THE EFFECTS OF CHRONIC UNPREDICTABLE STRESS ON THE ABILITY TO EXTINGUISH FEAR: ZINC AS A MEDIATOR

by

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DEDICATION

This is dedicated to my parents Boyd Knaack and Robin Chavez for all of their love and support.

ACKNOWLEDGEMENTS

I would like to thank many friends and relatives for their love, support, and patients even when I was stressed and unpleasant. My lab members assisted me in my research. Dr. Steve Lev and Dr. Mark Monk ran ICP on my blood and brain samples. My committee members Dr. Jane Flinn, Dr. Craig McDonald, and Dr. Nathalia Peixoto provided guidance and feedback. Finally, Franz Hamilton, Matlab guru, helped with my graphs and curve fitting recommendations.

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ABSTRACT

THE EFFECTS OF CHRONIC UNPREDICTABLE STRESS ON THE ABILITY TO EXTINGUISH FEAR: ZINC AS A MEDIATOR

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George Mason University, 2011

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Chronic stress can be deleterious to the brain and it is the unpredictability of these stressors that fails to permit habituation, the effects of which are exhibited through maladaptive behaviors such as Post Traumatic Stress Disorder (PTSD). Implementing a chronic unpredictable stress (CUS) paradigm as an animal model of PTSD may facilitate the examination of possible mechanisms relating prolonged stress to the cognitive deficits frequently observed. One factor may be zinc because it is decreased in the blood while increased in the brain during stress, and it has been previously demonstrated that excess zinc in the brain causes learning and memory deficits. To ascertain this possibility rats were raised pre- and post-natally on supplemented zinc water for four months, administered a 21 day CUS paradigm, and then underwent fear conditioning. Post stress blood samples were also analyzed. Results determined that excess zinc rats displayed learning and memory deficits on the ability to extinguish fear and recall that extinction 24 hours later. CUS decreased the level of zinc in the blood for animals that drank lab water,

but increased the amount of zinc in blood for animals that were supplemented with zinc. Furthermore, there was a positive correlation between the level of zinc in the blood and the ability to extinguish fear. These data suggest that zinc may be a mediator between chronic stress and anxiety disorders.

1. INTRODUCTION

Stress is a normal event experienced by humans in our daily lives and is a natural defense mechanism [1]. However, prolonged or chronic stress can be deleterious not only to our health, but to our cognitive functioning as well [2, 3]. Research has demonstrated these maladaptive cognitive deficits through chronic stress (CS) paradigms. However, CS models consist of the same single stressor repeated over time. It can be argued that since stress is a defense mechanism, it is possible that habituation would occur to the administration of the same stressor [4] because it becomes predictable, thus permitting adaptation. Additionally, this type of chronic stress does not emulate real life because, frequently, the stress a person is about to endure is unknown and even novel. Unfortunately, many animal models commonly used for assays of anxiety-like behavior are not valid models for human stress and anxiety [2]. Therefore, it is possible that a chronic unpredictable stress (CUS) paradigm would be more applicable to humans because of the randomization as well as the novelty of the stressors.

CUS has produced differing behavioral results depending on the measure implemented. One study determined that the CUS rats demonstrated an increase in anxiety-like behavior as observed in elevated plus maze [2]. On the contrary, other research concluded that CUS rats did not show any significant differences from controls and exhibited a trend toward an increase in open arm exploration [3], which would

suggest that the rats were less anxious than controls. However, when the CUS rats we re-exposed to the elevated plus maze for a second trial 72 hours later, results showed an increase in anxiety, possibly from consolidation [3]. This implies a conditioned phobic response that differs from generalized anxiety. Similarly, CUS rats displayed no significant difference in the time spent in the center of the open field box compared to controls [5]. This also suggests CUS does not produce a generalized anxiety, although it may induce a conditioned fear more similar to Post Traumatic Stress Disorder (PTSD).

This theory was additionally supported when CUS was tested on one conditioned (defensive burying) and two unconditioned (elevated plus maze and light-dark box) behavioral tasks. It was suggested that the unconditioned behavioral assays measured generalized anxiety [6], while the conditioned behavioral assays assessed disorders more similar PTSD. CUS rats demonstrated a significant increase in anxiety on the conditioned behavioral test compared to controls. On the contrary, no significant differences were detected between CUS rats and controls on any unconditioned response behaviors [6]. These results reinforce previous findings by explaining that a CUS model does not seem to produce symptoms of generalized anxiety, but does elicit signs of other anxiety disorders like PTSD.

Another pertinent aspect of CUS that parallels PTSD is the amount of time between the cessation of the stressor and appearance of the maladaptive behavior. CUS animals tested only one day after stress appeared less anxious than controls [6]. Although it may seem surprising that stressed rats would be less anxious, these findings matched elevated plus maze [3], open field , and MWM [5] data which all displayed less anxiety

for the CUS rats when tested only one day after stress. If CUS does model anxiety-like behavior in conditioned responses, then it could be theorized that similarly to PTSD, detrimental effects of stress would not emerge until weeks after the stress. Indeed, CUS rats assessed seven days after the cessation of stress expressed significantly more anxiety-like behaviors than both controls and CUS rats tested one day after. These differences were exacerbated in CUS rats examined 14 days after the stress [6].

All of the current CUS paradigms implemented 2 stressors daily for several consecutive days (approximately 14) that varied either by type or time. However, if the same number of stressors occurs every day for a specific number of days, is that actually unpredictable? Furthermore, is that a valid animal model of chronic stress since humans do not always experience stress every day? A better model of CUS may be one that extends longer, but still induces the same number of stressors combined with stress free days, thus adding to the unpredictability. Based on previous research, implementing a CUS paradigm may better emulate chronic stress in humans. This would then facilitate the examination of mechanisms to explain the deleterious effects on learning and memory as observed in patients with PTSD.

One possible factor linking stress to cognitive deficits is Zinc (Zn). Zn plays a vital role in the brain and body as an essential trace element in many physiological and biochemical functions [1, 7]. Based on the uptake and release of Zn in the hippocampus and amygdala, it is also theorized that Zn may play a role as a neuromodulator of synaptic plasticity in the brain [8]. This may occur because Zn^{2+} is concentrated in synaptic vesicles and is co-localized with glutamate. As such, Zn regulates long term

potentiation (LTP) in these areas by inhibiting GABA and NMDA, while enhancing activity at AMPA receptors [9]. Although Zn's interactions with several neurotransmitters have been established, the effects of stress on the levels of Zn in tissue as well as the relationship between Zn and brain functions during the stress response are still unclear.

Extinction of fear involves plasticity in the medial prefrontal cortex (mPFC) and the amygdala [10]. Glutamatergic efferents from the infralimbic (IL) region inhibit the activation of the basolateral amygdala (BLA) by synapsing on GABAergic intercalated cells in the amygdala, which hamper the autonomic response from the central nucleus (CE) to the hypothalamic-pituitary-adrenal axis (HPA) [10, 11]. This is particularly important because a subset of glutamatergic neurons located in the fear conditioning pathway, specifically the lateral nucleus of the amygdala (LA), are also zincergic [8, 12].

Given the location of Zn and the specific functions of these areas of the brain, it is plausible that Zn plays a vital role during fear conditioning. Administration of a Zn^{2+} chelator abolished LTP in the cortical input to the LA. Additionally, LTP was rescued by adding a GABA antagonist [8]. These results suggested that Zn^{2+} released during the induction procedure induced LTP at the cortico-amygdala synapses by decreasing feed-forward GABAergic inhibition of principal neurons. Moreover, the addition of exogenous Zn^{2+} also initiated LTP by reducing inhibition from GABA. Jointly, these data demonstrated that Zn^{2+} acts both presynaptically by decreasing the release of GABA and postsynaptically by decreasing postsynaptic sensitivity to GABA in the LA [8]. These data parallel the results from infusion of muscimol, a GABA agonist, into the BLA after

fear extinction which facilitated extinction [10]. This suggests that GABA in the BLA is responsible for the consolidation of fear extinction [10], and this role is possibly mediated by Zn.

Rats raised on excess Zn showed impairments in both reference and working memory coupled with increased levels of Zn in the brain [13]. Additionally, pre- and post-natal dosed rats tested at four months of age demonstrated higher freezing rates during contextual retention as well as during both contextual and cued extinction [14]. These data suggested that excess Zn may lead to overactivity in both the hippocampus and amygdala resulting from a lack of inhibition. Interestingly, increased freezing without a cue suggests increased generalized anxiety, which was also observed during MWM with increased thigmotaxicity [13], where as increased freezing to the tone (cue) implies a deficit in fear learning.

Recent data further support the role of excess Zn in the impairment of fear extinction as seen in the 129 genetic mouse strain, which demonstrated a significant deficit in extinction [15, 16]. When placed on a Zn deficient diet, the fear extinction impairment was rescued [15, 16] and was coupled with the normalization of c-Fos expression in the cortio-amygdala pathway of fear extinction [15]. Together, these data imply that excess Zn may cause extinction deficits resulting from a learning impairment.

Although stress is a normal experience and elicits a natural defense mechanism, long term or chronic stress can be detrimental as a result of neural, endocrine, or immune system dysfunction [1]. To examine some of these effects, both an acute and a chronic restraint stress were applied to mice, and Zn levels in the blood were measured. Although

the Zn levels decreased in both the acute and the chronic stress groups, the deficits were larger in the chronic group with a decrease of 31% and only a decrease of 13% in the acute stress group, both compared to controls [1]. These results suggest that chronic stress may be more harmful than acute. Furthermore, these findings imply that during stress, Zn is being reallocated from the blood to other tissue also affected by stress. One possible mechanism for this may be that the hormones released in the stress response mediate redistribution between tissues [1].

During a one hour acute restraint stress the levels of endogenous Zn and other neurotransmitters were measured in the hippocampus. Both the Zn and glutamate levels spiked significantly above baseline after 15 minutes in the restrainer [7]. This simultaneous increase of extracellular Zn and glutamate was also found specifically in the LA during footshock stress [12]. These results showed that acute stress may affect both Zn and glutamate similarly. This relationship may be explained by the fact that Zn^{2+} is located in synaptic vesicles and co-localized with glutamate [9]. However, more research is needed concerning the relationship between Zn and glutamate during stress.

Since findings determined that stress decreased levels of Zn in the blood [1] and other results demonstrated that stress increased Zn levels in the brain [7,12], these studies jointly suggest that Zn might be dispersed from the blood to the brain. Coupled with behavioral results that excess Zn in the brain caused increased anxiety as well as memory deficits [14], Zn may be a mediator between stress and the inability to extinguish fear. Essentially, Zn may play an important role in stress-related pathogenesis [1].

To study the effects of Zn and CUS in the brain, rats were raised on either Zn enhanced water or lab water, and administered a 21 day CUS paradigm followed by fear extinction. Zn levels in both the blood and the brain were measured.

2.1 METHODS: SUBJECTS

Thirty-two male Sprague-Dawley rats were raised pre- and post-natally on either tap water or water supplemented with Zn carbonate (10mg/kg $ZnCO_3$). Rats were housed with litter mates, 4 per cage in 12”X18” polycarbonate cages with Tek-FRESH bedding (Harlan Laboratories). Each cage also had three cotton squares and one nyla bone for enrichment. The colony was maintained on a 12 hour light/dark cycle with ad libitum accesss to food and water. Rats were handled three times a week to habituate them to human contact. At age three months, half of the rats from each water type were administered CUS and all rats were tested at four months. There were a total of four groups: Tap water + No stress (n = 8), Tap water + CUS (n = 8), Zn (10mg/kg $ZnCO_3$) + No stress (n = 8), Zn (10mg/kg $ZnCO_3$) + CUS (n = 8).

2.2 METHODS: WATER PREPARATION

Rats were supplemented with Zn carbonate via their drinking water. Tap water was flushed for 20 minutes prior to collection. A concentration of 10mg/kg Zn was prepared from Zn in 5% nitric acid and a neutral pH of 7.0 was obtained by the addition of a Na_2CO_3 buffer. All drinking water was maintained in polycarbonate carboys and administered to the rats in glass bottles. Water samples were taken from each carboy and analyzed by the United States Geological Survey (USGS, Reston, VA) to ensure the dosing was accurate.

2.3 METHODS: CUS PARADIGM

The CUS paradigm was adapted from a combination of those sited in the literature to accurately produce a stressed and anxious rat model [2, 17, 18]. There were 6 different stressful conditions, each administered twice, and days without stress in a random order over a total period of 21 days. This procedure began at age three months. The administration order of the stressful events was created by utilizing a random number generator in an effort to produce an unpredictable model that closely resembles real life stressors, such that the results obtained were more generalizable to the human population. Table 1 illustrates the order and explanation of the stressors administered throughout the 21 day paradigm. Days 0 and 20 are not actually part of the CUS paradigm, but have been added to the table 1 to clarify the order of procedures.

Table 1: CUS Paradigm

DAY	CONDITION	TREATMENT
0	Blood Withdraw	Blood Withdraw
1	Free	No stress
2	Restraint	30 min. restraint
3	Free	No stress
4	Isolation	Individually caged for 24 hrs
5	Free	No stress

6	Water Deprivation	Withdraw of water for 24 hrs
7	Free	No stress
8	Food Deprivation	Withdraw of food for 24 hrs
9	Forced Swim	Swim 10 min. with no rest
10	Free	No stress
11	Light Stress	Lights stay on overnight
12	Forced Swim	Swim 10 min. with no rest
13	Restraint	30 min. restraint
14	Isolation	Individually caged for 24 hrs
15	Free	No stress
16	Food Deprivation	Withdraw of food for 24 hrs
17	Light Stress	Lights stay on overnight
18	Water Deprivation	Withdraw of water for 24 hrs
19	Free	No stress
20	Blood Withdraw	Blood Withdraw
21	Free	No stress

2.4 METHODS: BLOOD WITHDRAW

Tail blood samples were collected from all rats before and after the CUS paradigm to determine the effects of CUS on levels of Zn in the blood. Rats were restrained in a decapicone. A local anesthetic cream of 20% benzocaine was applied to the tail site and a heat lamp was placed over the tail to facilitate vasodilatation of the tail vein. The area over the tail vein 5 cm from the base of the tail was cleaned with rubbing alcohol. A 22 gauge needle was partially inserted into the lateral tail vein and approximately 0.5 mL of blood was collected into a 1.0 mL syringe. All of the samples were immediately frozen on dry ice and then placed into a -80 freezer until analyzed.

2.5 METHODS: BLOOD ANALYSIS

Zn concentrations from blood samples taken both before and after the CUS paradigm were determined by inductively coupled plasma mass spectrometry (ICP-MS). 200 μL of each blood sample was digested by adding 1.5 mL of 7 normal nitric acid (7N HNO_3) and diluted to a total volume of 20 mL. 10 mL was then removed and spiked with 10 μL of Indium (1000 ppb In).

2.6 METHODS: FEAR CONDITIONING

After the 21 day CUS paradigm a 10 day delay period occurred so that all rats were tested at four months of age. Cued fear conditioning, extinction, and recall occurred in two identical clear Plexiglass (26 cm long x 26 wide cm x 18 cm high) fear conditioning chambers inside sound attenuating boxes (Coulbourn Instruments). FreezeScan software (Clever Sys, Inc.) monitored freezing behavior and administered 20 second 85 dB tones, each of which coterminated with 2 second 0.5 mA scrambled foot shock.

Training occurred on day one of fear conditioning. The animals habituated to the chambers for the first 160 seconds with no stimuli. After the acclimation period three tone/shock pairings were administered one minute apart. In between trials fear conditioning boxes were cleaned with acetic acid.

Twenty-four hours later animals underwent fear extinction. The fear conditioning boxes were covered will black and yellow strips as well as polka-dots in an effort to disguise the boxes from the training day. Black plexi glass was also placed on the floor covered with bedding to further modify the boxes. Lighting in the altered testing chambers and room was dimmed and a fan ran in the background to provide white noise. Similarly to the training day, animals habituated to the chambers for the first 160 seconds with no stimuli. At the end of the acclimation period 18 tones were administered, without

a shock, one minute apart. In between trials both fear conditioning boxes were cleaned with 70% alcohol.

Fourty-eight hours after training (24 hours after extinction) rats were placed back into the altered chambers to test extinction recall. The same extinction process that was conducted the day prior was again administered. Animals habituated to the boxes for the first 160 seconds without stimuli and then received 18 tones without a shock one minute apart. In between trials both fear conditioning boxes were cleaned with 70% alcohol.

2.7 METHODS: BRAIN ANALYSIS

A separate cohort of thirty-six animals received the same dosing, CUS paradigm, blood draw, and fear conditioning as the previously mentioned cohort except that there was no delay between the stress and behavioral testing. This cohort was raised to examine the level of Zn in the brain after CUS.

Twenty-four hours after fear extinction recall, all 36 rats were sacrificed with CO_2, followed by decapitation and brain extraction. After removal, all brains were immediately frozen on dry ice and then placed into a -80 freezer until analyzed.

The left hemisphere was removed from all thirty-six brains and dehydrated in an oven overnight. They were digested by adding 5.0 mL of of 7 normal nitric acid (7N HNO_3) and heated overnight. After drying down, they were diluted up to 50 mL with internal standard solution (2% HNO_3 + .1 ppb In + .001 ppb Bi). Values were determined utilizing ICP-MS.

3.1.1 RESULTS: TRAINING DAY

A 2x2 repeated measures ANOVA of the 3 tones administered during fear conditioning determined that there was a significant main effect on the average freezing rate across tones $F(2,54) = 11.15$, $p < .01$ (See Fig. 1). There were no significant differences between the groups, which indicated that all animals increased their freezing equally and were successfully conditioned.

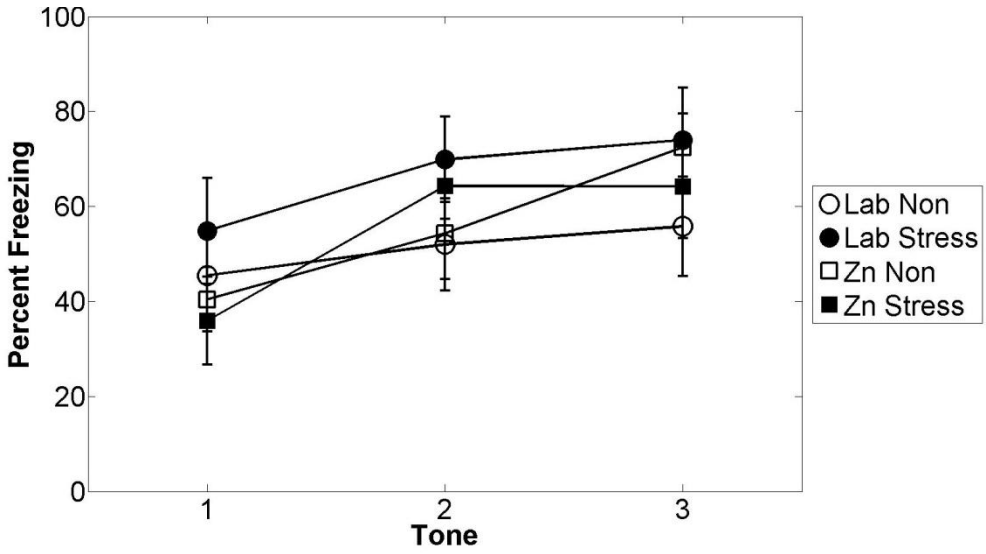

Figure 1: Fear Conditioning Training Day. Freezing significantly increased across tones similarly for all groups. Animals successfully conditioned to the tone. Error bars denote S.E.M.

3.1.2 RESULTS: PRIOR TO TONE ONSET

A 2x2 repeated measures ANOVA of the first 3 minutes prior to the administration of tones on extinction day, with the Greenhouse-Geisser correction, revealed that there was no significant effect across minutes or between groups (See Fig. 2). Since rats froze comparably and below 50 %, generalized anxiety was not observed.

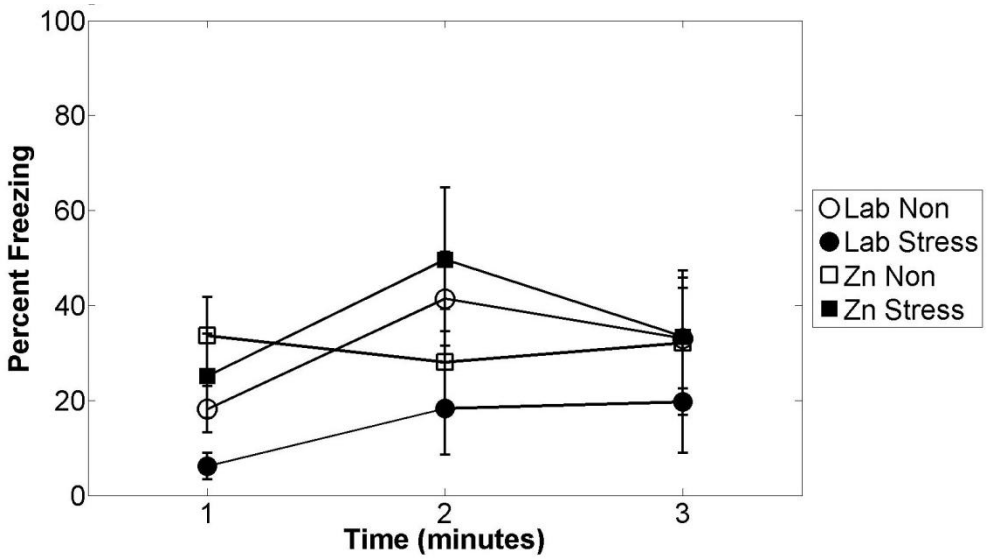

Figure 2: First Three Minutes of Extinction Before Tones. Animals generally froze less than 50% with no significant differences across time or between groups. There was no generalized anxiety detected. Error bars denoted S.E.M.

3.1.3 RESULTS: FEAR EXTINCTION

18 tones were administered during fear extinction, but only the first 15 were analyzed because after reviewing the videos it was discovered that several rats started sleeping and this lack of movement was miscoded by the software as freezing. A 2x2 repeated measures ANOVA of the first 15 tones, with the Greenhouse-Geisser correction, revealed a significant main effect across tones on the ability to extinguish fear, $F(5.88, 152.78) = 12.05$, $p < .05$ (See Fig. 3). Planned contrast indicated that all rats extinguished fear across tones as denoted by significantly freezing less at tone 15 compared to all other tones. There was a significant main effect for water type, on the ability to extinguish fear, $F(1, 26) = 4.72$, $p < .05$. The group supplemented with zinc (M = 81.90) displayed an impairment in the ability to extinguish fear by significantly freezing more than the controls (M = 61.53). There was also a significant interaction between tone and water type with the Greenhouse-Geisser correction, $F(5.88, 152.78) = 2.9$, $p < .05$. To better elucidate this interaction, follow up analysis of tone one was performed as an evaluation of conditioning recall and analysis of tones 3-15 were separated as a measure of extinction rate. There were no differences between groups at tone one. This suggests that all animals recalled conditioning similarly and have comparable baseline levels of anxiety. Comparable to tones 1-15, tones 3-15 also had a significant main effect for water type, $F(1, 26) = 5.28$, $p < .05$ and a significant interaction between tone and water

type $F(5.42, 2173.65) = 2.47$, $p < .05$. These analyses indicate that the difference between supplemented zinc groups and the controls across tones was due to the rate of extinction and not a difference in initial anxiety level of. Although all animals decreased the amount of freezing across time, the supplemented zinc groups decreased their freezing slower; therefore, they took longer to learn fear extinction. There was no significant main effect of stress on the ability to extinguish fear. There was also no significant interaction between water type and stress on the ability to extinguish fear. Inspection of the plot suggested there might be an effect of stress during the extinction phase in control animals; however, ANOVA failed to reveal a significant difference. Follow up analysis determined that there was no significant difference between groups on the final tone, thus permitting the examination of extinction recall 24 hours later.

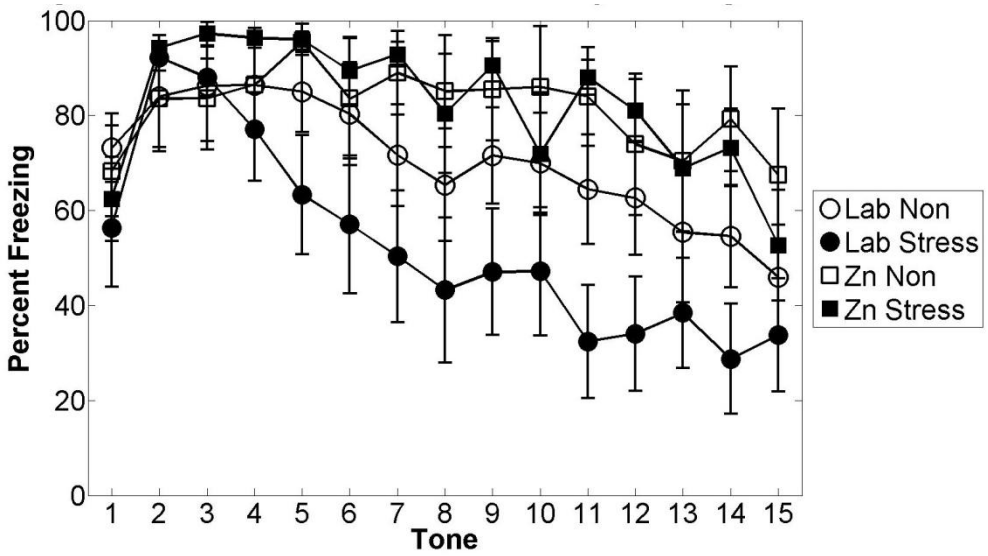

Figure 3: Excess Zn Decreases the Ability to Extinguish Fear. Freezing rate during the first 15 tones of fear extinction. There was a significant decrease across tones, but zinc animals took longer to extinguish and maintained a higher freezing rate than lab water animals. Error bars denote S.E.M.

3.1.4 RESULTS: EXTINCTION RECALL

18 tones were administered during extinction recall, but only the first 5 were analyzed because they were the most representative time frame for recall. A 2x2 repeated measures ANOVA of the first 5 tones, with the Greenhouse-Geisser correction, revealed a significant main effect across tones on the ability to recall fear extinction, $F(2.35, 61.06) = 5.76$, $p < .05$ (See Fig. 4). There was a significant main effect for water type regardless of tone, on the ability to recall fear extinction, $F(1, 26) = 4.32$, $p < .05$. The group supplemented with zinc ($M = 75.08$) displayed an impairment in the ability to recall fear extinction by significantly freezing more than the controls ($M = 51.37$). There was no significant main effect of stress on the ability to recall fear extinction. There was also no significant interaction between water type and stress on the ability to recall fear extinction.

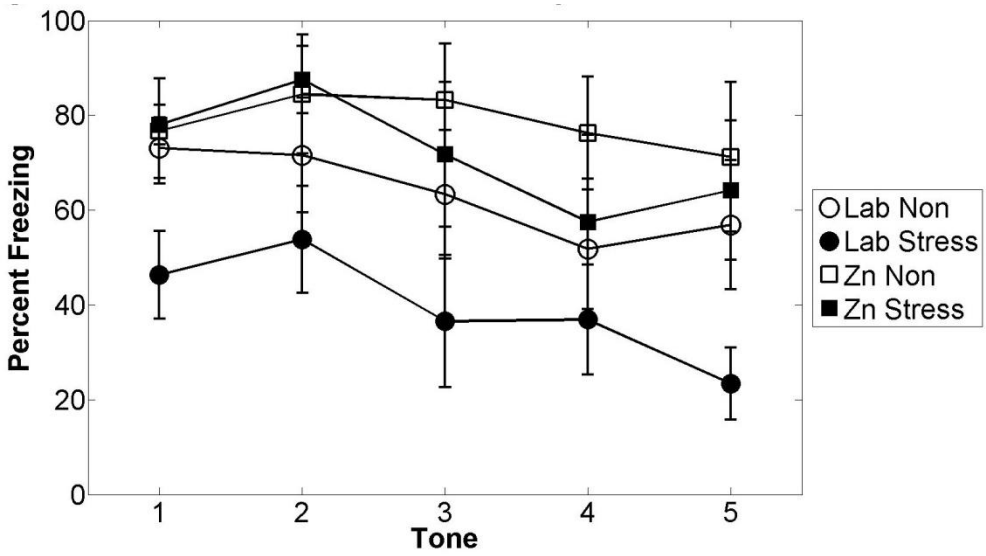

Figure 4: Excess Zn Results in an Inability to Recall Fear Extinction. Percent freezing for the first 5 tones during extinction recall. Zinc animals froze more than lab water animals, indicating a deficit in fear memory. Error bars denote S.E.M.

3.2.1 RESULTS: ZN LEVELS IN THE BLOOD

A 2x2 ANOVA of Zn levels in the blood after CUS was conducted. There was no significant main effect for water type nor stress on the level of Zn in the blood. However, there was a significant interaction between water type and stress on the level of Zn in the blood, $F(1, 24) = 4.45$, $p < .05$ (See Fig. 5). Follow up T-tests demonstrated a significant difference between CUS and non-stressed animals on Zn supplemented water $T(1,13) = -1.77$, p(one-tailed) $= .05$ and trend toward a difference for rats on lab water, $T(1,13) = 1.56$, p(one-tailed) $< .1$. These results indicated that the effect of CUS on the level of Zn in the blood depended on whether the animals were supplemented with zinc. CUS decreased the level of Zn in the blood for animals that drank lab water, but increased the amount of Zn in blood for animals that were supplemented with Zn.

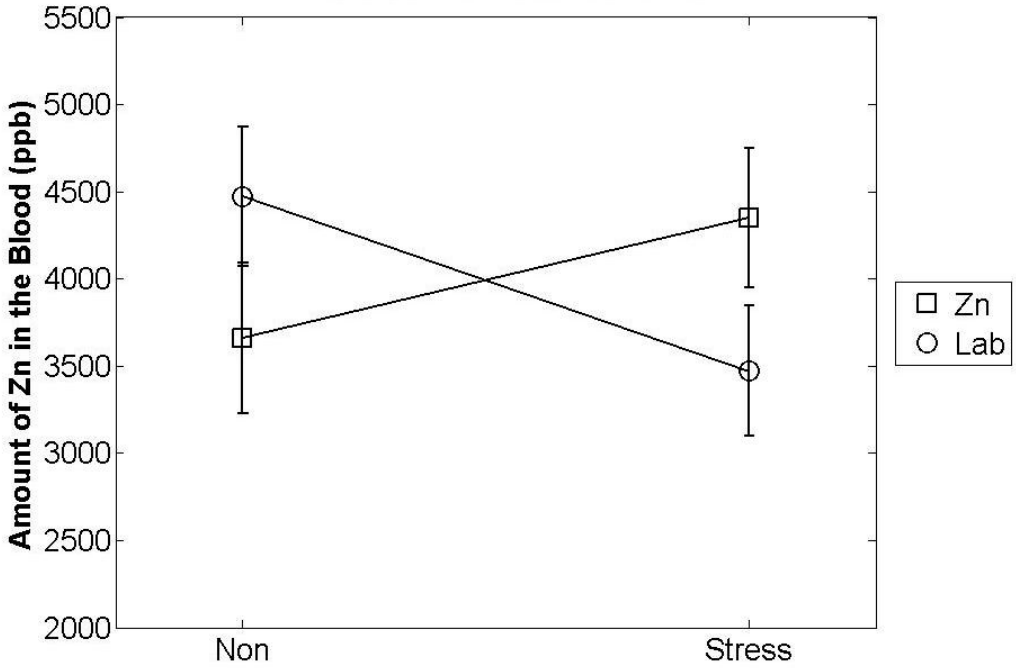

Figure 5: Interaction Between CUS and Zn Supplementation on the Level of Zn in the Blood. A significant interaction between CUS and zinc, such that CUS decreased zinc in the blood for lab water controls, but increased it for animals dosed on zinc. Errors bars denote S.E.M.

3.2.2 RESULTS: EXTNCTION RATE AND ZN IN THE BLOOD

Pearson's correlation showed that the relationship between the levels of Zn in the blood after CUS and the rate of fear extinction depended on the group. There was a significant positive correlation for animals on lab water that were stressed $r = .84$, p (one-tailed) $< .01$ (See Fig. 6a). There was a trend toward a positive relationship for animals on lab water that were not stressed, $r = .63$, p (one-tailed) $= .07$ (See Fig. 6b). This result indicated that as the level of Zn in the blood increases after CUS, the rate at which fear extinction occurs also increases. However, there was no significant relationship for animals supplemented with Zn for either of the stress groups.

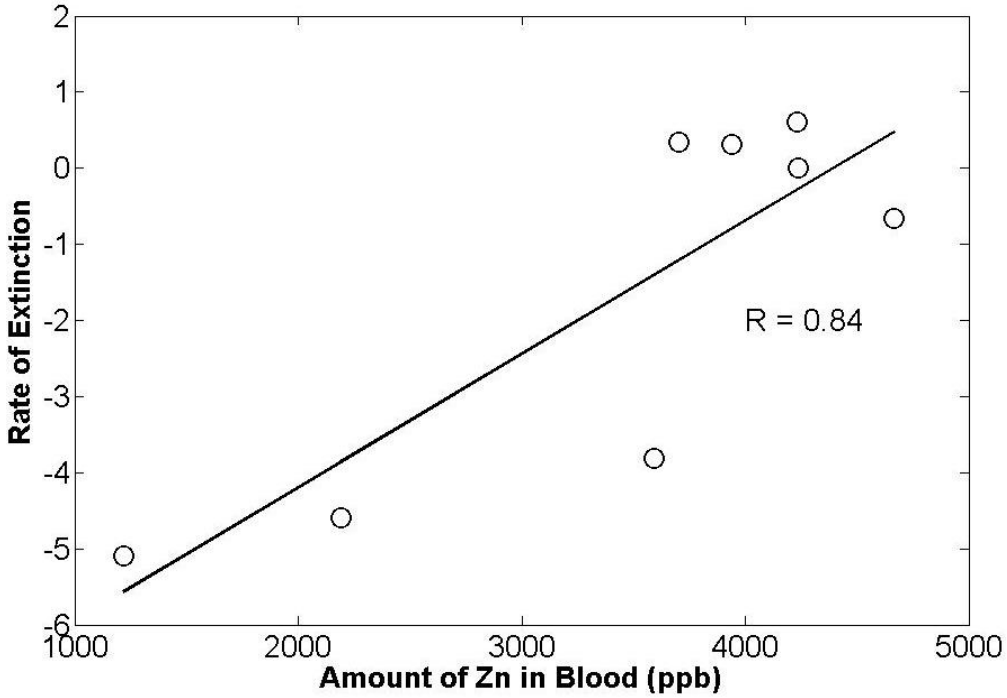

Figure 6a: Relationship Between Zn in Blood and Extinction Rate for Stressed Lab Animals. A significant positive correlation for CUS animals on lab water. As Zn increases in the blood, animals extinguish faster.

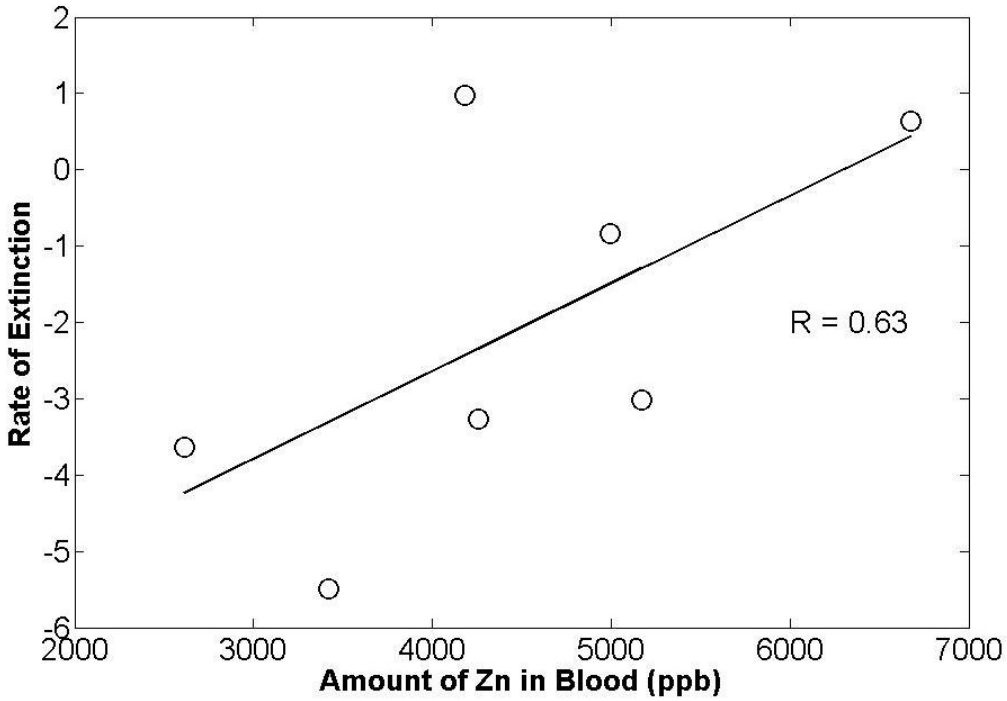

Figure 6b: Relationship Between Zn in Blood and Extinction Rate for Control Animals. A Positive trend for non-stressed animals on lab water (controls). As Zn increases in the blood, animals extinguish faster.

3.2.3 RESULTS: EXTINCTION RECALL AND ZN IN THE BLOOD

There were no significant correlations between the levels of zinc in the blood after CUS and the average recall of fear extinction.

3.3 RESULTS: BRAIN ANALYSIS

A 2x2 ANOVA was conducted to examine how CUS affected the level of zinc in the left hemisphere of the brain. There were no significant effects of CUS or water type on the level of Zn (See Table 2). All groups had similar levels of Zn in their left hemisphere.

Table 2: Level of Zinc in the Left Hemisphere After CUS

Experimental Group	Lab_Non	Lab_CUS	Zn_Non	Zn_Stress
Zn in Left Hemisphere (ppm)	45.93±.94	45.47±.95	45.67±.57	45.81±.65

4. DISCUSSION

Results from this study indicate that Zn is involved in a conditioned phobic response similar to PTSD. More specifically, excess Zn impedes fear extinction and recall, which suggests a learning and memory impairment related to cued-fear. The levels of Zn in the blood also signify that there is some interaction between CUS and Zn that elicits this abnormal behavioral response. These findings are pertinent because Zn is prevalent in the diet, including supplements and cold remedies [19, 20]. Furthermore, stress is a daily event in human lives [1].

Analysis of the three tones administered during fear conditioning training, demonstrated that animals learned the cued-fear response and successfully became conditioned to the tone, as denoted by their freezing rate significantly increasing across tones (See Fig 1). Additionally, there were no significant differences found between the groups, indicating that all animals learned this behavioral response similarly. It was essential that animals conditioned to the cue so that their ability to extinguish this fear could be further examined.

The freezing rate during the three minutes prior to tone onset, on extinction day, was the same across time and between all groups (See Fig. 2). Since all animals commonly froze less than 50% without the presentation of the conditioned cue, generalized anxiety was not exhibited. This finding supports prior conclusions that CUS

did not elicit generalized anxiety as determined by normal exploration in both elevated plus maze [3] and open field [5]. Paired with the cued-specific results from fear extinction, these data jointly suggest the involvement of Zn with pathologies comparable to PTSD.

Examination of the first 15 tones during fear extinction revealed that although all animals decreased their freezing across the trial, the Zn animals had a notably slower rate of extinction and continued to have a significantly higher freezing rate compared to the lab water controls (See Fig. 3). These data are consistent with previous findings where supplemented Zn also caused anxiety [14, 15] and a Zn deficient diet remediated extinction deficits [16]. An effect of CUS on fear extinction was not obtained despite the recommended delay between stress and behavior [6], which differs from other studies that determined CUS caused a cued phobic response [2, 3, 6]. This difference may have occurred because Zn levels in the blood were also examined in this study; therefore, blood samples were collected from the same animals as the behavioral assays.

Assessment of the first 5 tones during extinction recall determined that Zn animals had significantly higher rates of freezing than controls, suggesting that Zn rats failed to recall extinction 24 hours later (See Fig. 4). These results parallel the extinction data, demonstrating an impairment in both learning and memory for cued-fear. This is somewhat expected because if the task was originally challenging for Zn rats, then it is feasible that remembering the extinction would also be difficult. Previous studies examining the effects of CUS or Zn, on extinction, lack assessment of recall. However,

this time point is essential because even if extinction is successful, it is an individual's ability to remember this extinction that determines their behavioral response to the cue.

Zn in the blood after CUS showed a significant interaction between stress and Zn supplementation (See Fig. 5). The effect of CUS on the level of Zn in the blood depended on whether the animal consumed Zn or lab water. CUS decreased the amount of Zn in the blood, compared to non-stress, for lab water controls, but increased Zn levels, compared to non-stress, for Zn dosed animals. The reduction of Zn by CUS for lab water rats parallels a previous study demonstrating a decline of Zn in the blood after acute and chronic restraint stress [1]. Although CUS had a different effect on Zn animals than what has been previously discovered with stress, it may be explained by the role of the liver. It is possible that by dosing the animals daily with Zn, the liver filtered this excess from the body in the non-stress group before it could reach the bloodstream. Since stress breaks down the immune system [21], it is also feasible that CUS blocked the liver response, thus causing an increase in the Zn supplemented group.

There was a positive correlation discerned between the amount of Zn in the blood and the rate at which animals extinguished fear for the two lab water groups (See Fig. 6). As the level of Zn increased in the blood, the animals were able to extinguish fear more quickly. Similarly, as Zn decreased in the blood, it took longer to extinguish the conditioned phobic response; thus, was more difficult. Although a similar relationship was not discovered for the Zn supplemented groups, this may be due to a ceiling effect. An association would be difficult to obtain with the deficit in extinction. However, the relationship observed for the lab water groups is significant because it implies that Zn

may be a key factor between stress and the behavioral response exhibited by a cue. Moreover, the level of Zn in blood may be predictive of the rate at which an individual can extinguish fear.

Whole tissue analysis of the left hemisphere did not detect any significant difference in the level of Zn between groups (Table 2). This does not match previous work that found an increase in Zn levels after administering and acute stressor [7,12]. However, since behavioral [ ] and blood [1] data indicate differences between chronic and acute stress, than it is likely that the results obtained from these acute stressor studies would not generalize to the CUS findings. Additionally, these studies examined endogenous Zn levels in specific brains regions. This is a more sensitive measure than whole tissue analysis. Since only a subset of glutamatergic neurons in the fear pathway, specifically in the LA, also contain Zn [8, 12], it is possible that Zn was amplified in these particular cells enough to implicate a behavioral change. This increase may have been so precise that it was not discernable utilizing whole tissue analysis. Future studies should investigate the change in Zn levels of the LA in response to CUS.

The observed maladaptive behaviors in this study may result from an impairment in fear learning and memory. A learning deficit was supported with the investigation of c-Fos expression during fear extinction in the cortio-amygdala pathway of mice on a Zn deficient diet [15], but future studies should also examine these levels during extinction recall. Additional research can confirm this theory by investigating levels of similar biological markers such as phosphorylation of MAPK (pMAPK).

Another explanation for the cued-phobic response caused by Zn may be a heightened amygdala, heightened HPA axis, or hypoactive infralimbic cortex. A hyperactive amygdala may be a more probable premise since Zn is required to mediate LTP in the fear conditioning pathway, resulting from its co-localization with glutamate [9, 10, 13]. Moreover, not all neurons in the LA contain Zn [9, 13], which implies that Zn may also permit spatial specificity of LTP. This selective potentiation may contribute to the conditioned stimulus (CS) discrimination during recall of fear memory [9]. Interestingly, a small population of neurons within the dorsal region of the LA has been implicated as the location for synaptic plasticity of fear memory [22]. However, more research is needed to ascertain if these populations overlap. If the complete blockade of inhibition could unspecifically enhance LTP at the LA synapses, then it is possible that this lack of specificity could result in an overactive amygdala as seen with anxiety disorders. It is also plausible that if a specific amount of Zn is needed for spatial specificity of LTP, then an overabundance could cause the absolute blockade of inhibition and induce anxiety symptoms.

5. CONCLUSION

CUS altered the amount of Zn in the blood and these levels were directly related to the ability to extinguish fear. Additionally, animals with excess Zn experienced deficits in fear extinction and recall. These data jointly support the theory that Zn may be a mediator between stress and anxiety-like behaviors such as PTSD.

APPENDIX 1: EXTENDED INTRODUCTION

Based on the literature, I believed that zinc is a mediator between stress and learning/memory deficits. During chronic stress, zinc is being reallocated from the blood in the body to the brain. It is this excess zinc in the brain that is actually causing the learning and memory deficits frequently observed in stressed individuals. These deficits would be demonstrated by utilizing a chronic unpredictable stress (CUS) paradigm instead of a chronic immobilization stress paradigm (CIS), which would illustrate general anxiety behavior. Additionally, these behavioral differences are not obtained immediately after the stress, thus a time delay of 10 days between the completion of the stress paradigm and the behavioral measures must occur.

To test this hypothesis as well as any interaction effects between CUS and zinc, four groups of rats were bread, dosed for four months, and underwent fear conditioning: 1. Tap water + No stress, 2. Tap water + stress, 3. Zinc (10mg/kg $ZnCO_3$) + No stress, 4. Zinc (10mg/kg $ZnCO_3$) + stress. To ascertain the changes of zinc in the blood and the brain as a result of the stress, blood was drawn from all rats at three different time points: pre-stress, post-stress, and post-behavior. Zinc was also measured in the brains from all of the rats after sacrifice.

Research Questions:

1. How does chronic unpredictable stress affect the levels of zinc in the blood and brain?

   Hypothesis 1: CUS will decrease the level of zinc in the blood over time and increase the level of zinc in the brain.

2. How do zinc and CUS affect the ability to extinguish fear?

   Hypothesis 2: CUS will diminish the ability to extinguish fear and these effects will be exacerbated by zinc supplementation.

3. How do zinc and CUS affect generalized anxiety?

   Hypothesis 3: Zinc rats will freeze more during the first 3 minutes before a tone is administered, but CUS rats will show no difference in freezing rate, compared to control rats, during the first 3 minutes.

4. Are the behavioral differences observed in fear extinction learning correlated with the levels of zinc in the brain?

   Hypothesis 3: The behavioral differences in fear extinction learning will be correlated with the levels of zinc, such that there will be a positive correlation between the levels of zinc in the brain and the rate of freezing.

APPENDIX 2: EXTENDED METHODS

16 female Sprague-Dawley breeders were purchased from Charles River. 8 male Sprague-Dawley breeders were ordered from the same company, such that there was one male paired with two females.

When the breeders arrived, the females were taken out of their shipping boxes and placed into their new home cages in groups of four with the appropriate cage tags. Therefore, there were four cages of female breeders. This was done for two reasons. First, the offspring were both pre and post natally dosed with zinc, thus the breeders must also be dosed. Given that there were only two water groups (lab & zinc), there were two corresponding cages per water type. The second reason for this specific group housing is that housing females together coordinated their estrous cycles and permitted for simultaneous breeding. The males were also taken from their shipping boxes and placed in their new home cages. Males were also housed four to a cage, but were later individually housed to prevent fighting.

The rats were given two weeks to acclimate to the new housing environment. During this time the females were administered zinc through their drinking water. After two weeks, the estrous cycles were activated by splitting up the four females from one cage into two separate cages, and placing a male rat in each of the breeders' cages. The rat's estrous cycle is four to five days, but ovulation is on day three. For the optimum

pregnancy rates, the male rat remained with the two female rats for one week. After breeding pairing, the females were returned to group housing and male breeders will return to individual housing.

Rat gestation is 21 days. Therefore, after 14 days of pregnancy all female rat breeders were separated into individual housing to permit one week for nesting and other preparation before giving birth. During this time and throughout maternal care, the females received either tap or zinc water. On postnatal day 14, all litters were culled so that no litter was larger than 12 pups. This was to promote equal maternal care for each pup and to help prevent neglect.

The offspring were weaned from their moms on postnatal day 21. At this time the offspring were sexed. Females were sacrificed since this experiment only utilized males. To prevent excessive stress, the males were housed with their litter mates, such that there were four rats per cage and four cages per water group. Any extra males were kept and raised for pilot work. As always, all cages had appropriate cage tags. All breeding protocol was previously approved by IACUC under protocol number 2009-0175.

Cued fear conditioning and extinction occurred in two identical clear Plexiglass (26 cm long x 26 wide cm x 18 cm high) fear conditioning chambered inside sound attenuating boxes (Coulbourn Instruments). FreezeScan software (Clever Sys, Inc.) monitored freezing behavior and administered a 20 seconds 85 dB tone, which coterminated with 2 second 0.5 mA scrambled foot shock.

Training occurred on day one of fear conditioning. No habituation occurred. The first two animals were removed from their home cages, placed into separate,

appropriately labeled wire transport cages and moved to the fear conditioning room. They were taken out of the wire transport cages. One rat was placed in cage A and the other in cage B. The computer program was started, the cage doors were closed, and all experimenters left the room. The computer was programmed so that the first 160 seconds, there was no stimuli while the animals habituated to the cage. A 20 second 80dB tone started at 160 seconds and co-terminated with a 0.5 mA shock that was administered at 178 second. The second tone began at 220 seconds with the shock paired at 238 seconds. The final tone began at 280 seconds with the tone at 298 seconds. The entire trial lasted six minutes with a total of three foot shock/tone pairs. Once the trial was over, the experimenters re-entered the room, removed the rats from their boxes, and place them back into their corresponding wire transport cages. The cages were taken back into the colony where the rats were placed back into their home cages. During the five minutes between trials both fear conditioning boxes were cleaned with acetic acid. This process was repeated 15 more times for the remaining rats until all 16 trials and 31 rats were trained on Pavlovian cued fear conditioning.

Day two was extinction day. The first two animals were removed from their home cages, placed into the separate, appropriately labeled opaque polycarbonate transport cages with bedding. Whichever animal was on the right for the training day was on the left and vice versa. The transport cages were placed in a habituation room and left undisturbed for 10 minutes. After 10 minutes the transport cages were moved to the fear conditioning room. The animals were taken out of the transport cages. One rat was placed in cage A and the other in cage B. Again, the rat that was placed in cage A for training

was placed in cage B and conversely, the rat that was placed in cage B on training day was placed in cage A. The fear conditioning boxes were covered will black and yellow strips as well as poke-a-dots in an effort to disguise the boxes from the training day. There was also black plexi glass on the floor covered with bedding to further conceal the boxes. The computer program was started, the cage doors closed, and all experimenters left the room. A 20 second 80dB tone was administered at the end of minutes 3,4,5,6,7,8,9,10,11,12,13,14,15,16,&17. The trial will lasted 18 minutes with 15 tones total, but there were no shocks paired with them. Once the trial was over, the experimenters re-entered the room, removed the rats from their boxes, and placed them back into their corresponding transport cages. The cages were taken back into the colony where the rats were placed back into their home cages. During the five minutes between trials both fear conditioning boxes were cleaned with 70% alcohol. This process was repeated 15 more times for the remaining rats until all 16 trials and 31 rats underwent cued extinction.

Behavioral testing day three was extinction recall. The same extinction process exhibited in testing day two was administered during the extinction recall day as well.

APPENDIX 3: EXTENDED RESULTS

A 2x2 repeated measures ANOVA of the 3 tones administered during fear conditioning determined that there is a significant main effect across tones on the average freezing rate $F(2,54) = 11.15$, $p < .01$. There were no significant differences between the groups, which indicates that all animals increased their freezing and were successfully conditioned.

A 2x2 repeated measures ANOVA of the first 3 minutes prior to the administration of tones on extinction day, with the Greenhouse-Geisser correction, revealed that there was not a significant main effect across minutes on generalized anxiety, $F(1.63, 42.48) = 2.69$, $p > .05$. This signifies that all animals, regardless of water type or stress, froze similarly across minute 1 ($M = 20.75$), minute 2 ($M = 34.33$), and minute 3 ($M = 29.58$). There was not a significant main effect for water type on generalized anxiety, $F(1, 26) = 1.64$, $p > .05$. The group supplemented with zinc (M = 33.66) exhibited similar freezing levels compared to controls (M = 22.78). There was not a significant main effect of stress on generalized anxiety, $F(1, 26) = 0.56$, $p > .05$. The chronic unpredictable stress group (M = 25.39) froze similarly to the non-stress group (M = 31.05). There was also not a significant interaction between water type and stress on generalized anxiety, $F(1, 26) = 1.53$, $p > .05$.

A 2x2 repeated measures ANOVA of the first 15 tones, with the Greenhouse-Geisser correction, revealed a significant main effect across tones on the ability to extinguish fear, $F(5.88, 152.78) = 12.05$, $p < .05$. Planned contrast revealed that all rats extinguished fear across tones as denoted by significantly freezing less at tone 15 compared to all other tones. There was a significant main effect for water type regardless of tone, on the ability to extinguish fear, $F(1, 26) = 4.72$, $p < .05$. The group supplemented with zinc (M = 81.90) displayed an impairment in the ability to extinguish fear by significantly freezing more than the controls (M = 61.53). There was also a significant interaction between tone and water type with the Greenhouse-Geisser correction, $F(5.88, 152.78) = 2.9$, $p < .05$. This indicates that the extinction of fear across tones differed between the supplemented zinc groups and the controls. Although all animals decreased the amount of freezing across time, the supplemented zinc groups decreased their freezing slower; therefore, they took longer to learn fear extinction. There was no significant main effect of stress on the ability to extinguish fear, $F(1, 26) = 0.94$, $p > .05$. The chronic unpredictable stress group (M = 67.48) froze similarly to the non-stress group (M = 75.95). There was also no significant interaction between water type and stress on the ability to extinguish fear, $F(1, 26) = 1.00$, $p > .05$.

A 2x2 repeated measures ANOVA of the first 5 tones, with the Greenhouse-Geisser correction, revealed a significant main effect across tones on the ability to recall fear extinction, $F(2.35, 61.06) = 5.76$, $p < .05$. Planned contrasts revealed that rats increased their recall of fear extinction across tones regardless of water type or stress as denoted by significantly freezing less at tone 5 compared to tone 1, $F(1, 26) = 5.97$, $p <$

.05, and tone 2, $F(1, 26) = 11.11$, $p < .05$. There was a significant main effect for water type regardless of tone, on the ability to recall fear extinction, $F(1, 26) = 4.32$, $p < .05$. The group supplemented with zinc ($M = 75.08$) displayed an impairment in the ability to recall fear extinction by significantly freezing more than the controls ($M = 51.37$). There was no significant main effect of stress on the ability to recall fear extinction, $F(1, 26) = 1.94$, $p > .05$. The chronic unpredictable stress group ($M = 55.60$) froze similarly to the non-stress group ($M = 70.85$). There was also no significant interaction between water type and stress on the ability to recall fear extinction, $F(1, 26) = 0.58$, $p > .05$.

A 2x2 ANOVA of zinc levels in the blood after CUS was conducted. There was no significant main effect for water type on the level of zinc in the blood, $F(1, 24) = 0.06$, $p > .05$ (See Fig. 7). The group supplemented with zinc ($M = 4005.99$) contained similar levels of zinc in their blood compared to controls ($M = 3972.86$). There was no significant main effect of stress on the level of zinc in the blood, $F(1, 24) = 0.29$, $p > .05$ (See Fig. 7). The chronic unpredictable stress group ($M = 3912.07$) contained similar levels of zinc in their blood compared to the non-stress group ($M = 4066.77$). However, there was a significant interaction between water type and stress on the level of zinc in the blood, $F(1, 24) = 4.45$, $p < .05$. These results indicate that the effect of CUS on the level of zinc in the blood depended on whether the animals were supplemented with zinc. CUS decreased the level of zinc in the blood for animals that drank lab water, but increased the amount of zinc in blood for animals that were supplemented with zinc.

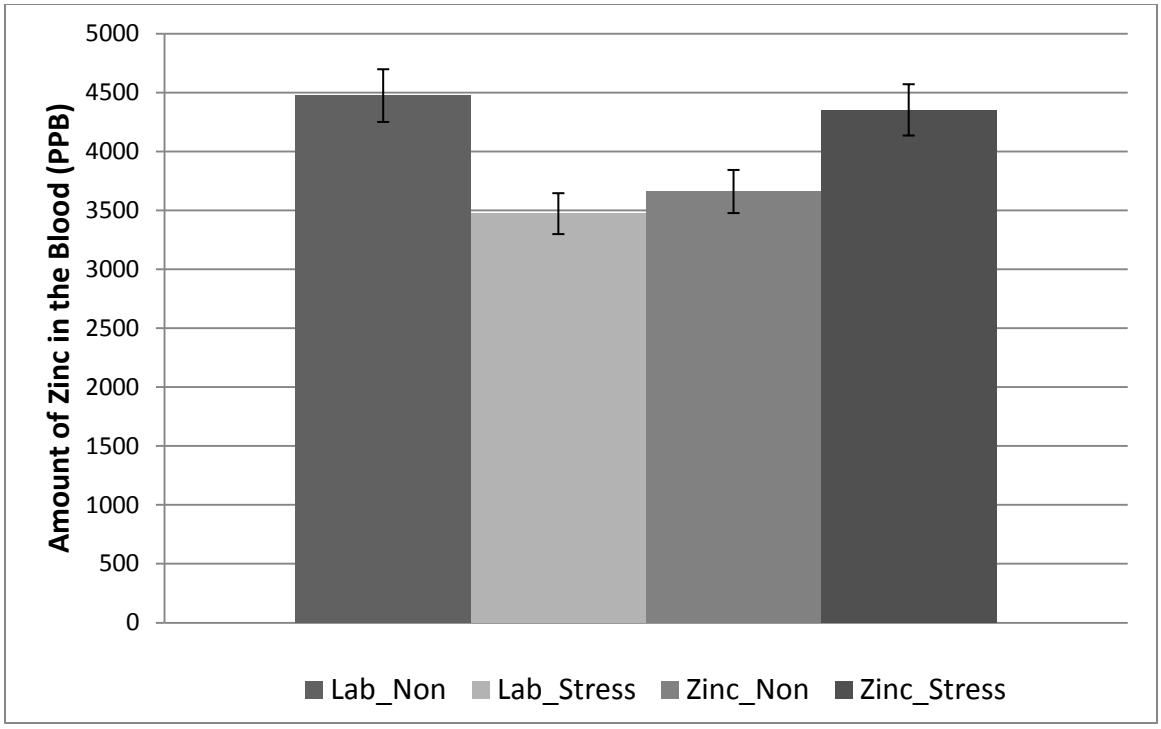

Figure 7: Level of Zn in the Blood from CUS

Pearson's correlation determined that the relationship between the levels of zinc in the blood after CUS and the rate of fear extinction depended on the group. There was a significant positive correlation for animals on lab water that were stressed $r = .84$, p (one-tailed) $< .01$. There was a trend toward a positive relationship for animals on lab water that were not stressed, $r = .63$, p (one-tailed) $= .07$. This result indicated that as the level of zinc in the blood increases after CUS, the rate at which fear extinction occurs also increases. However, there was not a relationship for animals supplemented with zinc that were not stressed $r = -.21$, p (one-tailed) $> .05$, nor animals supplemented with zinc that were stressed $r = .18$, p (one-tailed) $> .05$.

Pearson's correlation determined that there was not a significant relationship between the levels of zinc in the blood after CUS and the average recall of fear extinction for animals on lab water that were not stressed, $r = -.18$, p (one-tailed) $> .05$, animals on lab water that were stressed $r = -.18$, p (one-tailed) $> .05$, animals supplemented with zinc that were not stressed $r = -.46$, p (one-tailed) $> .05$, nor animals supplemented with zinc that were stressed $r = -.12$, p (one-tailed) $> .05$.

Utilizing the least squares method, several curves were fit to the data including linear, quadratic, and cubic. Further examination determined that attempting to fit polynomials of degree 3 or higher resulted in an ill-conditioned problem. Both quadratic and linear were appropriate, but linear illustrated the best relationship for the data.

Table 3: Curve Fitting Parameters

Equation	**Model Summary**					**Parameter Estimates**			
	R Squared	**F**	**df1**	**df2**	**Sig**	**Constant**	**b1**	**b2**	**b3**
Linear	.70	14.288	1	6	.01	-7.69	.002		
Quadratic	.71	5.97	2	5	.05	-7.25	.001	6.37e-8	
Cubic	.76	4.27	3	4	.1	3.38	-.01	4.95e-6	-5.41e-10

APPENDIX 4: FEAR CONDITIONING PATHWAY

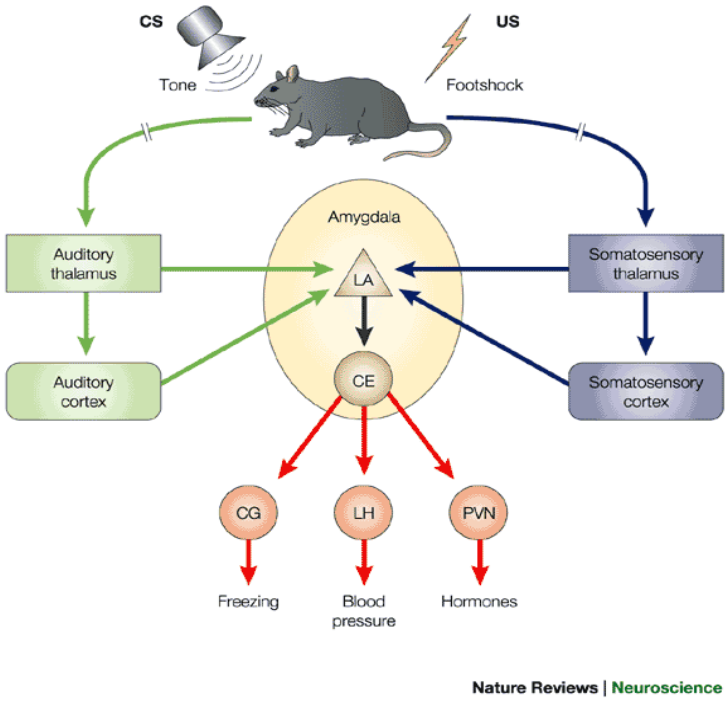

Figure 8: Fear Conditioning Pathway

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