INTRANASAL ZINC REMEDIATES DAILY ACTIVITY DEFICITS FOLLOWING STRESS AND TRAUMATIC BRAIN INJURY

by

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A thesis submitted in partial fulfillment of the requirements for the degree of
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ABSTRACT

INTRANASAL ZINC REMEDIATES DAILY ACTIVITY DEFICITS FOLLOWING STRESS AND TRAUMATIC BRAIN INJURY

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Mild traumatic brain injury (mTBI) has become the "signature wound" of the military population in recent combat operations. Repeated mTBI (rmTBI) produces long-term cognitive and behavioral deficits, including irregular circadian rhythm (CR). This can be exacerbated by the high-stress environment experienced by service members. This study employed a mouse model to examine the behavioral effects of rmTBI with chronic variable stress (CVS) on daily circadian activity. In addition, we tested the hypothesis that rmTBI + CVS effects would be ameliorated by supplemental zinc, a biometal that is known to be reduced in the brain following head trauma. Six-week old mice received two varied stressors (e.g., food deprivation, physical restraint, ice bath, predator urine) each day for 14 days. Four closed-head mild TBIs were concurrently administered, with each injury immediately followed by either intranasal zinc treatment or a vehicle control (saline solution). Mice underwent behavioral testing that assessed daily activity via 24-hour wheel-running activity for seven days, and by consecutive Morris water maze (MWM) testing for indices of spatial learning and anxiety. A significant interaction was found between stress and zinc in daily activity ($F(1.42, 56.82) = 3.610$, $p = .048$).

Stressed mice given vehicle treatment showed a significant deficit in wheel-running activity at the beginning of the dark cycle, but when zinc was administered, the deficit was corrected and activity for stressed mice nearly doubled. For non-stressed mice, zinc resulted in decreased wheel-running activity. MWM performance yielded consistent results, as the stressed-zinc group had the greatest number of platform crossings ($F(1,39) = 4.207$, $p = .047$) and reduced thigmotaxis ($F(1,39) = 3.222$, $p = .034$) compared to all other groups. Together, these findings suggest that zinc reduced behavioral deficits depending on the level of stress experienced with rmTBI, and that zinc may be efficacious under conditions of chronic stress. Additionally, zinc treatment increased TrkB receptor phosphorylation in the hippocampus in rmTBI animals, suggesting a TrkB-mediated neuroprotective mechanism for zinc's effect in stressed mice.

INTRODUCTION

Traumatic Brain Injury

Approximately 10 million people are affected by traumatic brain injury (TBI) every year (Hyder et al., 2007). Most frequently, military service personnel are exposed to repeated low-level blasts from improvised explosive devices, typically leading to mild-type TBI (mTBI) (Chandra & Sundaramurthy, 2015). For this reason, mTBI persists as the most common injury among U.S. service members and has quickly come to be known as the "signature injury" of the wars in Iraq and Afghanistan (Hoge et al., 2008).

Despite most blast-related TBIs being relatively mild, service members are at risk for sustaining repeated concussive injuries as they navigate through combat environments with wide-ranging blast pressure waves (Clark et al., 2018). A report from the Center for a New American Security (Fish & Scharre, 2018) suggests that repeated firing of heavy, shoulder-mounted weapons can also impose multiplicative damage on the brain, associated with transient impairments in cognition. Presently, it is documented that the damage from multiple repeated injuries has a cumulative effect on the brain (Slemmer et al., 2002).

TBI and Circadian Rhythm

Dysregulation of circadian rhythm (CR) is a common outcome in patients recovering from TBI (Zhanfeng et al., 2019). Previous research has demonstrated the cognitive deficits that follow TBI are closely linked to injury-induced circadian irregularities, as cognitive and memory dysfunction are correlated with impaired circadian rhythms in the

rodent hippocampus (Boone et al., 2012). The relationship between executive dysfunction and aberrant CR may be attributed to an overlap in molecular signaling cascades: cAMP, MAPK, and CREB proteins that underlie memory formation also mediate circadian clock regulation (Eckel-Mahan et al., 2008).

To compound this, TBI alone has also been found to induce disruptions in innate CR. Boone et al. (2012) attribute this to aberrant circadian gene expression in the suprachiasmatic nucleus and hippocampus: dysregulation of circadian clock genes Bmal1 and Cry1 (that are necessary for regular circadian timing) is common among TBI survivors. In turn, TBI research on injured rats with this dysregulation in circadian gene expression revealed disruptions in locomotor activity over a 48-hour period (Boone et al., 2012). Irregularities in CR have been documented to ultimately worsen TBI-related behavioral deficits and inhibit hippocampal neurogenesis (Li et al., 2016). This suggests that it is especially crucial that TBI patients maintain a regular CR to prevent the exacerbation of poor health outcomes.

TBI and Stress

In addition to TBI, U.S. military personnel are at high risk for chronically elevated stress. Service members are exposed to a high number of varied stressors, such as combat, terrorist attacks, violence, sexual assault, or serious injury (Reisman, 2016). Soldiers who experience a traumatic wartime event (including but not limited to TBI) may be susceptible to post-traumatic stress disorder (PTSD). In particular, high comorbidity rates of TBI and psychiatric stress disorders have been found in U.S. soldiers returning from recent combat operations in Afghanistan and Iraq (Hoge et al., 2008).

Stress and CR

Research has discerned a link between the stress response systems and functioning of the circadian clock, as the neurobiological pathways interact closely with one another (Koch et al., 2016). Physiological sensitivity to and rhythmic release of glucocorticoids (the hormone linked to stress response) are regulated by circadian clock genes (Landgraf et al., 2014). A high level of glucocorticoids is known to be neurotoxic to the hippocampus—a brain region implicated in PTSD—due to its rich concentration of glucocorticoid receptors (Sapolsky, 1985; Logue et al., 2016). Disruption in circadian clock genes also perturbs glucocorticoid activity in brain areas that are vulnerable to stress. In mice, for example, dysregulation of CR leads to increased fear conditioning as a result of greater corticosterone release (Loh et al., 2010). Inversely, the resulting disruptions in sleep/wake cycles can also contribute to elevated stress.

Zinc and the Nervous System

As one of the most abundant trace metals in the central nervous system (second only to iron), zinc is necessary for normal brain functioning, gene expression, cell signaling, and enzymatic activity (Gower-Winter & Levenson, 2012). The highest levels in the brain are in the hippocampus (Frederickson & Danscher, 1990; Levenson et al., 2011). Zinc finger proteins have also been found to be involved in neurogenesis throughout the hippocampus, which is largely responsible for spatial memory (Xie et al., 2010). For this reason, zinc is implicated in learning and memory processes as well as regulation of mood and emotion. Earlier studies have reported correlations between zinc deprivation and adverse behavioral side effects, such as enhanced depressive- and anxious-type

behaviors (Whittle et al., 2009). Zinc-deficient rats performing the Morris water maze task have also shown impaired ability of attention, learning, and memory in finding the platform, corresponding to increased apoptosis in hippocampal neurons (Yu et al., 2013). To further substantiate the effect of zinc, the researchers found that this deficiency-induced cell death was reversed when rats were treated with zinc supplementation. However, zinc has also been found to impair learning and memory (Flinn et al., 2005; Chrosniak et al., 2006). It is still highly disputed whether the role of zinc is neurotoxic or neuroprotective for conditions affecting the brain (Levenson, 2005; Maret & Sandstead, 2006; Sensi et al., 2011). Despite zinc's ambiguous role, several studies have demonstrated an unequivocal relationship between zinc and brain injury (Doering et al., 2010; Cope et al., 2012; Lucke-Wold et al., 2018), with TBI resulting in significant depletion of serum zinc. Following injury, zinc is shown to be excreted in the urine, with TBI severity being directly proportional to the amount of zinc excretion (McClain et al., 1986).

It follows that supplementing zinc may be useful in restoring the losses that follow TBI to improve neurological impairments (Young et al., 1996). Findings by Morris & Levenson (2013) support zinc supplementation for treating adverse symptoms associated with TBI, including depression and cognitive impairment. Furthermore, many studies have found synaptic zinc to be a requisite for increases in neurogenesis in the rodent hippocampus after TBI. For example, depletion of zinc leads to a significant reduction in post-TBI cell proliferation and neurogenesis, when compared to animals with normal zinc availability (Levenson & Morris, 2011; Choi et al., 2017).

Additionally, zinc is shown to be helpful in mitigating poor health outcomes, such as inflammation and suppressed immune responses that are typical of chronic stress (Gammoh & Rink, 2017). Similar to TBI patients, individuals with high psychological stress (i.e., anxiety disorders) have significantly decreased zinc availability in plasma levels, but with chronic zinc supplementation, anxiety symptoms are significantly improved (Russo, 2011). Zinc homeostasis is essential for cell maturation, cell differentiation, and proper immune functioning (Wessels, Maywald, & Rink, 2017). As TBI and chronic stress are related to compromised zinc levels in the nervous system, it is critical that individuals prone to a combination of injury and stress (i.e., military personnel) restore zinc loss to prevent further deleterious effects.

TrkB and BDNF

The neuroprotective effects that follow zinc supplementation may be closely related to the role that zinc takes in the activation of the tyrosine receptor kinase B (TrkB) pathways. TrkB is a receptor for brain-derived neurotrophic factor (BDNF) (Minichiello et al., 1999). When bound to TrkB, BDNF is known to trigger intracellular cascades that are linked to neuronal cell survival, dendritic stabilization, and long-term synaptic plasticity (Squinto et al., 1991; Klein et al., 1991; Yasuda et al., 2006). However, proBDNF—the precursor to mature BDNF—has a noted antithetical effect on cellular processes, as it is associated with neuronal apoptosis and long-term depression in the hippocampus when bound to nerve growth factor receptors (Teng et al., 2005; Woo et al., 2005). Furthermore, proBDNF levels are found to be elevated following central nervous system damage, resulting in cell death (Jansen et al., 2007).

To counter this, zinc binds to metalloproteinases and cleaves proBDNF to mature BDNF (Hwang et al., 2005). There is evidence that activation of TrkB pathways are neuroprotective after injury, and exercise following TBI has been correlated with recovering the cognitive deficits (i.e., spatial memory) due to increased BDNF activity (Wu et al., 2014; Griesbach et al., 2009). It is also possible for zinc to promote TrkB activation independent of BDNF binding by instead upregulating Src kinase activity as a separate mechanism for neuroprotection (Huang & McNamara, 2010). This BDNF-independent activation of TrkB receptors is the result of a zinc-induced intracellular cascade that ends with the autoinhibition of a Src kinase, which then phosphorylates TrkB (Huang et al., 2008).

Intranasal Administration

The use of enteral drug administration (i.e., oral consumption of zinc in food or water) is at a disadvantage when being studied during the acute window following TBI, as these drugs must firstly be taken up by the gastrointestinal tract before being absorbed into the body, which delays the effect of treatment (Neve et al., 1991). In addition to slowed onset, the absorption of orally administered zinc may be unpredictable due to degradation by stomach acid and enzymes. This phenomenon, known as first-pass metabolism, results in zinc's decreased bioavailability and a decreased therapeutic response. Cope et al. (2012) also indicated that zinc transporters in the digestive system (required for delivery of zinc across the blood-brain barrier) are less effective after TBI, adding to the oral route being a poor method for administering zinc treatment. Our study sought to bypass this effect by administering zinc intranasally to deliver rapid drug absorption via the highly-

vascularized mucous membrane, providing faster onset of action and improved bioavailability. When comparing intranasal and intraperitoneal zinc treatment, Persson et al. (2003) noted that zinc concentration was significantly higher in all areas of the brain except the cerebellum following intranasal deliver than with intraperitoneal. Therefore, it follows that intranasal is an optimal route of administration when considering the necessity of rapid delivery to the brain and an effective therapeutic response during the acute window that follows brain injury.

Our study investigated how the use of intranasal zinc as a therapeutic can moderate long-term effects of chronic stress on repeated mild traumatic brain injuries. To research behavioral outcomes of the drug, daily circadian activity and spatial memory were assessed in chronically stressed mice with rmTBI. Measures were then conducted to assess zinc and protein alterations in the brain, with a flurogenic Zn2+ reporter (Zinpyr-1) and western blot analyses of the BDNF/TrkB pathways, respectively.

METHODS

Animals

All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee at George Mason University (Fairfax). C57BL/6J mice ($N = 43$) were obtained from The Jackson Laboratory and housed in the university vivarium. This strain is commonly used in neurotrauma research and is practical for studies that do not necessitate transgenic animals. Animals were not singly-housed, as social isolation may cause disproportionate stress. Mice were provided enrichment (running wheels, igloos, nyla bones) with access to food (Teklad unautoclaved 7012 diet) and water ad libitum. Colony housing and testing rooms maintained a 12-hour light/12-hour dark cycle. Four groups of mice were studied, with respective sample sizes shown in Table 1.

Table 1 Experimental Groups and Conditions

	TBI + Zinc	TBI + Vehicle	Total
Stressed	11	11	22
Non-Stressed	10	11	21
Total	21	22	43

Materials and Procedure

At six-weeks of age, half of the mice were randomly assigned to a stressed group and subjected to one week of chronic variable stress (CVS). The following week, all stressed and non-stressed mice received four mTBI with a 48-hour inter-injury interval. Stressed mice were administered a second week of varied stressors concurrently with the four mTBI. Half of the mice in the stressed and non-stressed groups were then randomly selected and administered intranasal zinc treatment immediately after each TBI, while the remaining half was given a vehicle control. After concluding the rmTBI and CVS protocol, behavioral assays were conducted with 7 consecutive days of daily activity (DA) testing, followed by a 7-day Morris water maze paradigm. Upon completion of behavioral testing, mice were sacrificed with CO2, total protein extracted, and protein (Western) blots were performed to examine levels of TrkB, Phosphorylated TrkB proteins in the brain, and proBDNF and mature BDNF proteins. To ensure the presence and binding of zinc following intranasal administration, Zinpyr-1 staining was used to detect fluorescence of free-zinc in hippocampal slices. The timeline of all experimental procedures is shown in Figure 1.

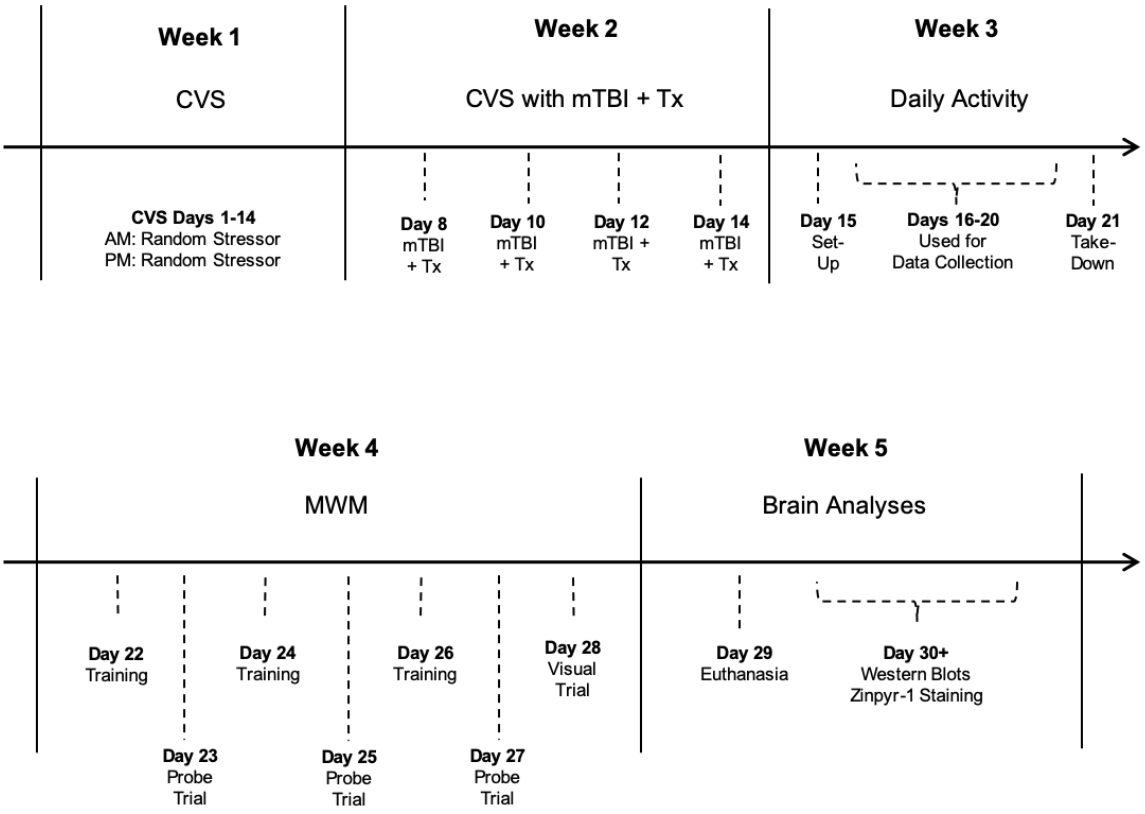

Figure 1 Experimental Timeline

Note. Timeline of study using chronic variable stress (CVS) in Weeks 1 and 2 for the stressed condition. Week 2 introduces four sessions of mild traumatic brain injury (mTBI) followed immediately with either intranasal zinc or control treatment (Tx), with a 48-hour interval. Daily activity (via wheel-running) and Morris water maze (MWM) testing was conducted in Week 3 and Week 4, respectively. Following behavioral tests, post-mortem analyses were conducted starting in Week 5.

Chronic Variable Stressors

Mice in the stress condition received a varied rotation of 8 different stressors, with two different stressors executed each day, for a period of 14 consecutive days. Each stressor has been validated in published CVS protocols (Lopez et al., 2011; Zhang et al., 2015). Any modifications to the tested stressors are based on viability of the procedure and accessibility of materials. Stressors were alternated between their daily presentation to prevent mice from acclimating to the given stimulus.

Orbital Shaker: For one hour, the animals' home cage was positioned on top of an orbital shaker, agitating the cage at a speed of 150 rotations per minute.

Cold Swim: For five minutes, mice were individually forced to swim in a container of 15°C water.

Bottle Restraint: For one hour, mice were individually restrained in a 50 ml conical falcon tube. The tube limits any mobility of the mouse, making the experience very stressful.

Strobe Light and Static Noise: For one hour, mice were exposed to a white strobe light and 100 decibels of white noise produced by a non-functioning radio channel.

Daytime Deprivation: For eight hours, all food, bedding, water, and enrichment (i.e., igloos and toys) was removed from the home cage. This was only done starting in the morning rather than overnight, with its completion time coinciding with the start of the next stressor.

Rat Bedding: A perforated tube containing soiled rat bedding was placed into each home cage for the entirety of time leading up to the next stressor. Cages subjected to this

stressor were placed outside of animal housing rooms so that no other animals were affected by the odor, with clean cages provided after completion. To avoid disturbing hormonal balances, male mice were only subjected to male rat bedding and female mice received female rat bedding. This stressor has been modified from the procedure used by Patterson et al. (2010), in which mice were transferred to a cage that rats were previously in.

Predator Urine: A perforated tube containing bobcat urine (purchased from predatorpee.com) was placed into each home cage. Like the rat bedding stressor, the tube remained in the cage until the start of the next stressor. Cages subjected to this stressor were placed outside of animal housing rooms so no other animals were affected by the odor, with clean cages provided afterward.

Wet Cage: Each home cage was flooded with 800 ml of room temperature water, supplied by the Edstrom lixit watering system. All bedding remained thoroughly soaked until the start of the next stressor, upon which a clean cage was provided.

CCI TBI Device

To induce reproducible mTBI with closed-head injury, the Flinn Laboratory uses the Impact One Stereotaxic Impactor for CCI developed by Leica, with the addition of a dropping platform. Before injury, the mouse was sufficiently anesthetized with isoflurane until they were non-responsive to forceful pinching of the tail or paw. Under anesthesia, the mouse was then placed on a small platform raised six inches above the stereotaxic base, with the center of impact being positioned on the scalp midline between bregma and lambda. CCI parameters have a preset velocity of 3.0 m/s to a cortical impact depth

of 3 mm. The baseline point of contact is determined by firstly lowering the extended impactor probe to the cortex. The impactor tip was then retracted and impact depth is adjusted by lowering the impactor depth by 1.0 mm. At this point, the mouse was removed from anesthesia for 30 seconds followed by the injury impact. As designed, when force is applied to the skull during impact, the hinged platform drops downward through a 90° angle, causing the mouse to fall six inches onto a foam pad below. The dropping of the platform permits a non-compressive injury while emulating a fall that is seen in human mTBI. This procedure was followed for each of the four mTBI with a 48-hour inter-injury interval.

Intranasal Zinc

For each day of zinc treatment, a fresh solution of 400 ppm zinc was made by diluting 10,000 ppm zinc with water as follows: 0.4 ml of 10,000 ppm zinc was diluted with 9.6 ml of distilled water, and then pH balanced with 0.12 mg of sodium bicarbonate. While mice were still anesthetized, intranasal treatment was administered immediately after falling from the TBI platform. The 4% zinc solution or vehicle control (saline) was dispensed in a 10 µl insufflation—delivered via pipette directly outside of the nare—to administer a dose of .4 µl zinc per mouse for each treatment, given every other day (see Figure 1 for timeline).

Daily Activity Testing

For the first day of daily activity (DA), animals were moved from colony housing to singly-housed cages, so data could be accurately attributed to individual activity. Each DA cage was equipped with a running wheel and directly connected to Actimetrics

Clocklab software for a continuous recording of each mouse's total wheel rotations at each hour. Consistent with animal housing rooms, the testing room was maintained on a 12-hour light/12-hour dark cycle, with light onset at 8 A.M. and offset at 8 P.M. Light offset/onset is also denoted in zeitgeber time (ZT) based on the 12:12 light:dark cycle, so that the 24-hour period starts with light offset at ZT 0 and onset commences at ZT 12 (see Figure 2). Based on procedures of a previous study (Graybeal et al., 2015), mice were singly-housed in DA testing for a period of seven days, excluding data from Day 1 and Day 7 to omit collection errors during experimental set-up and take-down. DA data from the median five days of the testing period were quantified by the mean wheel rotations for each hour of a 24-hour day. At the conclusion of the seven-day period, animals were returned back to their original colony housing with cage mates.

A three-way mixed ANOVA was conducted to analyze the within-subjects effects of time and between-subjects effects of stress and zinc on average daily activity. Because Mauchly's test indicated that sphericity was violated for the repeated measures variable of time, degrees of freedom were corrected using the Greenhouse-Geiser estimates of sphericity ($\varepsilon = .129$).

Morris Water Maze

The Morris water maze (MWM) is designed to assess spatial memory, a function of the hippocampus. Animals learn the location of a hidden escape platform relative to four visual cues positioned around the pool area. A four-foot diameter pool, surrounded on all sides by white curtains with a distinctive cue on each side, was used for this task. This test has been used for many TBI studies in the past and has been found to be reliable and

valid (Hellmich et al., 2008; Zuckerman et al., 2017; Marschner et al., 2016; Deng-Bryant et al., 2016). The MWM task assessed for several variables of interest, including 1) latency, which is the average amount of time required by each animal to locate the escape platform, 2) the number of crossings over the platform target area, and 3) thigmotaxis.

Thigmotaxis is considered a measure of anxiety (Herrero et al., 2006), and is defined as the percentage of time spent swimming in the outermost 10% of the pool closest to the wall. Thigmotaxis is seen in mice due to the fact that when placed in a new environment, mice prefer to remain close to the walls instead of within the open field. However, this gradually decreases as time goes on. MWM has been used in numerous studies (Flinn et al., 2005; Railey et al., 2010; Santacruz et al., 2005) and while it is mildly stressful to mice for a short period of time, it also encourages them to reach the goal of finding the platform.

Testing took place over a period of 7 days. Training days 1, 3, and 5 consisted of 3 trials/day with a fixed position for the platform. The platform was approximately 1 cm under water so that it could not clearly be seen (i.e., the mouse must use visual cues around the pool to remember the platform's location). Days 2, 4, and 6 also entailed 3 trials/day, with the difference being that the third trial of the day uses an "Atlantis" platform. For the "Atlantis" probe, the platform is completely submerged (and therefore unreachable) in order to examine further spatial aspects of the reference memory task, as mice can now be observed crossing over the target area where the platform previously was. All trials persisted for 60 seconds, with a 45 second inter-trial interval. Day 7

consisted of one visual platform trial, in which the platform is shown above the water and a tall plastic flag is placed on the platform. This is to ensure that no motor or vision deficits were present, with any mice discovered to have deficits being removed from the study. One three-way mixed ANOVA was conducted to analyze each of the following MWM tasks: (1) latency time to platform, (2) number of platform crossings, and (3) percent of time spent near walls (outer 10% of arena).

Western Blots

After the completion of MWM testing, mice were sacrificed with CO2 for brain extraction. Between each cohort, all brains were harvested at approximately 5 P.M. to maintain consistencies in daily protein levels. The cerebellum and left hemisphere were removed from each brain, with the right hemisphere being kept and homogenized in a cell lysis buffer (20 mM Tris HCl solution, Halt™ Protease/Phosphatase Inhibitor Cocktail). Homogenized tissue samples were then centrifuged for extraction of liquid supernatant. A BCA assay was used to determine total protein needed from each sample to be used for gel electrophoresis. Individual samples were prepared using 2.5 μL NuPAGE reducing agent, 6.25 μL LDS buffer, pre-determined protein lysate volume, and PBS for a total loading volume of 25 μL into each well of NuPAGE 4-12% Bis-Tris gels in MOPS running buffer, and the proteins fractionated with a current of 120 V. SeeBlue Plus2 protein ladder (Thermo Fisher Scientific) was used to visualize molecular weight standard.

The following primary antibodies were used: proBDNF, mature BDNF, TrkB, and Phospho-TrkB. GAPDH and Beta-Actin were used as loading-control antibodies.

Each primary antibody was conjugated with a goat anti-rabbit secondary antibody. All primary and secondary antibodies have been tested and validated for use in Western blots by the supplier (Thermo Fisher Scientific). Membranes were agitated with West Pico Chemiluminescent Substrate (SuperSignal) and then imaged with an exposure time of 10 seconds, to be semi-quantified using ImageJ (NIH). Each lane of the blot was analyzed to generate a value for the density (i.e., intensity) of its region of interest. To calculate individual protein densities, a relative value was computed = (Protein Signal) / (Lane Signal). Relative proBDNF and mature BDNF densities were each standardized to the corresponding density of their loading-control β-Actin, while TrkB and Phospho-TrkB bands were similarly controlled by GAPDH = (Relative Density for Protein of Interest) / (Relative Density for Loading Control). One 2 (stress) x 2 (zinc) ANOVA was conducted to compare each of the following protein ratios: (1) Phospho-TrkB / Total TrkB, and (2) ProBDNF / Mature BDNF.

Zynpyr-1

Flash frozen brain tissue samples were sliced into 16 μm sections using a Leica CM3050S cryostat and then mounted onto charged slides. A 1 mM stock solution of Zinpyr-1 (Abcam) was prepared with 0.9% saline to create a 17μM solution for incubation of hippocampal tissue. Slides were imaged by an Olympus BX51 fluorescence microscope with an FITC cube. Semi-quantification of images were performed by ImageJ software. Total fluorescence was determined by measuring the integrated density for each sample's selected region of interest, and then corrected by subtracting the mean

fluorescence of the background: Corrected Total Fluorescence = Integrated Density – (Selected Area of Interest * Mean Fluorescence of Sample Background).

One 2 (stress) x 2 (zinc) ANOVA was conducted to compare total fluorescence between the four experimental groups.

Binding for Zinpyr-1 only responds to "free" zinc, the subset of zinc ions that are bound to weak, rapidly exchangeable ligands like Cl- and water. Although some complications have been pointed out (Marszalek et al., 2019), it does not normally bind to protein-bound zinc ions as in zinc fingers (Thompson & Fierke, 2017). The zinc was delivered as $ZnCO_4$—and may bind to proteins, among them the Src family kinases—but a full assessment of total zinc would require an analysis using, for example, synchrotron X-ray fluorescence.

RESULTS

Daily Activity

Although there were no changes in onset and offset time of running, there were changes in daily activity. Despite non-significant main effects for stress and zinc groups, there was an interaction of stress and zinc over time, $F(1.42, 56.82) = 3.610$, $p = .048$, with zinc treatment significantly increasing activity for stressed mice when compared to the control treatment (Figure 2). A within-subjects contrast also indicated a significant linear $F(1,40) = 4.138$, $p = .049$, and cubic, $F(1,40) = 5.414$, $p = .025$, interaction of stress and zinc.

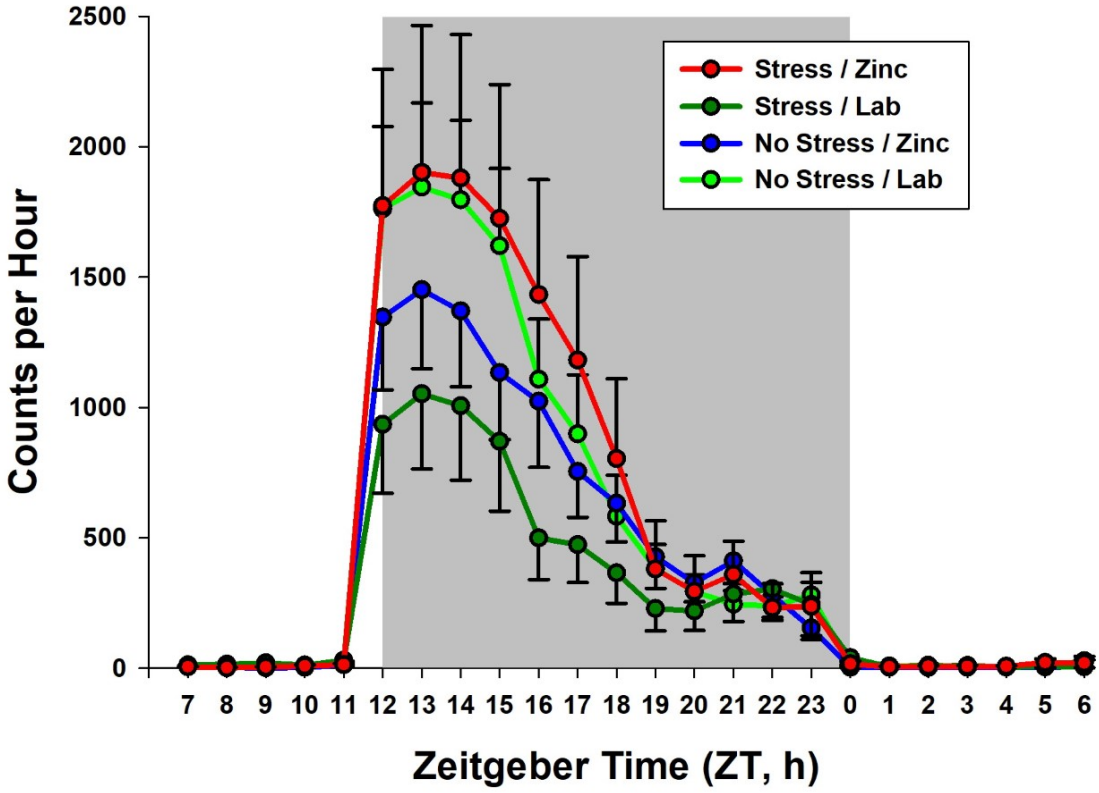

Figure 2 Hourly Running-Wheel Activity by Post-TBI Intranasal Treatment

Note. Hourly average count of running-wheel rotations for zinc- and vehicle-treated mice subjected to a protocol involving no stressors or chronic variable stress. In a 24-hour day, mice had a 12:12 light:dark cycle, with the dark phase shown in the shaded region (light offset at ZT 12).

Morris Water Maze

Our analysis revealed within- and between-subjects effects of stress and zinc on rmTBI mice while performing a Morris Water Maze paradigm for 7 days. The platform was fully submerged on Days 2, 4, and 6 for the third trial (probe). Following the probe trial on Day 6, 24 hours passed before testing on Day 7, in which only one trial was used to probe for navigation to the hidden platform.

Mice showed decreased latency in locating the maze platform over multiple days of training, $F(1,39) = 16.69$, $p < .001$, demonstrating spatial learning. However, time to locate the platform did not significantly differ between stressed and non-stressed mice, nor with zinc treatment and the vehicle control (Figure 3).

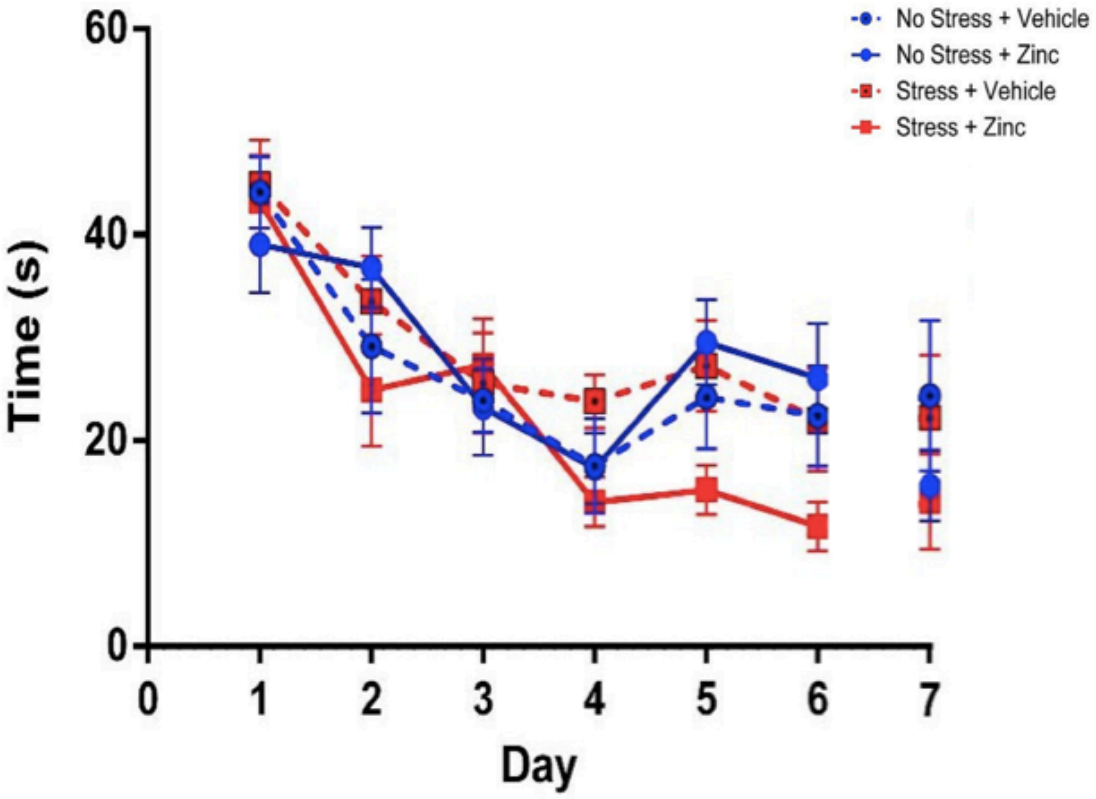

Figure 3 MWM Time to Platform

Note. Average time (s) to locate the platform in the Morris water maze (MWM). Days 1, 3, and 5 used a present but hidden platform for all three trials. Days 2, 4, and 6 removed the platform by fully submerging it for Trial 3. 24 hours later, Day 7 used one trial to probe for navigation to the hidden platform.

In the area of the pool containing the hidden platform, mice showed a significant increase in the number of platform crossings over time, $F(1,39) = 9.478$, $p < .001$. There was a significant interaction between stress and zinc, $F(1,39) = 4.207$, $p = .047$, as zinc treatment significantly increased the average number of crossings overall, but only in stressed mice (Figure 4).

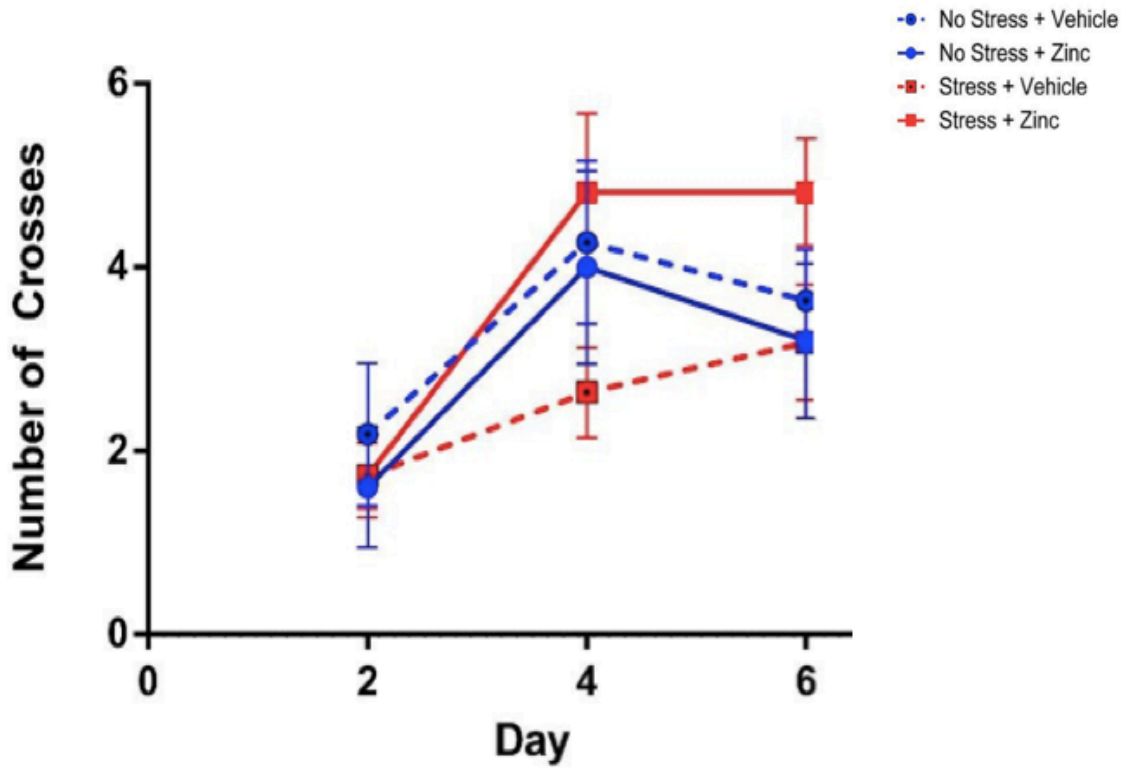

Figure 4 MWM Platform Crossings

Note. Average number of crossings over the Morris water maze (MWM) hidden platform area for probe trials (when platform was not present on Days 2, 4, and 6 on Trial 3).

An analysis for thigmotaxis showed that rmTBI mice had a decrease in the percentage of time spent near walls across probe trials, $F(1,39) = 16.053$, $p < .001$, as a possible index of reduced anxiety with subsequent days. There was a significant interaction between stress and zinc, $F(1,39) = 3.222$, $p = .034$, with stressed mice on zinc treatment spending the least amount of time near maze walls (Figure 5).

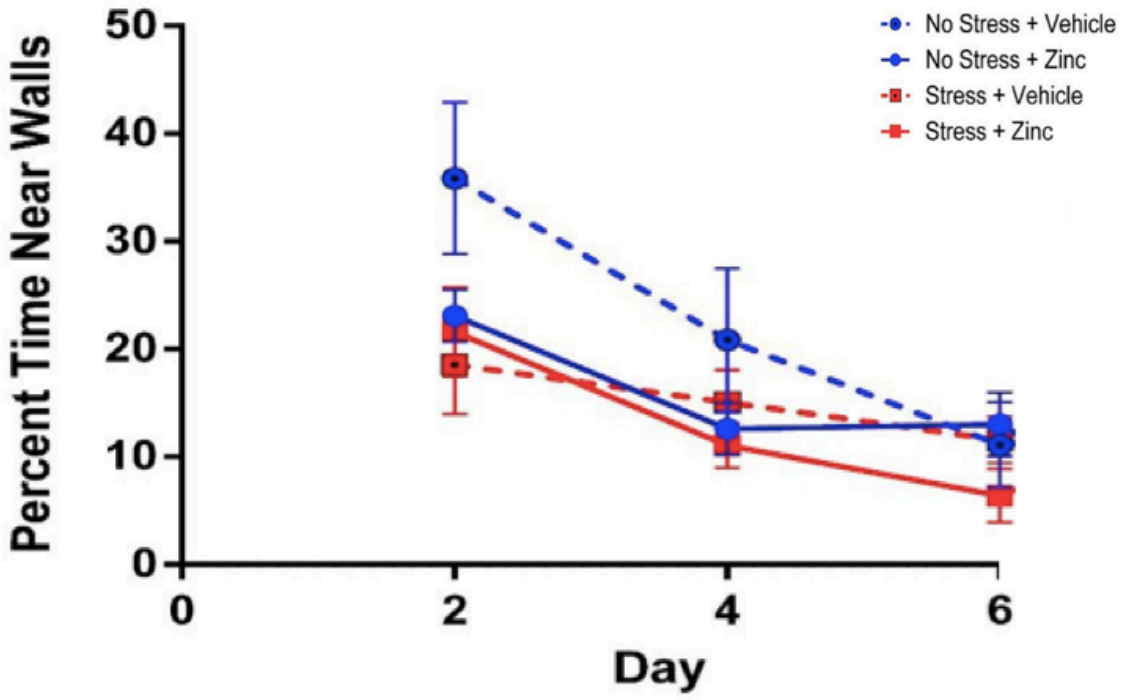

Figure 5 MWM Thigmotaxicity

Note. Percentage of time spent near Morris water maze walls (% = Time in Outer 10% of Pool / Total Time in Trial) during probe trials (when platform was fully submerged on Days 2, 4, and 6).

Free Zinc in the Brain

Mice that received the intranasal zinc solution had significantly more free zinc in the hippocampus (Table 2; Figure 6) than those that received the vehicle solution ($F(1, 12) = 337.79$, $p < .001$). Non-stressed mice also had greater hippocampal zinc than stressed mice ($F(1, 12) = 41.33$, $p < .001$). There was not a significant interaction effect.

Table 2 Means and Standard Deviations for Zinc Fluorescence (x 10^3)

	Vehicle		Zinc	
	M	(*SD*)	*M*	(*SD*)
Non-Stressed	560	(49)	1386	(160)
Stressed	265	(25)	1100	(64)

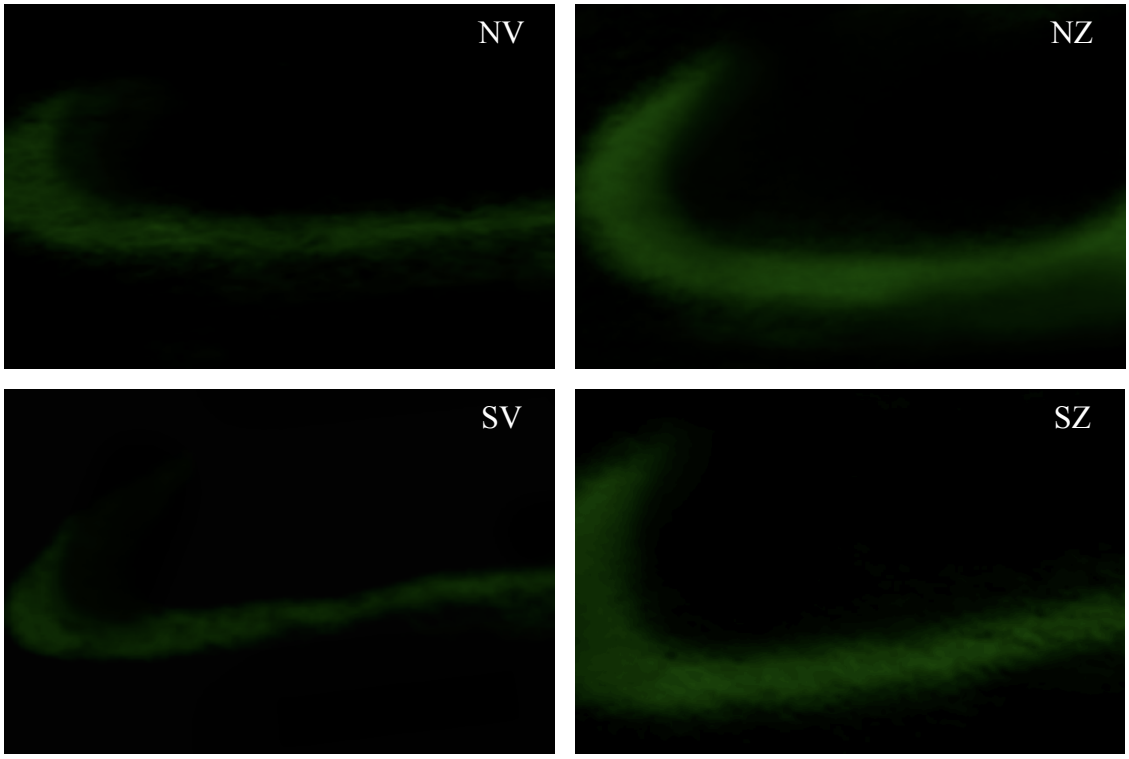

Figure 6 Hippocampal Free Zinc

Note. Zinpyr-1 staining in nonstress-vehicle (NV), nonstress-zinc (NZ), stress-vehicle (SV), and stress-zinc (SZ) mice in the hippocampal CA3 region. There were significant main effects ($p < .001$) for treatment and for stress on total fluorescence of Zn^{2+}.

Phosphorylated TrkB to Total TrkB

There was a significant effect of treatment with zinc increasing the relative amount of phosphorylation in TrkB receptors compared to the vehicle control, $F(1, 12) = 44.79$, $p < .001$ (Table 3; Figure 7). Stress conditions did not affect the number of phosphorylated receptors when examining at total TrkB levels.

Table 3 Densitometric Ratios for Phospho-TrkB/Total TrkB

	Vehicle		Zinc	
	M	(*SD*)	*M*	(*SD*)
Non-Stressed	0.44	(0.08)	0.86	(0.13)
Stressed	0.50	(0.04)	1.03	(0.24)

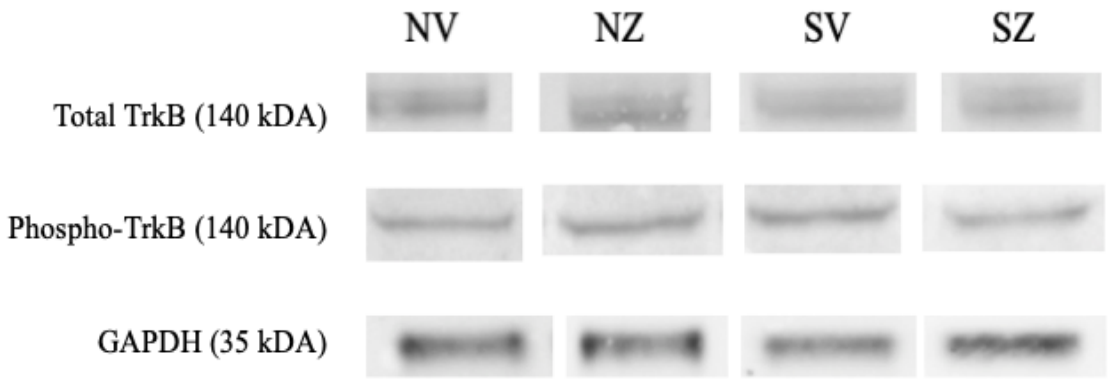

Figure 7 TrkB and Phosphorylated TrkB with GAPDH Control

Note. Western blot imaging of TrkB, Phosphorylated TrkB, and loading-control GAPDH antibodies in non-stressed vehicle-treated (NV), non-stressed zinc (NZ), stressed vehicle (SV), and stressed zinc (SZ) mice.

ProBDNF to Mature BDNF

Relative to the β-Actin loading control, stressed mice showed a higher level of the precursor molecule proBDNF, compared to mature BDNF, than non-stressed mice, $F(1, 12) = 9.365$, $p = 0.01$ (Table 4; Figure 8). Zinc treatment did not have a significant effect on proBDNF to mature BDNF levels compared to the vehicle control.

Table 4 Densitometric Ratios for ProBDNF/Mature BDNF

	Vehicle		Zinc	
	M	(*SD*)	*M*	(*SD*)
Non-Stressed	0.75	(0.18)	0.78	(0.23)
Stressed	1.04	(0.14)	1.10	(0.25)

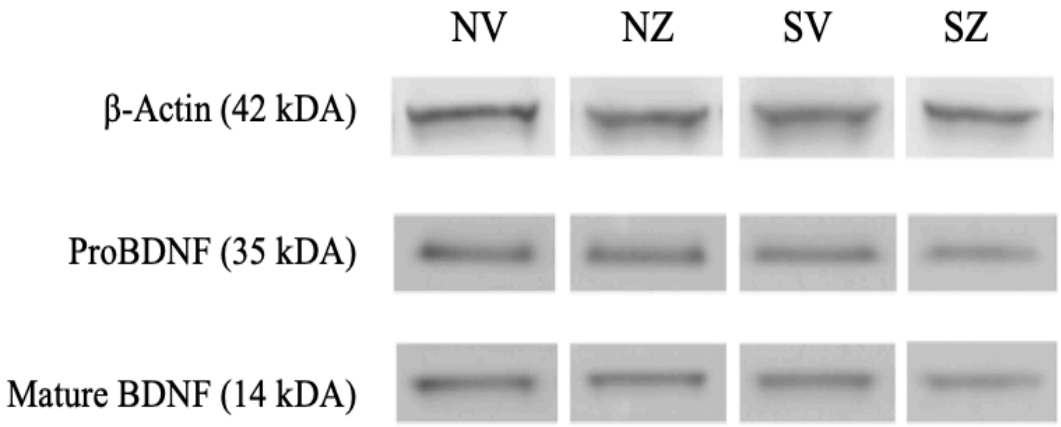

Figure 8 ProBDNF and Mature BDNF with β-Actin Control

Note. Western blot imaging of proBDNF, mature BDNF, and loading-control β-Actin antibodies in non-stressed vehicle-treated (NV), non-stressed zinc (NZ), stressed vehicle (SV), and stressed zinc (SZ) mice.

DISCUSSION

For each of the behavioral assays (daily activity and spatial memory) a significant interaction occurred between stress and zinc treatment over time. When comparing the stress conditions, post-injury zinc treatment yielded opposite effects for stressed and non-stressed mice. Post-mortem brain analyses revealed significant increases in hippocampal zinc concentrations and phosphorylated TrkB receptors for mice given zinc treatment, while stress decreased BDNF. Implications for all behavioral and brain analyses are discussed as follows.

Daily Activity

There were no changes in onset and offset time for wheel running, and thus no change in CR. There were, however, changes in daily activity, which have been shown to be a good predictor of cognitinve function: those with the highest levels of activity having the best outcome for cognitive performance (Tranah et al., 2011). For rmTBI mice that were administered a vehicle control, stress reduced the number of running-wheel rotations during the dark cycle compared to the non-stressed group. When intranasal zinc was administered, the deficit seen for stressed mice was remediated, as wheel-running activity nearly doubled in the first hour of light offset. Horwever, for the non-stressed group, the rmTBI mice demonstrated a significant reduction in wheel-running activity following zinc intervention.

The relationship between zinc and behavior is complex, both zinc deficiency and an excess of zinc can lead to impairments in behavior, and both zinc chelation and zinc

supplementation have been used to reduce the impairments associated with TBI (Levenson, 2020). In this experiment, zinc caused a reduction in activity compared to the mice given a vehicle control after rmTBI when no stress was present, although this difference was not significant. This reduction is somewhat surprising as other studies have reported that zinc administration had a positive effect following TBI (Cope et al. 2012). However, Frederickson et al. (2004) reported that zinc chelation was beneficial following head trauma.

As TBI is known to induce depletion of zinc stores, it should be noted that stress has comparable effects on zinc concentrations (Lopresti, 2020). Rats subjected to acute or chronic stress (i.e., restraint) had significantly lower blood zinc concentrations compared to non-stressed rats; repeated stress exposure has also been linked to decreased zinc concentrations in the rat hippocampus (Teng et al., 2008; Tao et al., 2013). Administering just one hour of restraint stress induced zinc release from the hippocampus, with a 167% increase during the first 15 minutes (Itoh et al., 1993), and a novel stressor (30-second tail suspension), "elicited a persistent decrease in extracellular zinc, which continued for 60 minutes" (Takeda et al., 2009).

Our data are consistent with these studies: stress reduced the levels of zinc in both conditions for vehicle and zinc administration. In the case of the stress + vehicle group, zinc levels were the lowest of all the four groups, thus the addition of the zinc may be returning the brain to more normal values, and an increase in activity. The non-stressed + zinc condition had the highest values of zinc, possibly higher than optimal, and a reduction in activity was seen. Persson et al. (2003) noted that zinc concentration was

significantly increased following intranasal delivery compared to intraperitoneal. In addition, administration of zinc through the oral route may be a poor method for administering zinc treatment (Cope et al.,2012), thus the amount of zinc received in the brain may be higher in the case of intranasal injection, leading to an inappropriately high level of zinc in cases of low stress.

Our results showed that the effect of zinc varied with the stress condition: the stressed group benefited from intranasal zinc, while the non-stressed animals experienced the opposite effect. Therefore, in this rmTBI model, repeated zinc treatment mitigated daily activity irregularities when chronic stress was present.

Morris Water Maze

Results of the MWM paralleled this interaction effect when examining the outcomes of zinc on stressed and non-stressed animals. Stressed mice with zinc treatment swam within the hidden platform area more than any other group. An increase in platform-crossing activity suggested that treatment with zinc augmented spatial memory for stressed mice.

Stressed-zinc mice also spent the least amount of time near the walls of the maze when compared to all other groups. This decreased thigmotaxis can serve as an index of reduced anxiety (Higaki et al., 2018). In the case of the stressed mice, this may be attributed to having repeated exposure to similar stressors during adolescence, which would allow these mice to develop an adaptive response to a stressful testing environment. A similar study, with a repeated 14-day stressor in juvenile mice, found decreased time spent immobile during the forced swim testing (Sadler & Bailey, 2016).

In contrast, our non-stressed mice given the vehicle control spent the highest percentage of time near maze walls. The observed thigmotaxis in the non-stressed group suggests heightened anxiety, as these mice were not previously exposed to a stress-inducing environment.

It was surprising that no effects in latency were seen as a result of stress or zinc as all groups learned the maze well despite having received TBI; this may have been due to the 4 ft diameter of the maze. Such a maze does show impairments in transgenic mice with neurological conditions, such as Alzheimer's disease (Craven et al., 2018), but not in wildtype mice on or off zinc supplementation.

Brain Analyses

In the brain, Zinpyr-1 staining corroborated the binding of zinc in the hippocampus of mice that were administered intranasal zinc. Additionally, this confirmed that stressed mice had depleted zinc stores in the brain, when contrasted with non-stressed mice.

Western blots also verified that zinc treatment alone correlated with an increase in Trk-B receptor phosphorylation. However, there was no association of zinc treatment on BDNF levels, a known mediator for Trk-B activation. When examining the main effect of the stress condition, Trk-B receptors were not affected, though chronic stress did lead to a decrease in BDNF. This effect of stress on BDNF was also reported in a 2019 study that used an equivalent model of chronic unpredictable mild stress, and saw a reduction of BDNF protein levels in the mouse frontal cortex and hippocampus (Szewczyk et al.).

The upregulation in phosphorylated receptors would indicate intranasal zinc is able to activate the Trk-B pathway independently of BDNF. Such a mechanism is

consistent with that of Huang et al. (2008), which asserted zinc is taken up into the presynaptic terminal of pyramidal cells in the hippocampus. Zinc would then activate a Src family kinase—as opposed to BDNF—and promote Trk-B activation (phosphorylation). However, it is known that increased release of mature BDNF can increase phosphorylated TrkB levels without requiring an increase in BDNF mRNA and protein synthesis (Marini et. al, 1998). Additional investigation is needed to determine a causal relationship between increased TrkB activation and zinc treatment following TBI.

Role of Zinc

Several animal studies have modeled zinc's vulnerability to psychological stress (Tao et al., 2013; Teng et al., 2008; Itoh et al., 1993; Takeda et al., 2009). Losses of zinc following TBI can be detected acutely, with changes in serum zinc and urinary zinc excretion being detected within the same day of injury (McClain et al., 1986). One TBI alone leads patients to experience heightened urinary zinc losses for several weeks post-injury, resulting in persistent decreases of serum zinc (Cope et al., 2012). As zinc depletion is cumulative and proportionate to the amount of damage incurred to the brain, it follows that our model of repeated injuries and chronic stressors would induce a major reduction in zinc over time, compounding with each successive injury or stressful event. This puts patients at risk for the development of a harmful zinc deficiency. However, acute changes in zinc levels can also be observed after supplementation (Young et al., 1996). For this reason, administering zinc supplementation during the acute post-injury window likely served as a favorable intervention for alleviating severe zinc losses, particularly if stress is present.

In one of the earliest studies on zinc and brain injury, Young et al. (1996) confirmed that post-injury zinc supplementation successfully recovered neurological function in patients with severe TBI. A more recent study found improved Glasgow Coma Scale outcomes, reduced organ failure and inflammation, and increased zinc concentrations in zinc-supplemented patients (Khazdouz et al., 2018).

Taken together, we surmise that zinc intervention may help to restore hippocampal functioning and zinc homeostasis that was previously compromised by both TBI and stress. In our study, the most notable effect of zinc-based activation was seen in stressed mice, i.e., the condition is predicted to yield greatest cumulative zinc loss after a combination of rmTBI and CVS. For this reason, the results suggest that the restorative effect of post-injury zinc is most profound when TBI-induced zinc depletion has been further exacerbated by chronic stress.

The results of our study support the use of intranasal zinc to help regulate daily activity in groups that experience the largest zinc imbalances, i.e., following rmTBI and chronic stress. The translational value of our work suggests that individuals with TBI be assessed for damaging zinc loss and its associated side effects, and then treated using supplementation within reason.

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