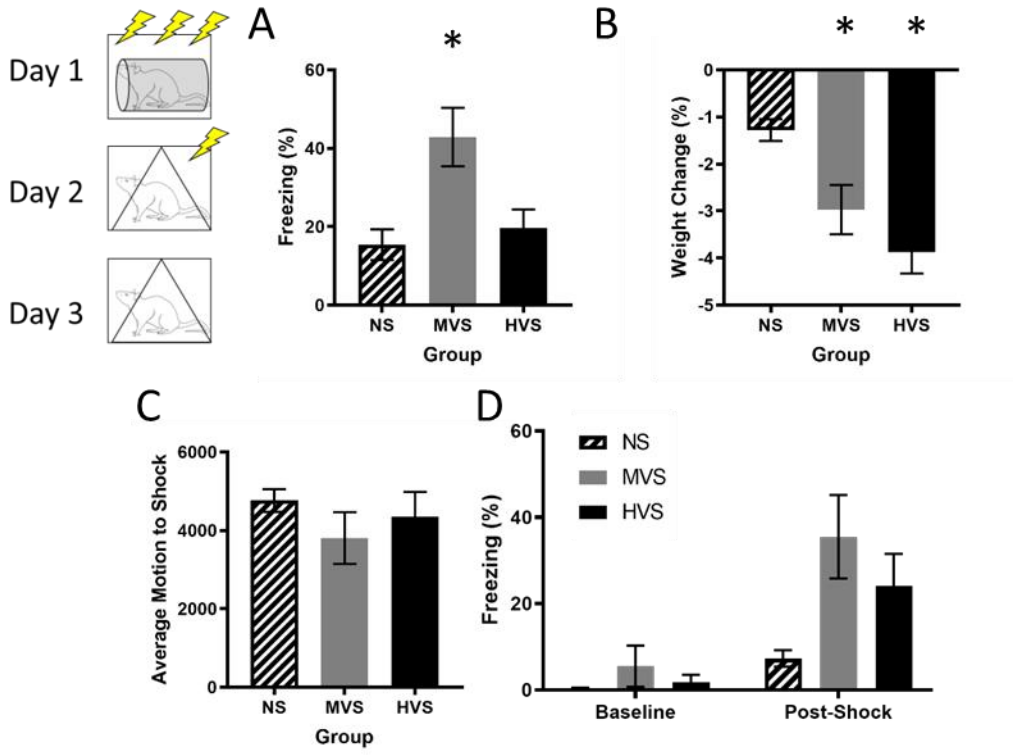
dissociable changes in AMPA and NMDA receptor density and morphology in the BLA and dorsal hippocampus.

There are a number of potential mechanisms through which stress volume exerts its effects on subsequent fear learning. One hypothesized mechanism is that the high-volume stressor is uniquely taxing energetically, such that the biological mechanism which gives rise to the non-associative effects underlying SEFL are inhibited. Shuttle-escape deficits produced by exposure to high-volume stress are reversed following the application of adenosine antagonists or the consumption of a highly concentrated glucose solution [1,17,19-23,33-37]. It has therefore been proposed that the depression-like behavior exhibited in animals exposed to high-volume shock is *conservation withdrawal* behavior due to an extreme challenge to energy homeostasis that occurs during and following exposure to the stressor [20-22]. Protein synthesis requires a large amount of energy [38]. It is possible that this challenge to energy homeostasis leads to the inhibition or dampening of the biological mechanisms responsible for SEFL. In the past, our lab has proposed that increases in BLA GluA1 are necessary for the induction of SEFL behavior. However, we saw that GluA1 levels were also elevated in the high-volume stress group. Therefore, it does not appear that this is the mechanism by which high-volume stress inhibits SEFL behavior.

Another possible explanation for the lack of SEFL is that high volume stress is producing a general deficit in contextual fear learning which masks the sensitization effect. Contextual learning critically depends on hippocampal processing [39-41]. An increase in circulating glucocorticoids during stress impairs glucose uptake transport into the hippocampus and severely impairs contextual processing [42-46]. The high-volume stress procedure used in our experiments produces deficits in contextual discrimination [30] and long-term effects on hippocampal spine density [47], neurogenesis [48], synaptic plasticity and long-term potentiation [49,50]. Deficits in

contextual learning are reversed by increasing hippocampal glucose concentrations by any of a number of means [51-53]. Therefore, while high-volume stress may still induce the non-associative fear sensitization process that occurs in moderate volume stress, the behavioral expression of this process may be nullified by an overall decrease in contextual fear learning. This is, in part, supported by our finding that high-volume stress *did* enhance fear conditioning to a tone. Evidence suggests that cued fear conditioning is hippocampal-independent [54]. Therefore, our finding that cued, but not contextual, fear conditioning is enhanced by high-volume stress suggests that hippocampal functioning may be impaired by exposure to a high-volume stressor. This hypothesis is further supported by our finding that high-volume, but not moderate-volume, stress decreases NR1 expression in the DH. NR1 is the obligatory NMDA receptor subunit, and therefore provides a reasonable estimate for NMDA receptor concentration [55]. Hippocampal NMDA receptor activity are essential for the acquisition of contextual fear [41,54,56]. Therefore, stress-enhanced contextual fear learning may be inhibited in high-volume stress by decreasing the hippocampus' ability to form new contextual memory.

These results present but a few examples of how the behavioral and biological outcomes of stress can be counter-intuitive. These studies explore the outer extremes of stress volume, and follow-up exploring intermediary values is clearly necessary. Furthermore, while we controlled for several factors, several procedural differences did remain. For example, while the moderate-volume and high-volume stress exposures occur over a relatively similar timeframe (90 and 114 minutes, respectively), this necessitates that the intervals between shocks are vastly different (six minutes and one minute, respectively). Length of individual shocks is also different between procedures. These aspects of shock undoubtedly impact subsequent behavior of the animal (in fact, see [57-59]) and can also be parametrically studied.



Supplemental Figure 1. Effects of stress volume on fear learning and weight maintenance when fear conditioning begins one day after stress pretreatment. Depicted: Percent freezing prior to and following exposure to one shock (panel D) and during context fear test the following day (panel A), weight change (panel B), and average motion to shock (panel C). Rats were exposed to 0 (NS), 15 (MVS), or 100 (HVS) tailshocks one day prior to fear conditioning testing. Testing consisted of exposure to a single, 1 mA shock in a novel context. Rats were then returned to this same context 24-hours later. Rats were weighed prior to stress exposure and prior to fear conditioning testing. The MVS group spent more time freezing during the context group when compared to NS and HVS groups. The HVS and MVS groups showed significantly less weight gain when compared to the NS group. There were no observed differences in shock reactivity or baseline freezing among groups. As is typically observed, post-shock freezing behavior exhibited a similar trend as that seen during the context test, but no statistically significant differences were found. Error bars denote mean ± SEM. * $p < .05$ (compared to NS).

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Chapter 4: Modeling Stress Disorders: Behavioral and neurobiological consequences of stress chronicity in the rat

In preparation for submission

Abstract

Exposure to traumatic stress results in a wide range of behavioral and biological effects. Many of the impacts of stress are deleterious, persistent, and often resistant to treatment. However, researchers have yet to identify factors that predict the apparent heterogeneity of stress-induced disorders. One dimension of stress disorders that likely impacts disease susceptibility and phenotypy is the chronicity of stress exposure. Unfortunately, there have been no published studies that investigate the effects of chronic stress while providing appropriate experimental controls to address this outstanding question. Here we investigate this question, using chronic and acute stress procedures that are identical across all other parameters. In a series of experiments, we use the stress-enhanced fear learning (SEFL) procedure to test the effects of chronic stress exposure on subsequent fear, anxiety, and depression-like behavior. Groups were exposed to 15, 1 mA footshocks over a single 90-minute session or across 15 days. Unstressed controls received identical context exposure without footshock. A subset of rats was given daily access to a 40% glucose solution following the termination chronic stress exposure. Following the termination chronic stress exposure, all groups received fear extinction training to the trauma context and/or preexposure to a novel context. This was done to mitigate fear generalizing from the stress to the test context. Following extinction training, all groups received a single footshock in this novel context and were tested for fear expression the following day. Additional subsets of rats underwent subsequent open field, elevated plus maze, and/or forced swim testing. We found that rats exposed to chronic stress exhibited greater generalized fear and slower fear extinction compared to their

acutely-stressed counterparts. Stress-enhanced fear learning was at comparable levels in both stress conditions when rats did not receive extinction training to the trauma context. However, rats exposed to chronic, but not acute, shock did show a decline in SEFL after they underwent extinction training. Furthermore, chronically stressed rats did not show the same enhancement in conditional fear to a tone as was observed in acutely-stress rats. Post-stress glucose enhanced generalized fear and enhanced fear learning in rats exposed to chronic, but not acute, stress pretreatment. Western blot analysis revealed that groups exposed to chronic stress exhibited an increase in basolateral GluA1 levels as previously seen in rats exposed to acute stress. However, chronically-stressed rats also exhibited increased levels of amygdalar NR1 not previously seen in acutely-stressed rats. Using our stress procedure, we were unable to replicate previous effects of chronic stress on forced swim float behavior. These findings taken together suggest that chronic stress exposure may induce a SEFL phenotype that involves a distinctive associative fear learning mechanism not present in rats exposed to acute stress. These findings open the door for future study on the effects of stress chronicity using comparable stress procedures.

Introduction

Following exposure to stress, a percentage of individuals go on to develop a particularly debilitating anxiety disorder referred to as post-traumatic stress disorder (PTSD). To develop a mechanistic understanding of why a single bout of intense stress can have such adverse consequences, we developed and characterized a rodent model, whereby a single bout of stress has a pronounced and prolonged impact on both behavior and physiology [1,2]. Rodents are exposed to a single 90 min session containing 15 (1mA, 1 sec) footshocks in a "stress context." Following this stressor, mild Pavlovian fear conditioning becomes sensitized for at least 90 days without remission, so that a context or tone paired with a single shock bestows the conditional stimulus

(CS) with a highly exaggerated, maladaptive level of fear [1]. The single shock may be the same as that used to cause the initial stress, but even a mild shock that would not normally support conditioning can now support strong conditioning after the stress [3]. Indeed, even a loud noise will support fear conditioning after this stressor [4]. We call the effect Stress-Enhanced Fear Learning (SEFL). Besides potentiated fear learning, SEFL is accompanied by several additional changes that share several commonalities with the effects of post-traumatic stress in humans. These effects include heightened anxiety, elevated baseline startle, a disturbed diurnal rhythm in basal corticosterone (CORT), increased alcohol intake, and decreased activity in rodent models of antidepressant activity [4-6]. Also like the symptomatology of PTSD, these are long-lasting changes. We have never seen the effects wane with time even with months between stress and testing [6,7]. While only a proportion of the people that experience trauma go on to develop PTSD (10-20% in the general population, considerably higher in combat veterans), about 92% of our rats show SEFL. However, if we decrease the number of stressor shocks, behavior becomes more heterogeneous, with 4 shocks producing SEFL in about 40% of the animals (for efficiency and cost considerations, we use the more effective protocol). We have shown that SEFL is robust in that we have produced the effect in males and females, rats (Sprague-Dawley and Long-Evans breeds) and mice, and over a range of modifications in the procedure.

We have also provided compelling evidence that SEFL is a non-associative phenomenon that does not depend on learning about, or even fear of, the trauma context. To be clear, what we are saying is that these effects of stress do not depend on associative learning about the stressful context; but the enhanced fear learning occurring during the test is associative. Therefore, SEFL is a non-associative enhancement in future associative fear learning. So, while we see increased fear learning in the single shock test when that single shock *follows* the stress, there is no

enhancement of fear learned *prior* to the stress [1]. We have made considerable effort to test the non-associative nature of this enhancement. For example, one associative account of SEFL would be generalization of fear from the stress (15 shock) context to the testing (1 shock) context. However, because we specifically choose stress and testing contexts that are markedly different, we see no significant baseline fear when the animals are first placed in the test (1 shock) context. Generalization is also ruled out by the fact that the 15 shocks used as a stressor are unsignaled, but will potentiate learning of fear to an auditory CS [1]. Additionally, a generalization account predicts that the order of stress and testing should not matter, but order is critical. This is not to say there is no generalization of fear in SEFL, the high levels of fear to the stress context readily generalize to similar contexts, but we employ markedly different contexts to eliminate generalization as an account of the effects we observe.

Further support for the non-associative nature of SEFL is that a variety of manipulations that eliminate fear of the stress context do not impact SEFL. We have completely extinguished the fear of the stress context, but SEFL remained as strong in the extinguished rats as it was in those that received no extinction, even across multiple extinction protocols [1,8]. We have given the shocks at an age before contextual fear learning has developed (P19), and despite a complete adult amnesia for fear of the stress context, the early life stressed adults show SEFL [6]. Perhaps most dramatically, when the N-methyl-D-aspartate (NMDA) antagonist (2R)-amino-5-phosphonovaleric acid (APV) was administered in a manner that targeted the hippocampus concurrent with stress, the rats showed no fear of the stress context but showed equivalent SEFL to rats that received vehicle during stress [1]. These studies point out one of the many advantages of the SEFL model—we can use the well-documented fear conditioning assay and freezing as a

behavioral index of the impact of the stressor. And by doing so, we show a clear dissociation between fear of the stress context and the changes in subsequent fear conditioning.

Our SEFL procedure is an acute stressor; a single 90 min treatment produces these profound and long-lasting effects. There is considerable interest, both clinically and experimentally, on whether chronic stress has similar or unique effects compared to acute stress. Will multiple and continuing bouts of mild stress have a similar or dissimilar psychiatric and mechanistic impact? Does sustained intense stress have a similar effect to just a brief stressor? Despite the large volume of research on chronic stress, the field has absolutely no answer to this important question! This is because every study that has attributed an effect to chronic stress contains one of two experimental confounds. The first is that the chronic stress condition is compared to an unstressed control. This comparison may allow conclusions about stress *per se*, but it says nothing about the factor of chronicity. In studies where chronic stress is compared to an acute stressor, there is also a very serious confound—the chronic stressor also provides considerably more total stress. This is because the chronic condition is compared to a more limited number of exposures to the same stressor. For example, one recent study purported to compare acute, subacute, and chronic stress by giving stress for 1, 7 or 21 days respectively [9]. The stress was 6 hours of restraint stress on the treatment days. Thus, besides chronicity, the groups also differed by having 6, 42 or 126 hours of restraint. Stress severity was completely confounded with chronicity. We provide this as an example, as all current studies of chronic stress suffer from at least one of these two confounds (eg. [10-15], and so on). Therefore, it is essential, both from a basic science and translational perspective, to develop a strategy that allows us to separate chronicity from severity. Just as chronicity may be a critical determinant of stress's impact,

severity may also be an important factor. Here, we modified our acute stress procedure to provide a chronic stressor with comparable stress severity.

An advantage of the stress we use in the SEFL model is that every aspect of it is quantifiable. The procedure itself is made of time (e.g., 90 min), number of shocks (e.g., 15), and their relationship (a variable rate of presentation averaging 1 shock every 6 min). This allows us to take our acute stress condition and carve it into a chronic treatment of 15 daily époques, where each époque contains a pre-shock interval and a single shock. The pre-shock interval for each époque is one of the interstimulus intervals from the acute treatment. Thus, time in the stress context, time to next shock, and amount of shock are completely equated in the two conditions, with the only distinction being distribution of the experiences. This manipulation will serve as our manipulation of chronicity.

The overarching hypotheses that guided this project is that we believe there are qualitative differences in the behavioral and physiological impact of chronic vs. acute stress. In other words, chronic and acute stress experiences likely cause different, albeit partially overlapping, phenotypes. Why should chronic and acute stress produce such different results? We propose a corollary to our chronicity hypothesis, that while the effects of acute stress primarily derive from non-associative processes, the additional effects of chronic stress derive from associative processes. We developed our empirically-based rational for the non-associative basis of acute stress above. We suggest that chronic stress's differential engagement of associative influences is an example of the ubiquitous rule that spaced experiences are more effective at promoting learning and memory than massed experiences. Obviously, the chronic condition administers stress in a more spaced manner. There is clear theoretical and empirical precedent for the premise that massed trials favor non-associative processes, and spaced trials favor associative processes in the

conditioning and habituation literatures [16-18].

Five experiments are reported that investigate the behavioral and neurobiological consequences of stress chronicity. Rats were exposed to 15 footshocks in a single, 90-minute session, 15 footshocks across 15 days, or context exposure without shock. A subset of rats received daily access to a glucose solution following the termination of the day's chronic stress procedure. Following stress pretreatment, all groups were assessed for generalization, extinction, enhanced fear learning, and/or sacrificed for tissue analysis. All rats were weighed throughout the study and the normal tendency for rats to gain weight over time was compared across groups.

Method

2.1. Subjects

Two-hundred and sixteen male and female Long-Evans rats (250-400g) from Envigo 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.

2.2. Apparatus

Rats were housed in metal hanging cages. Each cage was equipped with a standard glass (250 mL) water bottle with a rubber stopper and metal spout.

Stress pretreatment and testing occurred in Med Associates (St Alban, Vt) behavioral testing chambers. Each chamber was equipped with an infrared camera, speaker for tone delivery, shock scrambler, and fluorescent and infrared light sources. The behavioral testing chambers in each testing room were controlled by a PC using Med Associates Video Freeze software that also automatically scores motion and freezing of the animal during the test session. A rat is considered

freezing when image change is registered at less than 50 pixels for at least 1 second. Any baseline pixel change due to mechanical operation of the chamber or camera is measured before the animal's entry and subtracted from measurement during the trial. To create distinct contexts between stress pretreatment and subsequent fear conditioning and testing, the chamber's contextual features were modified using differential lighting, odors, ambient noise, grid floors, and wall inserts. Transport also differed among contexts. Transport either occurred in a mobile cage rack or black tubs divided into compartments and partially-filled with bedding. Unless otherwise stated, contexts differed on as many dimensions as possible to reduce generalization effects.

2.3. Procedure

In all experiments, stress pretreatment and subsequent testing occurred in Med Associates conditioning chambers. Light, fan, grids, odor and transport were adjusted to distinguish contexts. On Day 1 or 15, acute-stressed groups received 15, 1 mA unpredictable footshocks over a 90-minute session. These rats remained in their home cages for the remaining 14 days of chronic stress pretreatment. Chronic-stressed groups received 15, 1 mA unpredictable footshocks over 15 consecutive days (1 shock/day). Unstressed controls received identical context exposure without shock in either a single, 90-minute session or across 15 days. Following stress pretreatment, all groups underwent subsequent testing. Extinction to the trauma context included 30-minute sessions of trauma context exposure without shock. Context preexposure included 30-minute sessions of novel context exposure without shock. The single-shock exposure occurred in the preexposed context following the extinction of fear generalization (3-6 days). All shocks were one mA in intensity and one second in length. All tone presentations consisted of a 65 dB, 2800 Hz tone. All startle noise presentations consisted of a 100 msec, 115 dB white noise. Rats were returned to the single-shock/noise context 24 hours after exposure for an 8-minute context test. In

experiments involving fear testing to an associated tone, the tone test was preceded by context preexposure, just as in the one-shock context. On tone test days, three minutes preceded the first tone, to establish baseline freezing. Tone presentations lasted for 30 seconds each and one minute intervened between presentations.

In experiments involving the glucose intervention, every group was pre-exposed to a glucose cocktail over three consecutive days [19]. The cocktail consisted of 40% glucose and 5% sucrose dissolved in tap water (weight/volume). One group that received acute stress pretreatment (ASG) and one group that received chronic stress pretreatment (CSG) received fifteen days of free access to the glucose cocktail for 6 hours/day immediately following the end of the chronic stress session. The other three groups (CNW, ASW, & CSW) received only water during this time. We recorded total fluid consumption for all groups during this interval.

In experiments involving tissue analysis, all rats received the same stress schedule as previously described. One week following the termination of chronic stress, all rats were sacrificed. Dorsal hippocampus, ventral hippocampus, and basolateral amygdala were dissected and flash frozen for western blot analysis.

2.4. Western Blot Analyses

The dorsal hippocampus, ventral hippocampus, and basolateral amygdala were dissected and flash frozen for western blot analysis. Tissue was homogenized, spun to separate crude and synapto-neurosome homogenate, and diluted in a synaptic protein extraction reagent containing protease and phosphatase inhibitors (ThermoFisher, Cat #s 87793 & 78440). Protein concentrations of diluted homogenate were estimated using BCA assay (ThermoFisher, Cat # 23225). 15ug of protein was loaded into a 10% polyacrylamide gel for electrophoretic separation,

and then transferred to a PVDF membrane (Bio-Rad, Cat #s 5671035 & 1704157). Lanes were assessed for total protein using Ruby protein blot staining (ThermoFisher, Cat # S11791). Primary antibody was then applied overnight for approximately 16 hours. The following day, blots were washed and secondary antibody (fluorescent or chemiluminescent) was applied the following day for 1-2 hours. Tissue was analyzed for GluA1 (Millipore cat # ABN241, 1:5000), GluA2 (Millipore cat # MABN1189, 1:1000), NR1 (Millipore cat # AB9864, 1:5000), NR2a (Millipore cat # AB1555P, 1:10000), NR2b (Abcam cat # AB28373, 1:5000), and GAPDH (Abcam cat # AB8245, 1:5000). Secondary antibodies were applied at a 1:10000 to 1:5000 dilution depending on primary antibody specifications (Abcam cat # AB205719, Bio-Rad cat #s 12005867 & 12004162). Blots were imaged using a ChemiDoc MP imager and analyzed using Image Lab software (Bio-Rad, cat #s 17001402 & 1709690).

2.5. Statistical Analysis

Software package SPSS (SAS Institute, Inc., Version 16.0, Cary, NC, USA) was used for statistical analyses. One-way, two-way, three-way, and mixed-design ANOVAs were used when appropriate. Following significant interactions, Tukey post-hoc analyses are reported. Statistical significance was noted when p values were less than 0.05. Data is presented as group means with error bars denoting group mean +/− SEM. No statistical outliers were removed from the data; animals were excluded solely based on equipment malfunction or high baseline levels of freezing.

Results

Experiment 1: Impacts of Stress Chronicity and Sex on Subsequent Fear Learning

We have previously and extensively shown that exposure to 15, 1 mA shocks during a single, 90-minute session enhances future fear learning [1,7]. We have also shown that this effect is present regardless of sex [3]. In this experiment, we tested the hypothesis that we would see a similar fear learning enhancement when the 15 shocks were distributed across 15 days, rather than during a single session. We also tested our hypothesis that this would hold true, regardless of the rat's sex.

Figure 1 shows percent freezing during acquisition, generalization test, context preexposure, one-shock exposure, and context test. The group that experienced acute stress on the final day of the chronic stress procedure showed significantly less generalized fear when compared to chronically-stressed groups and the group that received acute stress exposure on the first day of the chronic stress procedure. However, the rate of generalized-fear extinction was slower in the chronically-stressed groups. Additionally, rats that received acute stress exposure on the first day of the chronic stress procedure exhibited significantly less freezing during the one-shock context test, when compared to groups that received chronic stress. No sex differences were observed (Fs < 1). No group differences were observed in chronic or acute groups during acquisition or baseline freezing prior to 1 shock exposure following preexposure (Fs < 1).

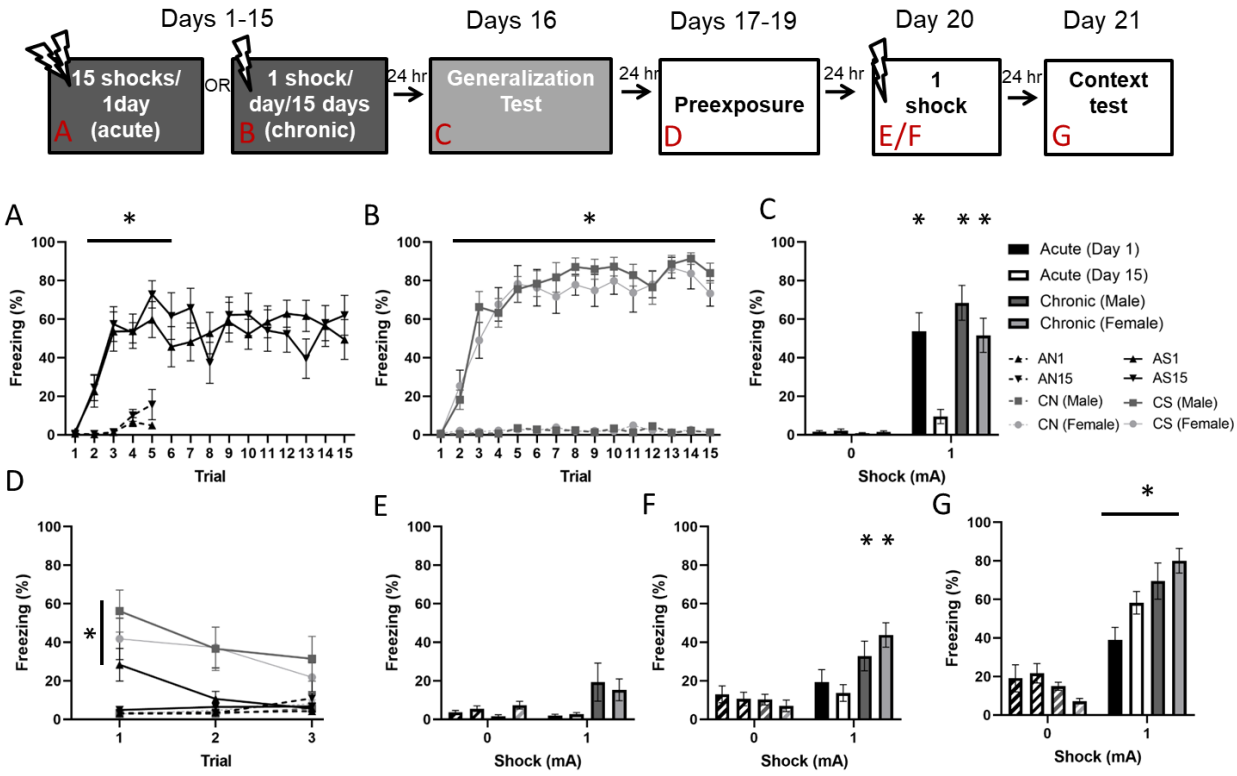

Figure 1. Impacts of stress chronicity and sex on subsequent fear learning. Depicted: Percent freezing during stress pretreatment (panels A & B), generalization testing (panel C), context preexposure (panel D), prior to and following exposure to one shock (panel E & F), and context test (panel G). Male (M) and female (F) rats received either acute (AS1 & AS15) or chronic (CS) exposure to 15 footshocks, or identical context exposure with no shock (AN & CN). Following stress pretreatment, all rats were exposed to a novel context that shared some similar dimensions to the stress pretreatment context. All groups were then preexposed to a completely novel environment for three consecutive days. Twenty-four hours after the termination of preexposure, all groups received a single footshock in the preexposed context. All groups were tested for contextual fear learning 24 hours later. As expected, all groups that received footshock during stress pretreatment readily reached asymptotic contextual fear conditioning. Groups that received chronic stress or that received acute stress on Day 1 of the chronic stress procedure showed greater generalized fear compared to all other groups in the similar and totally novel contexts. However, the AS1 group showed a greater rate of extinction compared to the CS groups. AS and CS groups showed greater freezing behavior during the context test when compared to unshocked controls. Error bars denote mean ± SEM. * denotes significance ($p < .05$; compared to No Shock).

A two-way ANOVA on freezing during the generalization test yielded a significant Chronicity x Shock interaction, $F(3,72) = 10.04$, $p < .0001$. Tukey post-hoc comparisons on group means indicated a relationship among groups, such that: AN1 = AN15 = CN (Male) = CN (Female) < AS15 < AS1 = CS (Male) = CS (Female). A mixed-design ANOVA on freezing across context preexposure trials yielded a significant Group x Trial interaction, $F(14, 144) = 3.419$, $p < .0001$. Tukey post-hoc comparisons on group means indicated a relationship among groups on Day 1 of Preexposure, such that: AN1 = AN15 = CN (Male) = CN (Female) = AS15 < AS1 = CS (Male) = CS (Female). A two-way ANOVA on freezing during the context test yielded a significant Chronicity x Shock interaction, $F(3, 72) = 7.256$, $p = .0003$. Tukey post-hoc comparisons on group means indicated a relationship among groups, such that: AN1 = AN15 = CN (Male) = CN (Female) < AS1 < AS15 = CS (Male) = CS (Female).

Experiment 2: Effects of Extinction on Subsequent Contextual and Cued Fear Learning

In the previous experiment, we showed that stress-enhanced fear learning occurs regardless of the stressor's chronicity and the animal's sex. Here we test the nature of this enhancement. We have provided substantial evidence that acute SEFL is due to non-associative mechanisms [1,6,8]. Here we test the hypothesis that when the stress is chronic, there are additional associative mechanisms at play. If chronic stress produces a SEFL phenotype with an associative component, we would expect that extinction of the trauma context would have an effect of subsequent fear learning. We also hypothesize that chronically-stressed animals will not show a similar enhancement of cued fear conditioning because there was no tone during stress pretreatment.

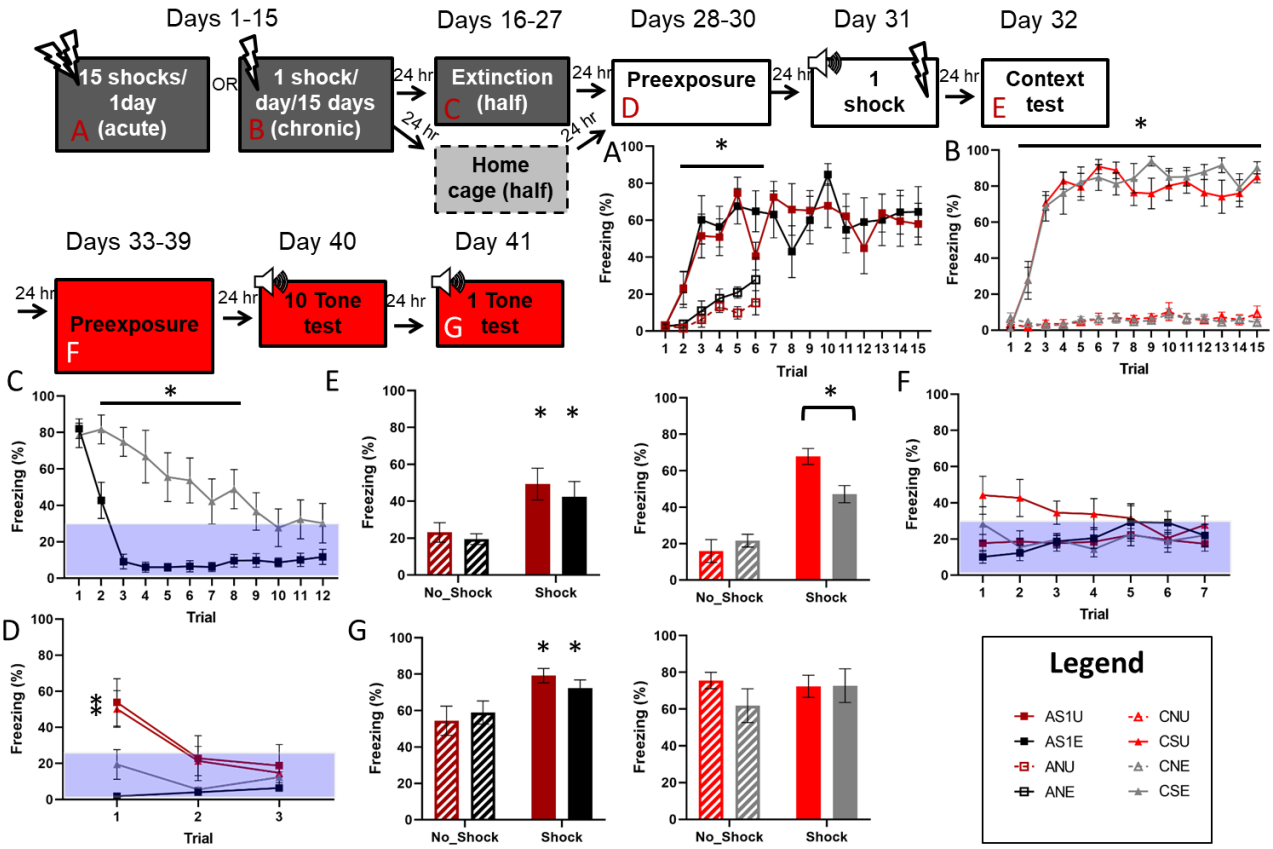

Figure 2. Effects of extinction on subsequent contextual and cued fear learning. Depicted: Percent freezing during stress pretreatment (panels A & B), extinction (panel C), context preexposures (panels D & F), context test (panel E), and tone test (panel G). Rats received either acute (AS) or chronic (CS) exposure to 15 footshocks, or identical context exposure with no shock (CN). Following stress pretreatment, half of the rats received extinction training to the trauma context (E), while half did not (U). Following extinction training, all rats were preexposed to a novel context for three consecutive days. Twenty-four hours after the termination of preexposure, all groups received a single footshock in the preexposed context. All groups were tested for contextual fear learning 24 hours later. All groups were then preexposed to a third context for 7 days, and the tested for fear conditioning to the tone in this context. Rats that had received chronic stress exhibited a slower rate of extinction. As expected, stressed rats that did not receive extinction training exhibited higher levels of fear expression to the novel context. As expected, stressed rats, regardless of stress chronicity, exhibited enhanced fear learning to the one-shock session. However, extinction training attenuated learning in the chronically-stress group only. Furthermore, only acutely-stressed groups exhibited enhanced fear learning to the tone. Error bars denote mean ± SEM. * denotes significance ($p < .05$; compared to No Shock).

Figure 2 shows percent freezing during across fear extinction days, context preexposure days, one-shock exposure, and context and tone tests. Indeed, we saw that chronically-stressed rats that underwent extinction training froze significantly less than their counterparts that received no extinction training; we found no effect of extinction in rats that received acute stress pretreatment. What's more, rats that had received acute stress pretreatment exhibited enhanced fear conditioning to the associated tone, but this same enhancement was not apparent in chronically-stressed groups.

As in the first reported experiment, chronically-stressed rats exhibited impaired fear extinction to the trauma context compared to acutely-stressed rats. All shocked rats that did not receive extinction training showed significantly higher levels of fear during preexposure to the novel context, regardless of chronicity. Chronically-stressed rats that did not receive extinction training, while lower than unextinguished groups, did show significantly higher levels of fear when compared to extinguished, acute-stressed rats. Relatively similar, but muted, effects were observed during preexposure to the third context. In both cases, all rats received context preexposure until freezing levels were comparable to unstressed controls. No group differences were observed in chronic or acute groups during acquisition ($Fs < 1$).

A mixed-design analysis of variance (ANOVA) on freezing during fear extinction yielded a significant Shock x Chronicity x Trial interaction, $F(11,297) = 6.042$, $p < 0.001$. Tukey post-hoc comparisons indicated significant group differences between acutely and chronically-stressed rats on trials 2-8. A mixed-design analysis of variance (ANOVA) on freezing during context preexposures 1 and 2 yielded significant Shock x Extinction x Trial interactions, $F(2,110) = 11.388$, $p < 0.001$ and $F(6,330) = 2.525$, $p = 0.021$, respectively. Tukey post-hoc comparisons indicated significant group differences during preexposure 1 such that rats that received stress pretreatment and no extinction training exhibited greater freezing during the first preexposure

session. A two-way ANOVA on freezing during the context test in acute groups yielded a significant main effect of shock, $F(1,27) = 13.72$, $p = 0.001$, but not extinction. A two-way ANOVA on freezing during the context test in chronic groups yielded a significant Shock x Extinction interaction, $F(1,25) = 7.153$, $p = 0.013$. Tukey post-hoc comparisons indicated significant group differences such that CSU > CSE > CNU = CNE. A two-way ANOVA on freezing during the tone test in acute groups yielded a significant main effect of shock, $F(1,27) = 10.01$, $p = .0038$. There were no significant effects found in chronic groups during the tone test.

Experiment 3: Impact of stress chronicity on subsequent fear learning to novel aversive stimulus

In the previous experiment we showed that chronic stress produces a SEFL phenotype which involves associative mechanisms. Here we test the hypothesis that this associative mechanism is reinstatement of generalized fear. Reinstatement is the phenomena whereby a previously extinguished association returns after presentation of the previously associated unconditional stimulus [20]. Importantly, the reinstatement effect does not appear, or is muted, when a novel unconditional stimulus is presented [21]. Here we used a nearly identical procedure to before, but with one important distinction- the rats were exposed to an aversive noise burst instead of a single footshock. We also tested the animals on the open field and elevated plus maze to see if chronically-stressed animals show similar levels of anxiety-like behavior when directly compared to acutely-stressed animals.

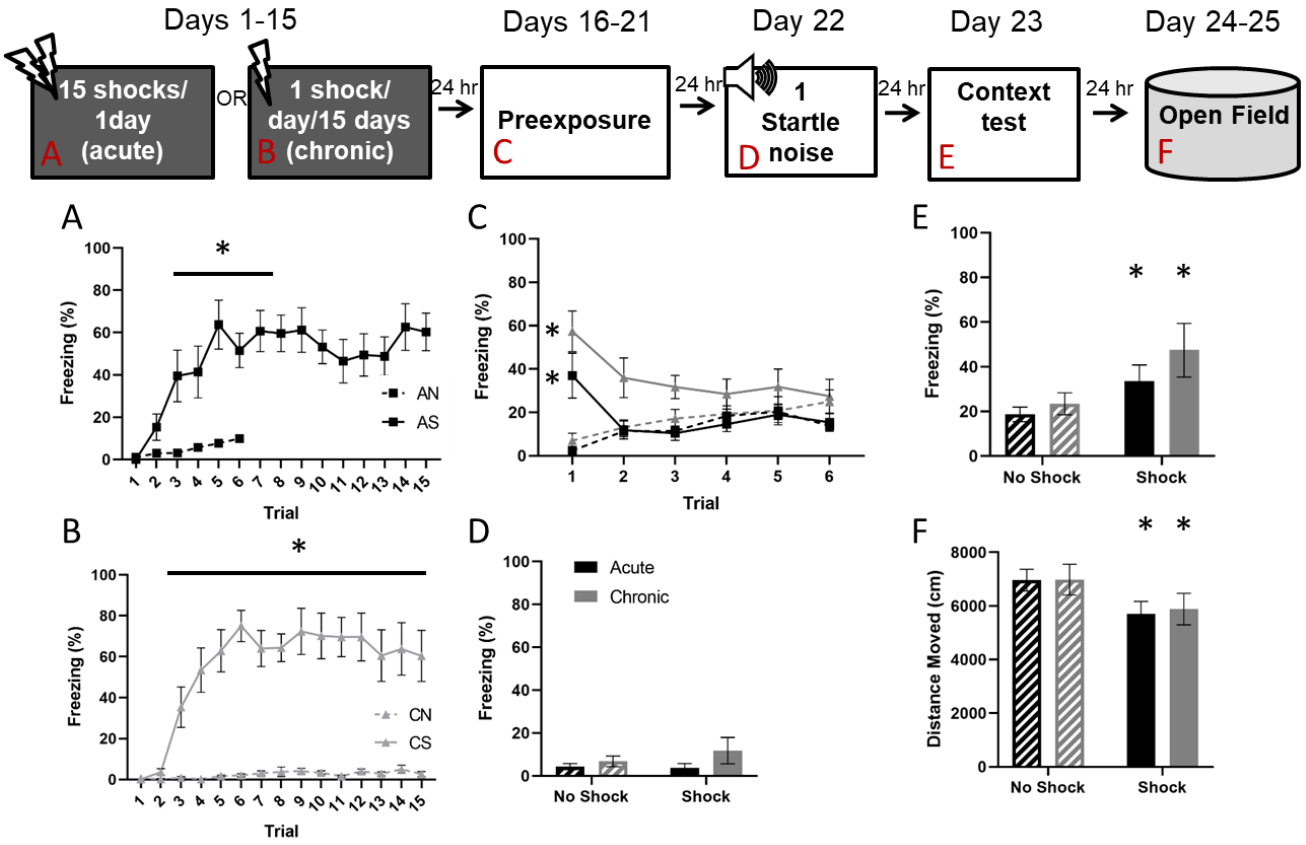

Figure 3. Impact of stress chronicity on subsequent fear learning to novel aversive stimulus. Depicted: Percent freezing during stress pretreatment (panels A & B), context preexposure (panel C), prior to and following exposure to one shock (panel D), context test (panel E), and open field test (panel F). Rats received either acute (AS) or chronic (CS) exposure to 15 footshocks, or identical context exposure with no shock (CN). All groups were preexposed to a novel environment for six consecutive days. Twenty-four hours after the termination of preexposure, all groups received a single startle-noise in the preexposed context. All groups were tested for contextual fear learning 24 hours later. As previously observed, exhibited higher levels of fear expression to the novel context. Importantly, stressed rats, regardless of stress chronicity, exhibited enhanced contextual fear learning to the startle-noise. Additionally, stressed rats, regardless of stress chronicity, exhibited decreased exploratory behavior in the open field test as indicated by a decrease in distance travelled. Error bars denote mean ± SEM. * denotes significance ($p < .05$; compared to No Shock).

Figure 3 shows percent freezing during acquisition, context preexposure, one startle noise exposure, and context test. Importantly, despite the change in aversive stimulus during fear conditioning, effects of stress pretreatment were similar to the previous experiment. Namely, both chronically- and acutely-stressed groups showed enhanced contextual fear conditioning to the loud noise. Stressed animals also showed less exploratory behavior in the open field test, regardless of stress chronicity. As seen in the previous experiment, chronically-stressed rats appeared to generalize between contexts more than acutely-stressed rats.

A mixed-design ANOVA on freezing during context preexposure yielded a significant Group x Trial interaction, $F(15, 140) = 5.982$, $p < .0001$. Tukey post-hoc comparisons on group means indicated a relationship among groups, such that: CN = AN < AS = CS on trial 1 and CN = AN = AS < CS on trial 3. A two-way ANOVA on freezing during the context test yielded a significant main effect of Shock, $F(1, 27) = 8.224$, $p = .0079$. A two-way ANOVA on distance moved during the open field test yielded a significant main effect of Shock, $F(1, 28) = 5.289$, $p = .0291$.

Experiment 4: Effect of post-stress glucose consumption on fear learning and expression

In the previous experiments, we showed that chronically-stressed rats exhibit a similar SEFL phenotype to acutely-stressed rats, but with an additional associative component. There is a large body of work showing that consumption of glucose enhances the hippocampal processing of associative memory in aging and/or stressed populations [22,23]. Here we test the hypothesis that the associative component SEFL will be further enhanced when the animal receives glucose following stress exposure. Specifically, we hypothesize that consumption of glucose following stress exposure will exclusively affect subsequent fear learning in chronically-stressed groups.

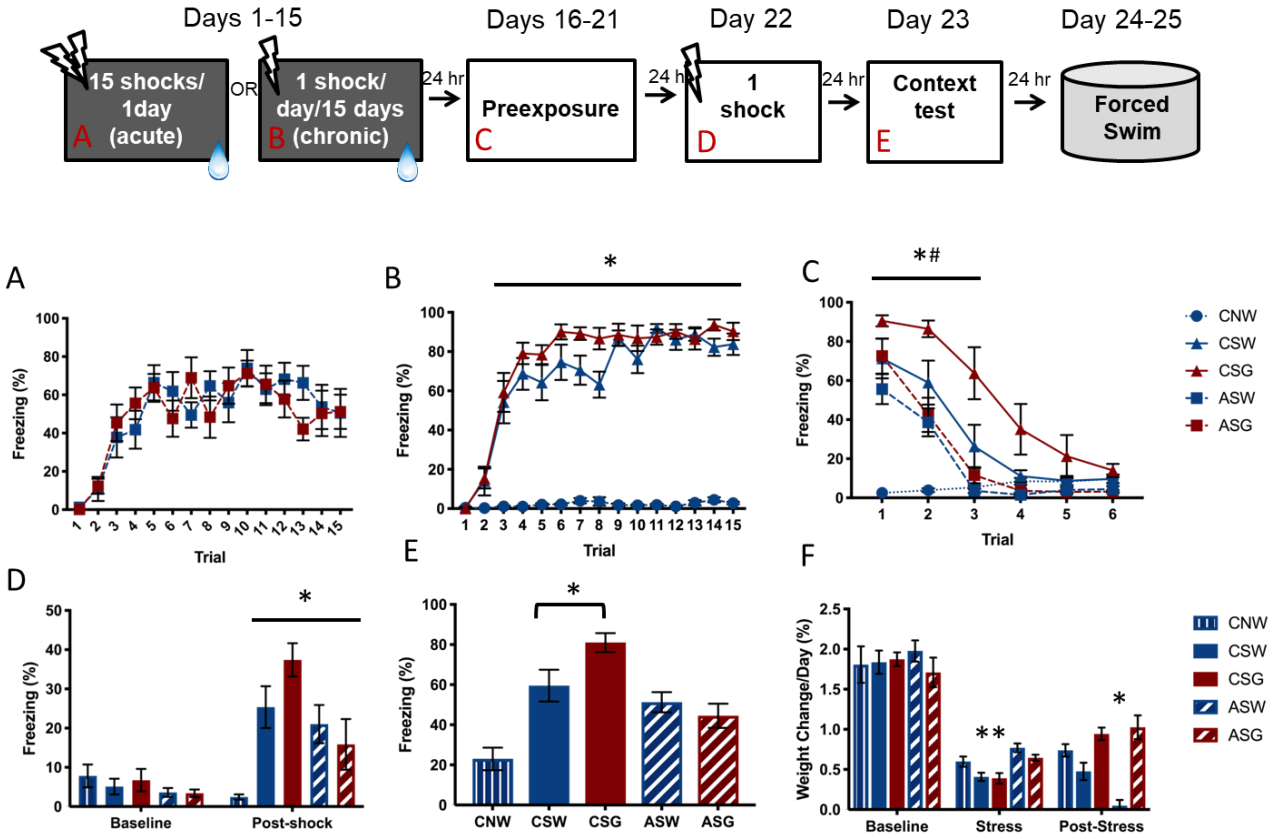

Figure 4. Post-stress glucose selectively enhances subsequent fear learning in chronically-stressed rats. Depicted: Percent freezing during stress pretreatment (panels A & B), context preexposure (panel C), prior to and following exposure to one shock (panel D), and context test (panel E). Weights prior to, during, and following stress pretreatment are also reported (panel F). Rats received either acute (AS) or chronic (CS) exposure to 15 footshocks, or identical context exposure with no shock (CN). Rats received daily access to a 40% glucose solution (G) or tap water (W) following the termination chronic stress pretreatment. All groups were preexposed to a novel environment for six consecutive days. Twenty-four hours after the termination of preexposure, all groups received a single footshock in the preexposed context. All groups were tested for contextual fear learning 24 hours later. As expected, all groups that received footshock during stress pretreatment readily reached asymptotic contextual fear conditioning. Groups that received chronic or acute stress showed greater generalized fear compared to the unstressed group. However, chronically stressed rats that received post-stress glucose exhibited markedly higher levels of generalized fear when compared to all other groups. An identical trend was observed during the 1-shock context test. CS groups showed decreased weight gain during stress pretreatment. Error bars denote mean ± SEM. * denotes significance ($p < .05$; compared to No Shock). # denotes significance ($p < .05$; CSG compared to CSW).

Figure 4 shows percent freezing during acquisition, context preexposure, one-shock exposure, and context test. It also shows weight change over the course of the experiment (and percent float time during forced swim test). As seen in the previous experiments, chronically-stressed rats appeared to generalize between contexts more than acutely-stressed rats. Interestingly, glucose appeared to heighten this generalization effect in chronically-stressed rats, but not acutely-stressed rats. Glucose similarly heightened freezing behavior specifically in chronically-stressed rats during the context test. Chronic stress reduced weight gain compared to all other groups during the 15 days of stress exposure, regardless of fluid condition. No effects of glucose were observed in chronic or acute groups during acquisition ($F < 1$). No group effects were observed for baseline freezing prior to 1 shock exposure or float time during the forced swim test ($Fs < 1$).

A mixed-design ANOVA on freezing during chronic acquisition yielded a significant Group x Trial interaction, $F(28, 378) = 18.225$, $p < .001$. Tukey post-hoc comparisons on group means indicated a relationship among groups, such that: CNW < CSW = CSG on trials 3-7 and 9-15 and CNW < CSW < CSG on trial 8. A mixed-design ANOVA on freezing during acute acquisition yielded a significant main effect of Trial, $F(14, 252) = 15.050$, $p < .001$. A mixed-design ANOVA on freezing during context preexposure yielded a significant Group x Trial interaction, $F(20, 225) = 8.993$, $p < .001$. Tukey post-hoc comparisons on group means indicated a relationship among groups, such that: CNW < ASW = ASG < CSW < CSG on trial 1, CNW < ASW = ASG = CSW < CSG on trial 2, and CNW = ASW = ASG = CSW < CSG on trials 3-4. A one-way ANOVA on freezing during context test yielded a significant main effect of Group, $F(4, 45) = 14.055$, $p < .0001$. Tukey post-hoc comparisons on group means indicated a relationship among groups, such that: CNW < ASW = ASG = CSW < CSG. A one-way ANOVA on percent weight gain during stress exposure yielded a significant main effect of Group, $F(4, 44) = 8.309$, $p < .0001$. Tukey

post-hoc comparisons on group means indicated a relationship among groups, such that: CSW = CSG < CNW = ASW = ASG. A one-way ANOVA on percent weight gain following stress exposure yielded a significant main effect of Group, $F(4, 45) = 15.61$, $p < .0001$. Tukey post-hoc comparisons on group means indicated a relationship among groups, such that: ASW < CSW < CNW = ASG = CSG.

Experiment 5: Neurobiological consequences of chronic stress

We found that chronic stress produces a SEFL phenotype with distinct behavioral characteristics. What's more, fear expression is enhanced by post-stress glucose consumption in chronically-stressed rats. Here we test the hypothesis that chronic stress produces distinct neurobiological effects in the basolateral amygdala (BLA). Our lab previously reported that acute stress enhances expression of the GluA1 subunit of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in the BLA [4]. However, under these circumstances, NMDA receptors remain unchanged. While NMDA receptor activation in necessary for the acquisition of associative fear learning [24,25], GluA1-containing AMPA receptors are more critical for the expression of fear [26]. Here we tested the hypothesis that neurobiological changes in the BLA will mirror the behavioral effects of stress chronicity. Specifically, we hypothesized that chronic stress will not only enhance GluA1 (thereby enhancing non-associative processes), but also NMDA (thereby enhancing associative processes), in the basolateral amygdala.

Figure 5 shows AMPA and NMDA receptor subunit protein quantification in the BLA one week after the termination of chronic stress treatment. Rats exposed to chronic footshock exhibited greater levels of GluA1, but not GluA2, in the BLA. They also exhibited increased levels or NR1.

Rats exposed to chronic stress exhibited dampened weight gain throughout stress exposure as previously seen. No differences in AMPA or NMDA receptor subunits were found in the DH or VH (Fs < 1).

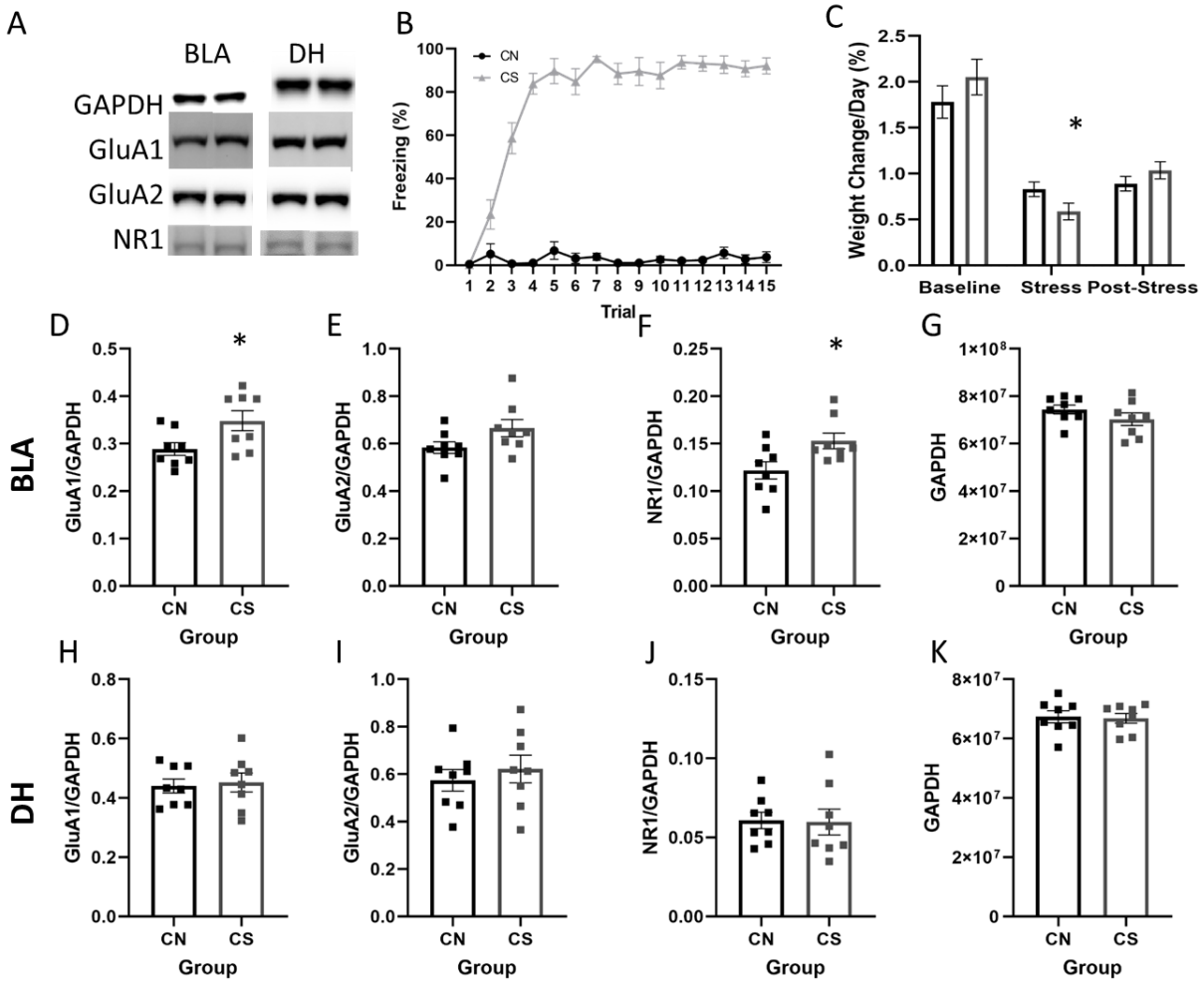

Figure 5. Neurobiological effects of chronic stress exposure. Depicted: Basolateral amygdalar (BLA) and dorsal hippocampal (DH) concentrations of GluA1 (panels D & H), GluA2 (panels E & I), and NR1 (panels F & J), as determined by western blot analysis. GluA1, GluA2, and NR1 are depicted as a ratio over GAPDH concentrations (panels G & K). Rats received chronic (CS) exposure to 15 footshocks, or identical context exposure with no shock (CN). One week later, rats were sacrificed for tissue analysis. Chronically-stressed rats exhibited greater concentrations of GluA1 and NR1 in the BLA. No significant differences were found in the DH. Error bars denote mean ± SEM. * denotes significance ($p < .05$; compared to No Shock).

An independent samples t-test on BLA protein analysis yielded a significant difference between Groups on GluA1/GAPDH, $t(14) = 2.381$, $p = .0320$, and NR1/GAPDH, $t(14) = 2.512$, $p = .0248$. A mixed-design ANOVA on Weight Change (%) yielded a significant Group x Timepoint interaction, $F(2,28) = 3.750$, $p < .0361$. Tukey post-hoc on group means indicated a relationship among groups, such that: CN > CS following stress exposure.

Discussion

The experiments described above provide evidence that the chronicity of stress exposure belies substantive differences in subsequent neurobiology and behavioral phenotypy. Specifically, we found that rats exposed to chronic stress exhibited greater generalized fear and deficits in the extinction of fear when compared to rats exposed to an acute stress of comparable severity. Chronically stressed rats were also uniquely affected by fear extinction training to the trauma context, and interestingly did not exhibit SEFL to a tone associated with shock. Several of these effects were further exaggerated if the rats were given glucose following each day of stress exposure. Furthermore, while chronically-stressed rats exhibited an elevation in BLA GluA1, as previously seen in acute-stressed rats [27], chronically-stressed animals also exhibited an elevation in BLA NR1, which has not been previously reported for acute-stressed rats using this procedure [27]. This provides us with a potential neurobiological mechanism for the observed associative effects of chronic stress exposure. It should be noted that we did not replicate previous effects of chronic stress on forced swim behavior, but did find that chronic stress exposure suppressed typical weight gain.

The apparent effects of our chronic stress procedure on associative learning are in line with the previously described hypothesis: spaced training will lead to greater associative learning when compared to massed training. However, it is important to recognize that the massed vs. spaced rule

is a descriptive law that belies multiple mechanisms that converge on a similar pattern. The enhanced impact of spaced training has been demonstrated for virtually every type of conditioning, skill learning and cognitive phenomenon, and the effects range across a vast temporal space [28-30]. It is found in both vertebrates and invertebrates [31]. Additionally, it derives from multiple psychological processes and biological mechanisms. As an example of psychological process, while spaced training enhances both cued and contextual fear conditioning, the psychological processes underlying the two are completely different: competitive error-correction in the former and behavioral inhibition in the later [32,33]. In terms of biological mechanism, there is evidence that maximization of CREB activity [34,35] and the dynamics of actin polymerization [36] underlie the beneficial effects of spacing on long-term potentiation and learning, but both of these mechanisms act in very different time domains (min vs. hours). Thus, while the massed/distributed idea provided a rationale for our hypothesis it does not provide a complete explanation, which will require further direct investigation.

There are several explanations for our forced swim null findings. Chronic variable stress has been shown to induce increased floating behavior during the forced swim test [37,38]. However, none of these studies have used a comparable acute stress group. It is possible that these effects are not due to chronic stress exposure, but exposure to a highly variable and severe stressor. In fact, when the stressor is severe, acute shock stress has also been shown to increase float times during forced swim [39]. Another consideration, is that the forced swim test is sensitive to pharmacological manipulation [40], but is not particularly sensitive to stress manipulation. In fact, stress effects are variable, depending on strain, gender, and stress procedure [41,42]. Therefore, it may be that the forced swim task is a relatively effective model for antidepressant activity, but not as a model of stress-induced depression-like behavior. An obvious follow-up is to use this stress

procedure to test the effects of stress chronicity on several other behaviors that model depression. In fact, rats exposed to chronic stressors have exhibited a wide array of other depression-like phenotypes [37]. Future studies should use chronic and acute stressors of comparable severity to investigate previously reported chronic stress effects, such as heightened anxiety [43-45] and physiological and functional impairments of the hippocampus [43,45-54], prefrontal cortex [44,55-57], and BLA [58,59].

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Chapter 5: Conclusions

Most studies aimed at understanding the mechanisms of stress-induced psychiatric disease focus on a very specific stress protocol and behavioral outcome. Very few studies have provided appropriate controls to examine whether the biological mechanisms underlying the behavioral effects are specific to the stressor used, or can be generalized to stress exposure of all types. Here, we tested the hypothesis that stress volume and chronicity are determining factors for stress' behavioral impacts and the mediating biology. We also tested the peripheral effects of glucose in order to understand why its prophylactic effects appear specific to animals exposed to high-volume stress.

In the first set of studies, we sought to interrogate the physiological mechanism by which post-stress glucose produces its prophylactic effect. Specifically, we investigated the peripheral effects of glucose consumption following exposure to high-volume stress on energy homeostasis and peripheral glucocorticoid expression. We were specifically interested in effects that occurred exclusively in stressed animals, but not unstressed controls, appeared to be exclusive to glucose, but not fructose, consumption, and appeared within the critical intervention time window of 3-6 hours after stress exposure. We found that post-stress glucose did not significantly affect concentrations of corticosterone or its binding globulin, CBG, as predicted. However, post-shock glucose exclusively and transiently increased blood glucose levels 3-6 hours following shock and repleted liver glycogen stores 24-hours after stress exposure. Taken together, this suggests that glucose may be exerting its effects by stabilizing energy availability after the extremely energy-taxing high-volume stress exposure. However, the exact route by which this energy stabilization effect impacts behavior still remains unclear.

The next set of studies tested whether there are divergent effects of stress volume on behavior, neurobiology, and the efficacy of glucose treatment. Specifically, we tested the effects of 0, 15 and 100 shock protocols on stress-enhanced fear learning (SEFL) behavior, weight maintenance, basolateral amygalar (BLA) and dorsal hippocampal (DH) ionotropic glutamate receptor density. We also examined the effects of post-stress glucose on SEFL among these varying stress protocols. We found that the 15-shock protocol produced a robust context and cued SEFL, but no persistent changes in weight maintenance. We also found that elevations in BLA GluA1 concentration accompanied these behavioral effects. Conversely, we found that the 100-shock protocol produced a robust effect on weight maintenance, but SEFL behavior was not observed in contextual fear conditioning. However, rats exposed to the high-volumes stressor did show SEFL to a tone paired with shock. These behavioral effects were accompanied by increases in both GluA1 and GluA2 in the BLA, as well as a reduction in DH NR1. Interestingly, we found that the SEFL behavior appeared in 100-shocked rats if they were given access to glucose following the stress pretreatment session. However, glucoprivation did not inhibit the formation of SEFL behavior in rats exposed to the 15-shock stress protocol. These findings suggest a complex relationship between stress volume and the psychological sequelae following trauma.

In the final set of studies, we investigated the behavioral and biological impacts of another dimension of stress: chronicity. Specifically, we tested the nature of the SEFL effect when the stress pretreatment is given in a single 90-minute session compared to when the session is divided across 15 days. We also tested the effects of post-stress glucose consumption in chronically-stressed rats. We found that chronically-stressed rats tended to exhibit more generalized fear and impairments in fear extinction. Unlike acute stress exposure, SEFL following chronic stress appeared impacted by extinction of fear to the trauma context and did not appear when the

conditional stimulus was a tone. However, SEFL did still appear when the unconditional stimulus (US) during subsequent fear conditioning did not match the trauma US in both acute and chronic conditions. Furthermore, we saw that glucose consumption following chronic, but not acute, stress enhanced generalized fear and subsequent fear learning in a novel context. While we found that chronic stress similarly elevated concentrations of GluA1 in the BLA, we also found elevations in BLA NR1 concentrations exclusively in rats that were exposed to chronic stress. Taken together, these data suggest a role of associative learning in the SEFL phenotype following chronic stress treatment, in addition to the non-associative effects that follow acute stress exposure. Finally, we found that while chronic stress did not induce depression-like effects when measured in the forced swim test, it did suppress weight gain. This suggests that the chronicity of stress may impact depression-like behavior when all other dimensions of the stressor are consistent among groups.

Discussion of results and future directions

The effects of stress exposure are variable in severity and quality [1,2]. Treatment efficacy for patients suffering from stress-induced psychiatric disease continues to remain similarly variable, with only a small percent of the population seeing a persistent quelling of symptoms [3,4]. One hypothesis is that the observed variability in treatment effectiveness may correlate with variability in stress exposure. In other words, the quality, severity, and chronicity of the experienced trauma may have direct impacts on symptoms expressed and the probability of a positive treatment outcome. While parametric study of trauma exposure is impossible in a clinical population, animal models provide us with the necessary tools to interrogate this hypothesis. Surprisingly, the experiments described in this thesis are some of the first, if not *the* first, to directly study the effects of stress volume and chronicity using appropriate controls. Therefore, despite an enormous literature devoted to the effects of stress, we are one of the first to provide evidence that

the dimensions of the stressor used can have direct impact on the subsequent behavioral and biological profiles.

There remains a wide host of questions regarding the effects of stress volume. Firstly, the above studies were restrictive in that they compared stressors of greatly differing volumes (15 and 800 seconds of shock). Parametric study of stress volume, which utilizes a series of intermediary stress volumes, will be necessary to understand the impact at higher resolution. Furthermore, while we controlled for several factors, procedural differences in inter-shock interval and length of individual shocks differed between stressors. These aspects of stress can also be parametrically studied in order to understand their impacts on subsequent behavior and biological change. There also remains a wide host of questions regarding the biological differences that mediate the resultant behavior of high and moderate volume stress. For example, there is a rich research literature that examines the effects of high volume stress on serotonin (5-HT) signaling in the dorsal raphe nuclei [5-12]. 5-HT signaling has also been implicated in standard fear conditioning [13]. However, no studies have tested to see if 5-HT signaling plays a mediating role in stress-enhanced fear learning. This is also true of other brain regions and neurotransmitters implicated in the resultant behavior of high-volume stress, such as the nucleus accumbens [14], dorsal striatum [10], bed nucleus of the stria terminalis [15,16], habenula [11], norepinephrine [6,17], and adenosine [14,18-20]. Of course, the impacts of stress volume are likely not as simple as a brain region or neurotransmitter being involved in one, but not the other. It is likely a much more complex relationship. For example, stress-induced secretion of corticosterone is necessary, but not sufficient, for both LH and SEFL-specific phenotypes [21,22]. However, the amount of corticosterone released by high-volume stress is 100-fold of that released by moderate-volume stress [22,23]. This suggests that while corticosterone plays a permissive role in the development of maladaptive behavior in both stress

procedures, the mechanism may not be similar. Another example is the differential role of the ventro-medial prefrontal cortex (vmPFC) in the development of LH and SEFL phenotypes. While evidence suggests that *activation* of the vmPFC is necessary for the development of SEFL [24], studies have shown that the *inactivation* of vmPFC is necessary for the development of LH [12]. This suggests that unique vmPFC neurocircuitry is involved in these dissociable behavioral consequences of differential stress volumes.

Several important research questions regarding effects of stress chronicity also remain open. We focused the scope of our research to the impacts of stress chronicity on subsequent fear learning. However, the literature suggests a wide range of chronic stress effects, which remain uncertain due to ubiquitous control group flaws. Specifically, a variety of chronic stressors have been shown to induce physiological and functional impairments of the hippocampus [25-35] and prefrontal cortex [36-39]. Furthermore, rats exposed to chronic stressors have exhibited a wide array of depression-like phenotypes [40], heightened anxiety [26,28,36], SEFL [41], and physiological changes in the BLA [41,42]. Interestingly, several of the effects reported following chronic stress exposure mirror effect differences observed between moderate and high-volume acute stress exposure. For example, exposure to acute, high-volume stress induces an array of depression-like behaviors [43,44] and physiological impairment of the hippocampus [45-49]. Though it should be noted that research regarding high-volume stress effects on hippocampal *function* are scant [50,51]. Perhaps the behavioral impacts of chronic stress exposure are due to differences in stress severity and not chronicity. In order to disentangle this issue, the field needs to test the reproducibility of these chronicity effects using a procedure that controls for stress severity. It is also possible that the chronicity effects we see are specific to an electric shock stressor. Therefore, it is imperative

to design similar procedures using other stressors. Finally, it is possible that stress chronicity and stress severity/volume interact in a meaningful way.

The eventual goal of this effortful, parametric work on the effects of various stress dimensions is to demystify clinical stress disorder. For example, I hope that one day clinicians may have access to a series of charts, informed by the various dimensions of stress, that outline expected psychiatric consequences of, and effective treatments for, different stressors. For example, *Patient A* was exposed to two months of systematic bullying by a peer in school. In patients that are chronically bullied in this way, we see *X*% develop symptoms *A*, *B*, and *C* and have found *Y* treatment the most effective in ameliorating these symptoms. *Patient B*'s house was burglarized while they were sleeping. In patients that have been the victims of a hot-prowl burglary, *X* % develop symptoms *A*, *C*, and *D* and have found Z treatment to be the most effective. We are able to accurately diagnose and treat many other diseases in this way, yet this data-driven approach remains largely missing in diagnoses of stress disorders. It is time for that to change.

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