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Research Paper

Effect of zinc supplementation on neuronal precursor proliferation in the rat hippocampus after traumatic brain injury

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ABSTRACT

There is great deal of debate about the possible role of adult-born hippocampal cells in the prevention of depression and related mood disorders. We first showed that zinc supplementation prevents the development of the depression-like behavior anhedonia associated with an animal model of traumatic brain injury (TBI). This work then examined the effect of zinc supplementation on the proliferation of new cells in the hippocampus that have the potential to participate in neurogenesis. Rats were fed a zinc adequate (ZA, 30 ppm) or zinc supplemented (ZS, 180 ppm) diet for 4 wk followed by TBI using controlled cortical impact. Stereological counts of EdU-positive cells showed that TBI doubled the density of proliferating cells 24 h post-injury ($p < 0.05$), and supplemental zinc significantly increased this by an additional 2-fold ($p < 0.0001$). While the survival of these proliferating cells decreased at the same rate in ZA and in ZS rats after injury, the total density of newly born cells was approximately 60% higher in supplemented rats 1 wk after TBI. Furthermore, chronic zinc supplementation resulted in significant increases in the density of new doublecortin-positive neurons one week post-TBI that were maintained for 4 wk after injury ($p < 0.01$). While the effect of zinc supplementation on neuronal precursor cells in the hippocampus was robust, use of targeted irradiation to eliminate these cells after zinc supplementation and TBI revealed that these cells are not the sole mechanism through which zinc acts to prevent depression associated with brain injury, and suggest that other zinc dependent mechanisms are needed for the antidepressant effect of zinc in this model of TBI.

1. Introduction

Depression is the most common life-long complication of traumatic brain injury (TBI), affecting as many as 40% of hospitalized patients (Jorge and Starkstein, 2005). Unfortunately, the increased risk of depression is not limited to those with severe brain injuries. It also affects patients with milder forms of TBI such as concussion (Levin et al., 2005). As a result, classical antidepressant drugs are often prescribed to TBI patients suffering from depression. However, an important report cautioned that the currently available antidepressant medications may be ineffective in treating TBI-associated depression (Fann et al., 2009).

Despite the well-recognized problem of depression in TBI patients, most of the work to date, both clinical and pre-clinical, has focused on the treatment of cognitive deficits after TBI, with little attention to the potentially debilitating effects of depression. For example, when patients with moderate to severe TBI were given supplemental zinc, not only was there improved visceral protein status, but there was also significant improvements in Glasgow Coma Scale scores (Young et al., 1996). Additionally, animal work has shown that zinc specifically targets hippocampally-mediated behavioral outcomes associated with brain injury, improving depression-like behaviors and spatial learning and memory (Cope et al., 2011).

The hippocampal formation, specifically the subgranular zone of the dentate gyrus, is one of the two areas of the brain known to continuously give rise to adult stem cells (Eriksson et al., 1998). These adult-generated proliferative cells have been shown to differentiate into all types of neural cells, with the majority becoming neurons that incorporate into the hippocampal circuitry (Gage, 2000; van Praag et al., 2002). Several studies have reported an increase in stem cell proliferation and neuronal differentiation in the dentate gyrus after TBI (Dash et al., 2001; Gao et al., 2009a; Sun et al., 2007). While the function of these neuronal

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precursor cells, particularly in regulating behavior, is currently being vigorously debated in the literature (Lazic et al., 2014; Yu et al., 2016), it has been hypothesized that proliferative cells in the hippocampus and adult neurogenesis participate in the regulation of mood and may open new doors to the treatment of depression (Snyder et al., 2011).

We have already shown that zinc may be an effective approach to prevent the behavioral deficits associated with brain injury (Cope et al., 2011). While the mechanisms of zinc action are not fully understood, there is mounting evidence that zinc regulates adult-born cells of the hippocampus. Several reports have now shown that zinc deficiency decreases stem cell proliferation, survival, and neuronal differentiation (Corniola et al., 2008; Gao et al., 2009b; Gower-Winter et al., 2013, Xu et al., 2011). Additionally, we know that antidepressant drugs stimulate proliferation of cells in this area of the brain (Malberg et al., 2000). Thus, using zinc supplementation, a well-established model of TBI, and targeted hippocampal irradiation, this work was designed to directly test the hypotheses that zinc supplementation enhances TBI-induced cell proliferation in the hippocampus and that the ability of zinc supplementation to prevent TBI-induced anhedonia is dependent on these neuronal precursor cells. We also examined the role of zinc in the survival of new cells after TBI, as well as the ability of zinc to induce neuronal differentiation.

2. Materials and methods

2.1. Animal care

Young adult 6 wk old male Sprague–Dawley rats (Charles Rivers Laboratories, Wilmington, MA) were individually housed in temperature-controlled rooms with a 12 h light-dark cycle. Animals were fed a commercially prepared, semi-purified diet using zinc carbonate as the zinc source (Research Diets Inc., New Brunswick, NJ). Rats were randomly assigned to a zinc supplemented (ZS, 180 ppm) or zinc adequate (ZA, 30 ppm) diet for 4 wk prior to TBI and throughout the behavioral testing period. The Florida State University Animal Care and Use Committee (IACUC) approved all animal experiments (#1317).

2.2. Traumatic brain injury

At the end of the 4 wk dietary period, TBI was induced by a controlled cortical impact (CCI) to the medial frontal cortex. Prior to CCI, rats were anesthetized using isoflurane gas. Aseptic techniques were used throughout the surgical procedures and body temperatures were maintained using a homeothermic blanket. Following stereotaxic placement and a midline incision, a 6 mm diameter mid-sagittal bilateral craniotomy 3 mm rostral to bregma was performed. A 5 mm diameter pneumatic cortical contusion device (MyNeuroLab, Inc., Richmond, IL) was used to produce a 3 mm deep contusion using an impact velocity of 2.25 m/s and an impact time of 500 ms (Cope et al., 2011, 2012). Following TBI, the incision was immediately sutured. Additional sham-operated controls underwent the anesthesia and received the midline incision but not the craniotomy or impact. Animals from both the TBI and Sham-operated groups were fed either the ZA or ZS diet described above.

2.3. Cranial irradiation

A cranial irradiation paradigm was used to suppress adult hippocampal neurogenesis according to previously described methods (Snyder et al., 2005; Winocur et al., 2006). Prior to the TBI, rats (n = 9–10/group) were anesthetized with isoflurane gas and placed into a stereotaxic device inside the irradiator chamber. Immediately following the first day of irradiation, rats were subjected to a TBI using the methods described previously. For 2 consecutive days, rats were exposed to 10 Gy of irradiation (IRR rats) for a period of 10 min at 250 kV, 12 mA, and 63 FSD via an X-RAD 320 self-contained irradiation system (Precision X-Ray, North Branford, CT). An additional group of rats (n = 8–9/group) were only given a TBI and anesthesia, but not the irradiation (TBI rats). Two devices were used to focus the irradiation to the hippocampus. First, using stereotaxic placement, an adjustable collimator was focused directly over the hippocampus (Paxinos and Watson, 1998). Secondly, a half-inch thick, custom-made lead shield was used to block any scattered irradiation to the rest of the body and other brain regions.

2.4. EdU labeling

At 1 h and 5 h post-TBI, animals were injected with 50 mg/kg EdU (in 0.9% saline, 0.007 N NaOH) to label proliferating newly born cells. Rats (n = 3/group) were perfused 24 h after injury to examine cell proliferation. An additional group of rats (n = 3/group) were perfused 7 d following injury to examine survival of the labeled cells. Rats were deeply anesthetized and brains perfused via the left ventricle with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), pH 7.4. Brains were post-fixed in PFA for 48 h followed by sucrose cryoprotection for 48 h. 40 μm thick coronal sections were collected by microtome (Microm HM 430; Thermo Scientific, Waltham, MA). EdU-positive cells were visualized using the manufacturer's protocol (Invitrogen, Carlsbad, CA). Briefly, the slices (4–6/animal) were washed in PBS to remove the fixative solution and blocked twice with 3% bovine serum albumin (BSA). Slices were then permeabilized with 0.5% Triton X-100 in PBS for 20 min and blocked again with BSA. To detect EdU, sections were incubated for 30 min protected from light with Click-it EdU reaction cocktail containing Click-It reaction buffer, $CuSO_4$, Alexa Fluor azide 488, and reaction buffer additive. For DNA staining, slices were washed with PBS and then incubated with 5 μg/mL Hoechst 33342 solution for 30 min.

2.5. Immunohistochemistry

Four weeks following TBI and cranial irradiation, rats were perfused using methods described above. Coronal tissue sections (40 μm) were collected throughout the dorsal hippocampus. Tissue sections (4–6/animal) were washed in PBS, permeabilized in 0.5% Triton X-100 in PBS, and blocked for 1 h in 3% BSA. They were then incubated for 2 h at 37 °C with rabbit anti-human doublecortin polyclonal IgG (Abcam, Cambridge, MA) or a mouse anti-CD68 monoclonal antibody (ED-1; Abcam, Cambridge, MA). After washes with PBS and blocking in BSA, slices were incubated overnight at 4 °C with anti-rabbit secondary antibody labeled with the fluorescent dye Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA). Slices were then washed in PBS and incubated with 10 μM of Hoechst 33342 (Sigma-Aldrich, St. Louis, MO) in PBS for 30 min.

2.6. Confocal microscopy

To measure co-localization of EdU-positive and DCX-positive cells, Z-stacks were captured with a Nikon A1 inverted confocal microscope equipped with a 40×/NA 0.75 objective. The percentage of EdU-labeled cells that co-express with DCX was analyzed using NIS-Elements software.

2.7. Unbiased stereology

EdU and DCX were visualized and recorded by microscopy (Leica DM5000B, Buffalo Grove, IL), and quantified in the dentate gyrus using unbiased stereology. The subgranular zone, granular cell layer, and hilar regions of the dentate gyrus were defined in accordance with the rat brain atlas of Paxinos and Watson (1998). EdU+ and DCX+ were estimated using the optical fractionator technique assisted by a computer-based system (not investigator blinded), StereoInvestigator software version 9.13 and 11.03.1 (MicroBrightfield, Inc., Williston,

VT) using methods similar to those described previously (Bregy et al., 2012). Cells throughout the rostro-caudal extent of the hippocampus were counted. For each saline-mounted section, the reference space (contour) was delineated by outlining at low power (5× objective); identification of EdU- or DCX-positive cells was accomplished at high power (40× objective). A 120 × 120 μm counting grid was placed over the outlined contour, and a 60 × 60 μm counting frame was used to count the randomly placed sampling sites. Mounted section thickness was 40 μm and the guard zone height for each section was 10 μm. Cell densities (estimated number of cells/mm^3) were calculated by dividing the number of estimated EdU- or DCX-positive cells by the total volume sampled of the contour. Representative hippocampal slices were imaged at 10× or 40× using a Nikon TE-2000 E2 eclipse C-1si with a Cool SNAP HQ2 monochrome camera (Nikon Instruments, Inc, Melville, NY).

2.8. Saccharin preference

As a measure of depression-like behavior, a standard two-bottle choice paradigm (not investigator blinded) for saccharin preference was used to examine the role of adult-born cells on the development of anhedonia after traumatic brain injury (Cope et al., 2011, 2012). Before the start of the experiment, rats (n = 8–10/group) were habituated to drink water from two bottles. One week following injury, rats were given a choice between saccharin and deionized water for a 4-day period; 2 days at 0.025% saccharin followed by 2 days at 0.05% saccharin. Intake of water and saccharin was measured daily. To avoid preferences associated with bottle placement, the position of the bottles were changed daily.

2.9. Statistical analysis

Graph Pad Prism 6 (Graph Pad Software, Inc., San Diego, CA) was used for the statistical analyses and graph preparation. All data sets are expressed as the mean ± SEM and statistically significant at $p < 0.05$ with 95% confidence. Data analysis was conducted using a one-way ANOVA comparison of groups followed by a Newman Keuls *post-hoc* comparison or two-way ANOVA.

Employing the GLM procedure in SAS Software version 9.4, we used linear regression to model saccharin preference and investigate its relationship to TBI, zinc treatment, and irradiation. Indicators of TBI, treatment, and irradiation were included as main effects. Pairwise interactions between treatment and TBI and between treatment and irradiation were also included in the initial model. We used an F test to formally test whether the interaction terms improved the model.

3. Results

3.1. Effect of zinc supplementation on proliferation

To examine cell proliferation, we injected rats fed a zinc adequate or a zinc supplemented diet with the thymidine analogue ethynyl deoxyuridine (EdU) at 1 h and 5 h following TBI (Fig. 1A) and evaluated EdU-positive cells in the subgranular zone and hilus of the dentate gyrus (Fig. 1B). Stereological quantification of EdU-positive cells revealed that TBI resulted in a significant increase in cell proliferation 24 h after injury, doubling the density of EdU-labeled cells in the dentate gyrus compared to ZA sham-operated controls ($p < 0.05$, Fig. 1C). Fig. 1 also shows that 4 wk of dietary zinc supplementation significantly increased (2-fold) hippocampal cell proliferation in the absence of injury ($p < 0.01$). Analysis of the mean cell count data also showed a significant increase in cell number (45%) independent of hippocampal volume ($p < 0.05$). When TBI animals were supplemented with zinc, this combination resulted in an additional increase that was approximately 2-fold ($p < 0.001$) greater than injured animals that were fed the ZA diet and over 3 times the density found in uninjured ZA sham controls ($p < 0.0001$, Fig. 1C). Likewise, analysis of the data using mean total counted cells, independent of volume, produced the same outcomes, with ZS TBI having

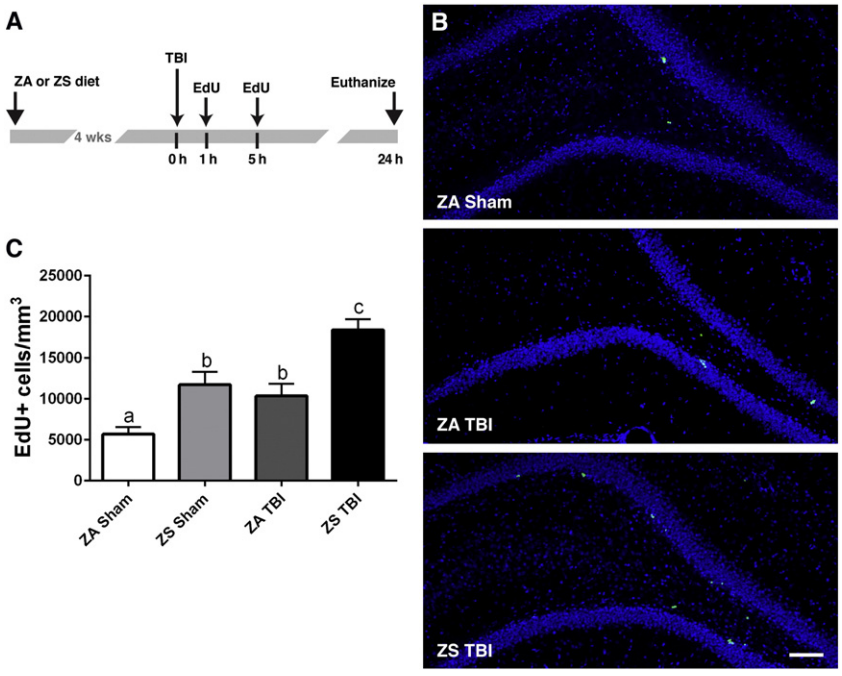

Fig. 1. Effect of zinc supplementation on cell proliferation after TBI. (A) Rats were fed a zinc supplemented (ZS) or zinc adequate (ZA) diet for 4 wk and then subjected to a traumatic brain injury (TBI) or sham surgery (Sham). At 1 h and 5 h post-TBI, rats were injected with EdU to label proliferating cells. Twenty-four hours after TBI, the number of proliferating cells in the dentate gyrus were visualized and quantified by stereology. (B) Representative hippocampal sections showing the subgranular zone and hilus of the hippocampus 24 h post-TBI in ZA Sham, ZA TBI, and ZS TBI rats. EdU (green), Hoechst 33342 (blue). Scale bar = 100 μM. (C) Stereological quantification of EdU-positive cells (cells/mm^3). Bars represent mean ± SEM. Bars with different letters are significantly different. a *vs.* b, $p < 0.05$; a *vs.* c, $p < 0.001$; b *vs.* c, $p < 0.01$.

significantly higher cell numbers compared to all other treatment groups. Mean cell counts are reported in Supplemental Table 1.

3.2. Effect of zinc supplementation on EdU + cell survival

To assess the long-term fate of these proliferating hippocampal cells, an additional group of rats was euthanized 1 wk after brain injury (Fig. 2A). There were no differences in cell densities (cells/mm^3) between zinc supplemented and zinc adequate sham (uninjured) controls (Fig. 2C). The density of EdU-positive cells was approximately 60% higher in ZS TBI compared to TBI rats fed the zinc adequate diet ($p < 0.05$, Fig. 2C). This difference was maintained when data were analyzed independently of hippocampal volume (Supplemental Table 1, $p < 0.05$).

These differences led us to compare the density of EdU-positive cells at 24 h and 7 d after TBI as an indicator of the survival of newly born cells. When animals were fed the ZA diet, 68% of the EdU+ cells survived to day 7 post-injury. Similarly, in animals fed the ZS diet, there was a 61% cell survival rate after injury. Despite these comparable survival rates, supplemented rats (ZS TBI) still had a significantly larger total density of EdU-labeled cells at day 7, compared to ZA TBI rats (11,085 ± 1372/mm^3 *vs.* 7033 ± 830/mm^3; $p < 0.05$).

3.3. Effect of zinc supplementation on TBI-induced neurogenesis

We used DCX-labeling to examine the extent to which supplemental zinc enhances neurogenesis after TBI. Immunohistochemistry at day 7 revealed that on average, 85% ± 4 of EdU+ cells were also DCX+ and that this density was consistent in all groups (no effect of diet or injury on percent DCX expression) (Fig. 3). However, Fig. 4 shows that when TBI followed zinc supplementation, there was a 37% increase in DCX-labeled cells in the dentate ($p < 0.01$) 7 days after injury. Furthermore, zinc supplementation alone significantly increased DCX expression when compared to all other groups (Fig. 4).

3.4. Targeted irradiation inhibits hippocampal neurogenesis

Fig. 5 shows that our method of targeted irradiation of the hippocampus successfully eliminates hippocampal neurogenesis by more than 90% ($p < 0.0001$, Fig. 5), an effect that lasts for at least 4 wk post-TBI. Irradiation did not alter mean body weights nor did it induce detectable expression of the inflammatory marker CD86 (ED-1) in the hippocampus at 7 days post irradiation (data not shown).

3.5. Role of neuronal precursor cells in TBI-associated depression-like behavior

As shown in Fig. 6A, elimination of newly born cells in the hippocampus was accomplished by irradiation prior to TBI and at 24 h post-TBI. Open field testing revealed no difference in line crossing in any group (Supplemental Fig. 1). Additionally animals were subjected to the Porsolt forced swim test that measures the amount of time spent immobile in a swim tank, to evaluate the responsiveness to antidepressant drugs (Slattery and Cryan, 2012). This test, as illustrated in Supplemental Fig. 2, is not appropriate for this model of TBI because animals with TBI displayed significantly increased swimming behavior and almost no immobility.

Fig. 6B illustrates the level of saccharin preference for each level of injury, zinc supplementation, and hippocampal irradiation status. Using multiple linear regression to model saccharin preference, the main effects of zinc treatment, injury, and irradiation status were all included, as well as interaction effects between zinc treatment and injury and between zinc treatment and irradiation status. The interactions terms did not significantly improve the model ($p = 0.6984$); consequently the interaction terms were removed and the model was refit with only main effect terms. Fitting the model with only main effects showed that after controlling for injury and irradiation, zinc supplementation was associated with higher saccharin preference ($p = 0.0013$). Similarly, even after controlling for zinc status, the elimination of proliferating cells in the hippocampus by irradiation is associated with a decrease in saccharin preference ($p = 0.0059$).

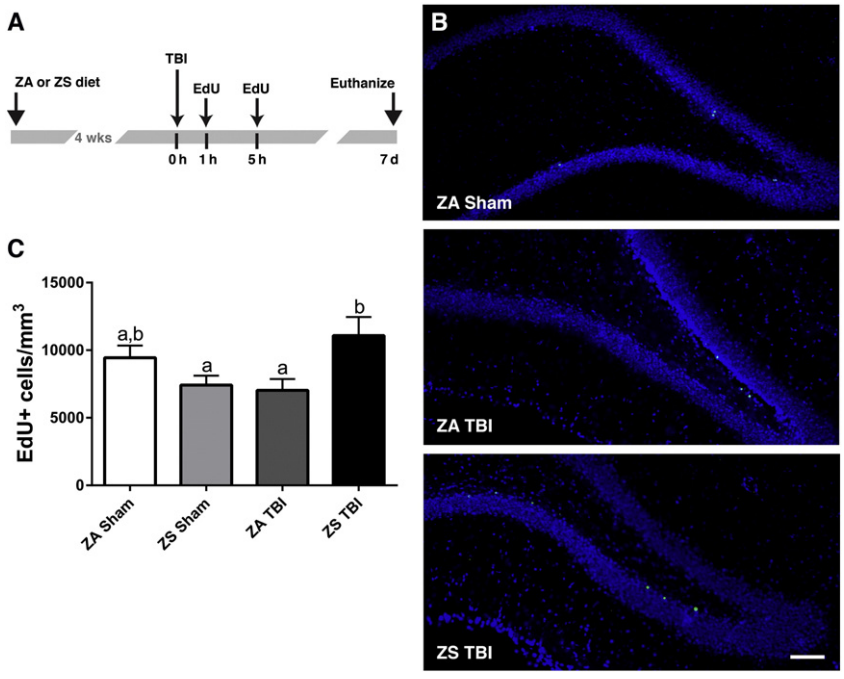

Fig. 2. Effect of zinc supplementation on cell survival after TBI. (A) Rats were fed a zinc supplemented (ZS) or zinc adequate (ZA) diet for 4 wk and then subjected to a traumatic brain injury (TBI) or sham surgery (Sham). At 1 h and 5 h post-TBI, rats were injected with EdU to label proliferating cells. One wk after TBI, the number of newly born cells in the dentate gyrus were visualized and quantified by stereology. (B) Representative sections of ZA Sham, ZA TBI, and ZS TBI rats. EdU (green), Hoechst 33342 (blue). Scale bar = 100 μM. (C) Stereological quantification of EdU-positive cells (cells/mm^3). Bars represent mean ± SEM. Bars with different letters are significantly different. a *vs.* b, $p < 0.05$.

4. Discussion

A role for zinc in the prevention and treatment of depression is supported by clinical work showing that daily zinc can be used to augment the efficacy of antidepressant drug therapy in patients with major depression (Nowak et al., 2003; Siwek et al., 2009). In humans, anhedonia, characterized by a decrease in the ability to experience pleasure, is a well-recognized symptom of depression (Kessler et al., 1994). In pre-clinical models, the development of depression-like behaviors can be monitored using the 2-bottle saccharin (or sucrose) preference test for anhedonia. Using this method, a reduction in saccharin preference is indicative of anhedonia (Katz, 1981). Recent findings, confirmed in the current report, show that chronic zinc supplementation prevents TBI-associated depression-like behaviors in a pre-clinical model (Cope et al., 2011). This is particularly significant because not only is depression the single most common consequence of all forms of TBI (Jorge and Starkstein, 2005), but TBI-associated depression also appears to be particularly resistant to antidepressant drug therapy (Fann et al., 2009).

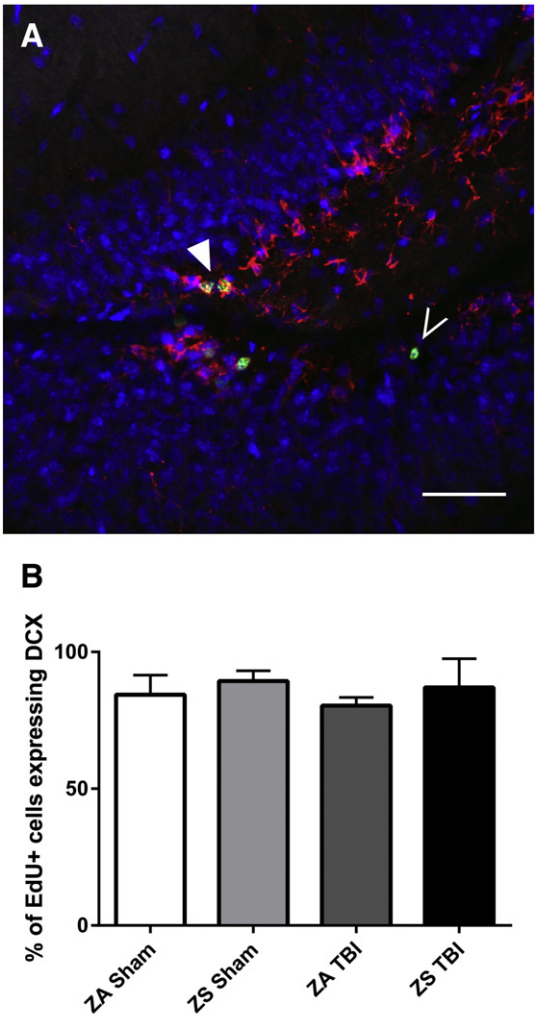

Fig. 3. Effect of zinc supplementation on the percentage of EdU-positive cells that express doublecortin (DCX) after TBI. (A) Rats were fed a zinc supplemented (ZS) or zinc adequate (ZA) diet for 4 wk and then subjected to a traumatic brain injury (TBI) or sham surgery (Sham). At 1 h and 5 h post-TBI, rats were injected with EdU to label proliferating cells. One wk after TBI or Sham, brain sections were labeled and immunostained with EdU (green), doublecortin (DCX, red), and Hoechst 33342 (blue). In the representative section, the closed arrowhead indicates EdU-positive cells co-localized with DCX. The open arrowhead indicates an EdU-positive cell not labeled with DCX. Scale bar = 50 μM. (B) Bars represent mean ± SD.

Unfortunately, there is almost no information about how zinc acts to prevent depression in humans or animals. To address this gap in knowledge, we hypothesized a role for the proliferating neuronal precursor cells that reside in the subgranular zone and hilar region of the dentate gyrus. The rationale for targeting these cells for study in our model of TBI is based on the hypothesis that inhibition of neurogenesis leads to depression (Snyder et al., 2011), while factors that increase adult neurogenesis such as antidepressant drugs, exercise, and environmental enrichment have all been associated with reductions in depression-like behaviors (Malberg et al., 2000; Schloesser et al., 2010; Yau et al., 2011). While there is published evidence to support the dependence of antidepressant drug efficacy on this increase in neuronal precursor proliferation (Santarelli et al., 2003), a recent meta-analysis of studies concluded that there is a negligible contribution of neurogenesis to behaviors, suggesting that other factors significantly outweigh the role of newly born neurons (Lazic et al., 2014). Thus, this study not only sought to examine the effect of zinc supplementation on newly born cells and their differentiation into neurons in the hippocampus in a model of TBI, but also to use targeted irradiation to specifically address the possible role of these cells in the zinc-induced reductions in TBI-associated depression-like behaviors.

4.1. Effect of zinc on cell proliferation and survival

We have known for some time that brain injury produces an increase in newly born cells in the dentate gyrus (Dash et al., 2001; Choi et al., 2014). The work reported here supports this finding by showing that 24 h after TBI there was a significant increase in the density of proliferating cells. At day 7 post-injury the percentage of these cells that express DCX, an early marker of neuronal commitment, was not different in the treatment groups.

In addition to TBI-mediated increases in newly-born hippocampal cells, the data reported here show that chronic zinc supplementation prior to TBI significantly enhances the density of proliferating cells. Not only did zinc increase the density of adult-born cells 24 h post-injury, supplementation also significantly increased the density of cells independently of injury (i.e. in uninjured animals). This is consistent with previous work, using a model of zinc deficiency, showing that adequate zinc availability is needed for neural stem cell proliferation and neurogenesis (Corniola et al., 2008; Suh et al., 2009; Choi et al., 2014) and the zinc-regulation of specific molecular mechanisms associated with neuronal proliferation and survival both in vivo and in vitro (Gower-Winter et al., 2013; Seth et al., 2015).

Despite the early burst in cell proliferation after TBI, others have documented TBI results in a reduction in new cells compared to uninjured controls at the 2 wk time point following injury (Rice et al., 2003; Rola et al., 2006). This pattern of an increase in BrdU-labeling,

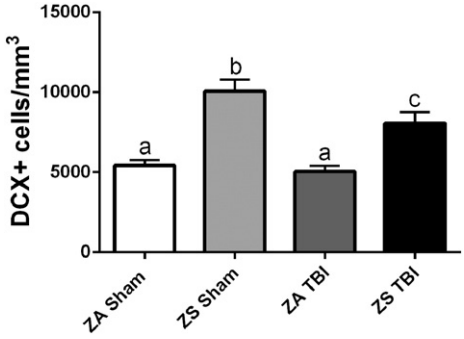

Fig. 4. Effect of zinc supplementation on doublecortin (DCX) expression after TBI. Rats were fed a zinc supplemented (ZS) or zinc adequate (ZA) diet for 4 wk and then subjected to a traumatic brain injury (TBI) or sham surgery (Sham). One week following TBI or Sham, DCX-positive cells (cells/mm^3) in the dentate gyrus were quantified by stereology. Bars represent mean ± SEM. Bars with different letters are significantly different. a *vs.* b, $p < 0.001$; a *vs.* c, $p < 0.01$; b *vs.* c, $p < 0.05$.

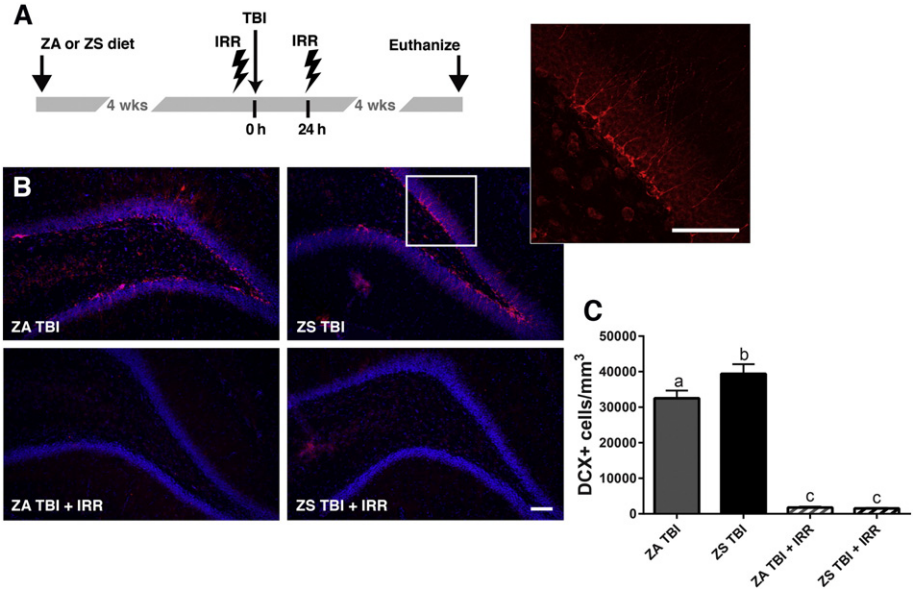

Fig. 5. Targeted hippocampal irradiation eliminates neurogenesis. (A) Rats were fed a zinc supplemented (ZS) or zinc adequate (ZA) diet for 4 wk and then subjected to targeted hippocampal irradiation (IRR) and traumatic brain injury (TBI). Two additional groups of rats received TBI, but not the hippocampal irradiation. Four weeks following the TBI and IRR, hippocampal slices were visualized and quantified by stereology. (B) Representative sections immunostained with doublecortin (DCX, red) and counterstained with Hoechst 33342 (blue); Scale bar = 100 μM. (C) Stereological quantification of DCX-positive cells (cells/mm^3) in the dentate gyrus. Bars represent mean ± SEM. Bars with different letters are significantly different. a *vs.* b, $p < 0.01$; b *vs.* c, $p < 0.001$; a *vs.* c, $p < 0.001$.

followed by a decrease in labeling in the dentate, has also been reported in ischemic models of injury (Jin et al., 2001). Our work shows that this decline in newly born cell density occurs even earlier than previously reported. Our survival study showed a reduction in EdU-labeled cells by 1 wk after TBI. Interestingly, the rate of decline in the first week after injury was similar in the zinc supplemented and zinc adequate groups, suggesting that while zinc enhances precursor cell proliferation, its effect on survival of these adult-born cells is minimal, if any. However, because of the robust enhancement of zinc-induced proliferation, there was still a significantly higher density of TBI-induced cells in the zinc-supplemented group 1 wk after injury. Thus, at the one-week time point when we began behavioral testing, there were approximately 60% more newly born cells in the dentate of zinc supplemented animals.

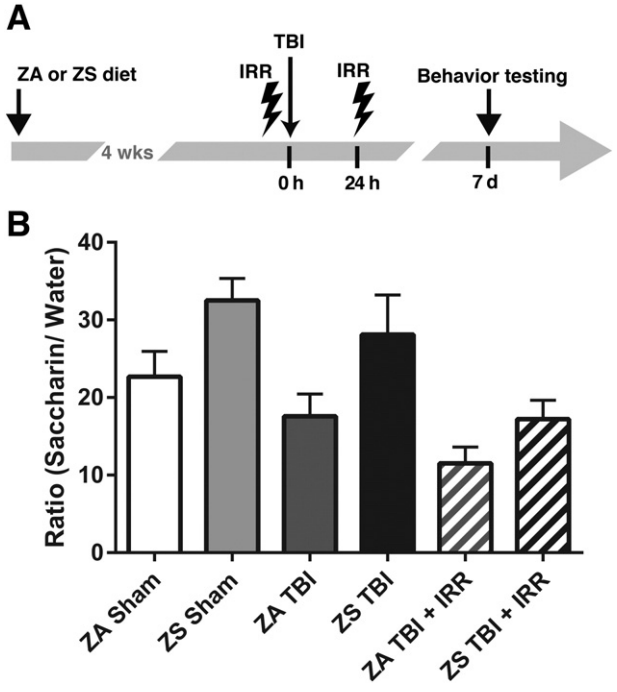

Fig. 6. Effect of targeted irradiation on the ability of zinc supplementation to prevent anhedonia. Rats (n = 8–10/group) were fed a zinc supplemented (ZS) or zinc adequate (ZA) diet for 4 wk and then subjected to traumatic brain injury (TBI) or sham surgery (Sham). Rats from each dietary group that received TBI were also subjected to hippocampal irradiation (IRR) to eliminate TBI-induced cell proliferation and neurogenesis. Additionally rats from each dietary group received TBI, but not hippocampal irradiation. Bars represent saccharin to water intake ratios measured over a 4-day period (mean ± SEM). These data were then subjected to analysis by linear regression.

4.2. Effect of zinc on early neuronal differentiation

Previous work has implicated zinc in the molecular mechanisms associated with neuronal differentiation (Gower-Winter et al., 2013; Morris and Levenson, 2013). Here we explored the impact of zinc on the fate of neuronal precursor cells after TBI, particularly at the 1 wk time point when there are zinc-induced improvements in anhedonia. To do this, we first examined cells that were double-labeled for EdU and DCX. This pool of cells represents those that proliferated during the first day after injury and then went on to commit to a neuronal phenotype by 1 wk post-injury. We found that the percentage of EdU-labeled cells that expressed this marker of early neuronal differentiation at 1 wk post-TBI was not changed by zinc. Regardless of diet, approximately 85% of the EdU-positive cells had committed to a neuronal phenotype, a percentage that is consistent with other reports on the timing of neurogenesis (Snyder et al., 2009). However, because there were more total EdU-positive cells in the zinc-supplemented group, supplemented rats had more DCX+ cells after injury than zinc adequate rats post-injury.

4.3. Role of adult-born cells in zinc efficacy

Given that a majority of adult-born cells appear to commit to a neuronal phenotype in this model, we next sought to determine the degree to which the efficacy of zinc is dependent on these newly born cells. To accomplish this, we used targeted hippocampal irradiation used previously by a number of investigators (Mizumatsu et al., 2003;

Snyder et al., 2005; Winocur et al., 2006; Rola et al., 2004; Tada et al., 2000). Consistent with previous reports, we showed that not only does this approach robustly decrease hippocampal neurogenesis, but it is also sufficient to inhibit new neuron production for at least a month after exposure. An advantage of this method is that it targets neural progenitors while still maintaining the integrity of mature neurons and other cell types in the hippocampus (Airan et al., 2007; McGinn et al., 2008; Monje et al., 2002). Mitotic inhibitors are unable to specifically target neural stem cells in the hippocampus without suppressing neurogenesis in the other areas of the brain (e.g. the subventricular zone). Moreover, unlike the method reported here, chemical methods often result in reductions in body weight and sickness (Dupret et al., 2005; Jayatissa et al., 2009). While there have been reports of inflammation following irradiation (Monje et al., 2002), our work confirms work showing, at most, modest and transient increases in inflammation (Noonan et al., 2010; Wojtowicz, 2006).

After controlling for injury and irradiation, zinc supplementation was associated with higher saccharin preference. Furthermore, controlling for zinc treatment revealed that saccharin preference was related to the presence or absence of newly born cells in the hippocampus. While the main effects are robust, caution is warranted in the interpretation of these results because the model revealed no significant interactions between zinc and irradiation. Specifically, in rats that received the injury zinc supplementation was associated with increased saccharin preference regardless of irradiation status. Thus, on the basis of the main effects analysis it appears that stem cells may well play a role, but it would be inappropriate to ascribe behavioral improvements after zinc supplementation solely to the proliferation of stem cells or neurogenesis in the hippocampus.

5. Conclusions

The current research shows that zinc supplementation enhances the density of adult-born cells in the dentate gyrus after cortical injury. Survival data suggest that zinc acts via an enhancement of proliferation, rather than by increasing survival. A majority of zinc- and TBI-induced cells go on to exhibit cell markers consistent with neuronal differentiation and the total density of immature cells committed to a neuronal phenotype is increased after TBI when animals were provided with chronic zinc supplementation.

The question this work raises is about the function of these cells. While chronic zinc supplementation clearly prevents the onset of depression-like symptoms in this model of TBI, use of targeted irradiation to eliminate the presence of newly born cells in the hippocampus failed to show clear evidence of a dependence of these cells on zinc for full efficacy. Future work will be needed to explore alternative mechanisms by which zinc is known to act in the hippocampus such as the role of synaptically active zinc (Szewczyk et al., 2015), the role of zinc in synaptic plasticity (Izumi et al., 2006), and the role of zinc in the structural integrity of a large number of transcription factors (Klug, 2010).

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