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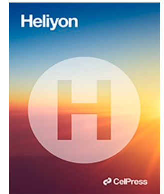

Sub-region expression of brain-derived neurotrophic factor in the dorsal hippocampus and amygdala is Affected by mild traumatic brain injury and stress in male rats

Aaron W. Fleischer[a,b,c], Laura C. Fox[a,b], Daniel R. Davies[a,b,d], Nathan J. Vinzant[a,b,e], Jamie L. Scholl[a,b,*], Gina L. Forster[f,1]

[a] Center for Brain and Behavior Research, 414 East Clark St, Vermillion, SD, USA
[b] Division of Basic Biomedical Sciences, Sanford School of Medicine at the University of South Dakota, 414 East Clark St, Vermillion, SD, USA
[c] Department of Psychology, University of Wisconsin-Milwaukee, 2441 East Hartford Ave., Milwaukee, WI, USA
[d] Mayo Clinic School of Graduate Education, Rochester, MN, USA
[e] Department of Anesthesiology & Perioperative Medicine, Mayo Clinic College of Medicine and Science, Rochester, MN, USA
[f] Department of Anatomy, University of Otago, PO Box 56, Dunedin 9054, New Zealand

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ABSTRACT

The US population suffers 1.5 million head injuries annually, of which mild traumatic brain injuries (mTBI) comprise 75%. Many individuals subsequently experience long-lasting negative symptoms, including anxiety. Previous rat-based work in our laboratory has shown that mTBI changes neuronal counts in the hippocampus and amygdala, regions associated with anxiety. Specifically, mTBI increased neuronal death in the dorsal CA1 sub-region of the hippocampus, but attenuated it in the medial (MeA) and the basolateral nuclei of the amygdala nine days following injury, which was associated with greater anxiety. We have also shown that glucocorticoid receptor (GR) antagonism prior to concomitant stress and mTBI extinguishes anxiety-like behaviors. Using immunohistochemistry, this study examines the expression of brain-derived neurotrophic factor (BDNF) following social defeat and mTBI, and whether this is affected by prior glucocorticoid receptor antagonism as a potential mechanism behind these anxiety and neuronal differences. Here, stress and mTBI upregulate BDNF in the MeA, and both GR and mineralocorticoid receptor antagonism downregulate BDNF in the dorsal hippocampal CA1 and dentate gyrus, as well as the central nucleus of the amygdala. These findings suggest BDNF plays a role in the mechanism underlying neuronal changes following mTBI in amygdalar and hippocampal sub-regions, and may participate in stress elicited changes to neural plasticity in these regions. Taken together, these results suggest an essential role for BDNF in the development of anxiety behaviors following concurrent stress and mTBI.

* Corresponding author. Center for Brain and Behavior Research Department of Basic Biomedical Sciences Sanford School of Medicine University of South Dakota, USA.

E-mail addresses: Fleisc54@uwm.edu (A.W. Fleischer), lfox@eptha.com (L.C. Fox), Daniel.davies@mayo.edu (D.R. Davies), vinzant.nathan@mayo.edu (N.J. Vinzant), jscholl@usd.edu (J.L. Scholl), gina.forster@otago.ac.nz (G.L. Forster).

[1] In memory of Dr. Gina Förster, who passed away December 23, 2020.

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1. Introduction

Mild traumatic brain injuries (mTBIs) make up 75% of the 1.5 million head injuries in the United States annually [1], and resulting head trauma often occurs during stressful situations, such as combat or sporting events [1,2]. To be classified as mild, the head injury may not result in a skull fracture, internal bleeding, or more than 30 min of loss of consciousness [2]. Following these events, up to 60% of individuals may experience negative psychological symptoms, such as anxiety, for upwards of one-year post-injury [2–5]. Mild traumatic brain injuries have recently come under much media coverage, due to the prevalence of chronic traumatic encephalopathy (CTE), a neurodegenerative disorder associated with reports of negative behavioral outcomes, such as aggression, anxiety, depression, and dementia [6,7] resulting from repeated incidents of mTBI [6,7]. Despite the recent exposure, and the knowledge that CTE can result from multiple mTBIs, the neural and molecular mechanisms underlying development of prolonged/delayed psychological states, such as anxiety, following a mTBI are not well understood.

Many TBI induction methods do not fit the criteria for mild injuries (for reviews, see Bolouri and Zetterberg, 2015, and Morales et al., 2005). We have previously used a modified weight drop to induce mTBI in Sprague-Dawley rats [8–10], which results in a precise impact with minimal internal bleeding, lack of skull fracture or loss of consciousness, making it a suitable model for mTBI [8–11]. Utilizing this method, we have shown that mTBI in male rats results in increased anxiety-like behavior, decreased neurons in the dorsal hippocampal CA1 sub-region and increased neurons in the medial (MeA) and basolateral and lateral (BLA/LA) nuclei of the amygdala [10]. The hippocampus is generally thought to reduce anxiety behaviors, whereas the amygdala is thought to promote them, though the two regions' actions are certainly more complex [12–15]. Due to their location in the temporal lobe, the hippocampus and amygdala are particularly vulnerable to coup-contrecoup mechanical forces, where injury occurs at the site of impact as well as the opposing side, exerted by head impact [10,16]. Altogether, this suggests a mechanism whereby mTBI specifically decreases neurons – and thereby activity – in an anxiety-reducing brain region; while increasing neurons and activity in an anxiety-promoting region, with the overall result promoting anxiety states, including the behavioral results directly related to the current study which were previously published [9].

One candidate protein for these neuronal changes is brain-derived neurotrophic factor (BDNF), due to its critical roles in neurogenesis, synapse formation and strengthening, and neuronal survival [17–20]. Indeed, other studies have examined BDNF in different forms of TBI, showing increases in BDNF mRNA in the hippocampus following fluid percussion, cortical impact, and penetrating methods of TBI induction [21–24]. All of these studies utilized moderate or severe forms of TBI, however, one previous study examined BDNF in the amygdala following mTBI, finding that injury leads to a decrease in the expressions of specific exons of the *bdnf* gene throughout the entire region [25]. Overall, this suggests a need to further explore the role of BDNF in mTBI.

As mTBI is often associated with stressful events, the effects of social defeat (SD) concurrent with TBI on anxiety states have shown that a single episode of SD immediately prior to injury resulted in both elevated corticosterone levels and anxiety behaviors compared to either TBI or SD alone [8]. Further evaluation with glucocorticoid (GR) and mineralocorticoid receptors (MR), have shown that antagonizing GR, but not MR, prior to SD + mTBI returned anxiety-like behaviors to control levels [9], suggesting that GR are part of the mechanism underlying anxiety states following mTBI induction, perhaps related to BDNF expression. Previous studies have shown that corticosterone administration to adrenalectomized (ADX) rats reduces BDNF mRNA in the hippocampus [26–28]. Similarly, studies using adrenal-intact animals have shown that acute stress promotes BDNF expression in several regions, including the BLA [29] and sub-regions of the hypothalamus and pituitary, and it decreases expression in the CA3 and dentate gyrus (DG) subfields of the hippocampus [30], whereas chronic stress further inhibits BDNF expression in the hippocampal CA1 [29]. Together, these studies suggest that differing levels of stress uniquely affects BDNF expression in distinct brain regions.

The current study used adrenal-intact rats to test the hypothesis that naturally upregulated corticosterone via SD stress, concurrent with mTBI would decrease BDNF in the CA1 sub-field of the dorsal hippocampus, and increase BDNF in the MeA and BLA, thereby promoting the down- and upregulation of neurons in these regions, respectively. We predicted these changes in BDNF would be accompanied by a reduction in neuronal cell numbers in the dorsal CA1, while increased neuronal counts would be seen in the BLA and MeA, in line with our previous findings [10] and the known role of BDNF in neuronal survival [19]. Additionally, we hypothesized that antagonizing GR, but not MR, prior to SD + mTBI would maintain basal-level BDNF expression in these regions. Together, suggesting that overall regulation of BDNF expression following stress and injury could underlie the development of anxiety-like behaviors.

2. Methods and materials

2.1. Animals

The experiments conducted were approved by the Institutional Animal Care and Use Committee of South Dakota, Care and Use Protocol 07-03-16-19D; and followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering, and a minimal number of animals were used for this study.

Thirty-six adult male Sprague-Dawley rats (8–10 weeks old, Animal Resource Center, The University of South Dakota, Vermillion, SD) were pair-housed at constant room temperature (22 °C, 60% relative humidity) on a reverse light cycle (12L:12D, lights off 10:00 a.m.) with food and water available *ad libitum*.

2.2. Social defeat + mild traumatic brain injury surgery and drug treatment

In preparation for SD, all rats were acclimated to a solitary cage in the testing room for 40 min per day for three days prior to

treatment day [9]. On treatment day, rats were randomly assigned to receive a single subcutaneous injection of either vehicle (propylene glycol, 1 mL/kg [9]), mineralocorticoid receptor (MR) antagonist (spironolactone, 50 mg/kg [9]), or glucocorticoid receptor (GR) antagonist (mifepristone, 20 mg/kg [9]) (Sigma-Aldrich, St. Louis, MO, United States) 40 min prior to either control handling (no SD, no mTBI) or SD + mTBI (n = 6/group). Concentrations of drugs were chosen based on their efficacy in reducing anxiety-like behaviors in pre-stressed rats [9].

Following vehicle, GR antagonism, or MR antagonism, animals assigned to SD + mTBI underwent a single episode of SD by an aggressive, larger male rat (more than 100 g larger than the subject), thereby elevating plasma corticosterone to promote desired conditions for mTBI incurred under a stressed state [8]. Control rats were placed in an empty cage similar to the cage used during acclimation [9].

Details regarding the SD model concurrent with weight drop mTBI may be found in Davies et al. [8]; and details regarding surgical procedure to induce mTBI may be found in Meyer et al. [10]. Briefly, immediately following the defeat episode, each rat was anesthetized with isoflurane (3–4% in 3.0 L/min O_2). Then, the skin atop the skull was shaved and disinfected, and a 1-inch-long incision was made to expose the skull. Next, a 175 g weight was dropped from 42 cm via a calibrated weight-drop device to produce a 5477 N/m^2 impact to the exposed skull directly behind bregma, and the force was distributed across a 10 mm diameter area via a vertical transducer rod placed just posterior to bregma and centered over the intraparietal suture. Delayed righting-reflex time post-surgery and the absence of skull fracture and gross brain and vascular damage at the conclusion of behavioral testing were determinants used to confirm mTBI [8–10]. Control (sham) animals did not receive head injury but otherwise underwent the same surgical

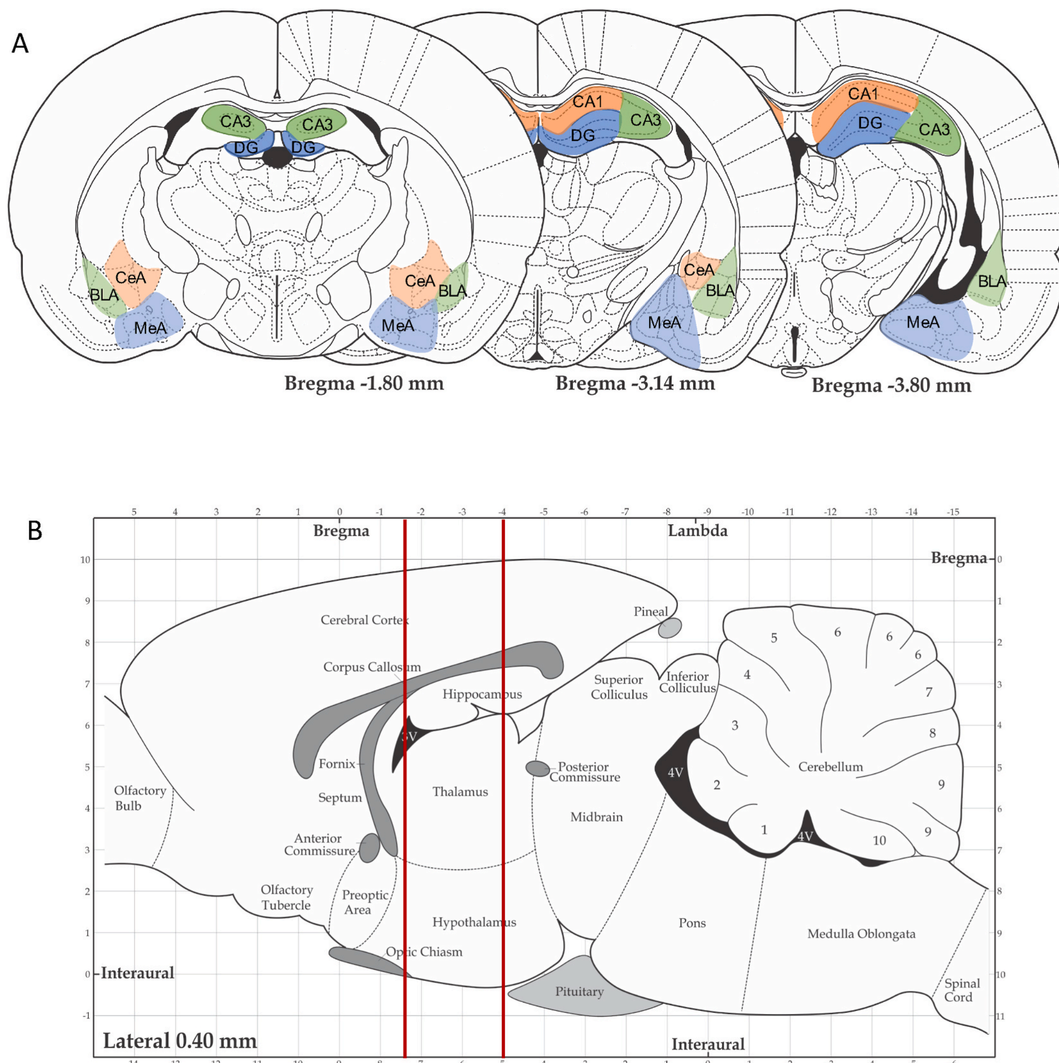

Fig. 1. Diagrams Illustrating Regions from which Sections were Selected for Imaging. A) Coronal sections showing the anterior-posterior progression of amygdala and dorsal hippocampal sub-regions. B) Sagittal view showing beginning and end of region from which every fifth sections was selected. *Abbreviations:* DG = dentate gyrus; CA1 = cornu ammonis sub-region 1; CA3 = cornu ammonis sub-region 3; MeA = medial nucleus of the amygdala; BLA = basolateral nucleus of the amygdala; CeA = central nucleus of the amygdala. Adapted from Paxinos and Watson [31].

procedures for the same length of time under isoflurane.

To evaluate generalized anxiety-like behavior, rats completed an elevated-plus maze (EPM) eight to nine days post-surgery, the results of which were previously published [9]. Importantly, Fox et al. (2016) found that animals receiving SD concurrent with mTBI spent less time in the open arms of the EPM compared to their control counterparts, and that antagonizing GR, but not MR, prior to SD + mTBI was sufficient to restore time spent in the open arms back to control levels. Therefore, GR were demonstrated to be more important than MR in the expression of anxiety-like behavior following SD + mTBI [9].

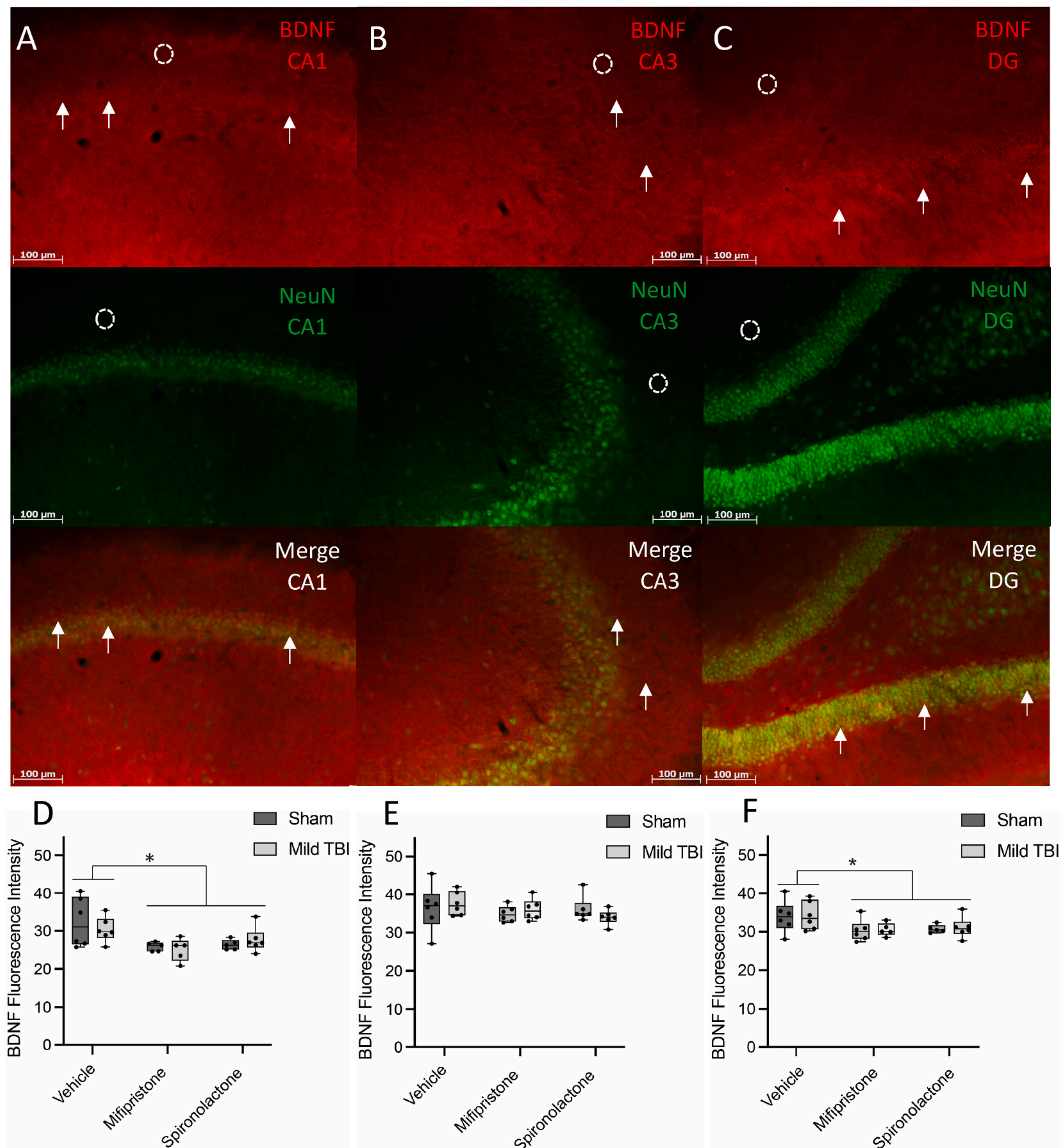

Fig. 2. Effects of mTBI and stress on BDNF expression in the Dorsal Hippocampus: Representative epifluorescent image (20x) of labeling of BDNF (red) and NeuN (green) in the Cornu ammonis sub-region 1 (CA1) of the dorsal hippocampus (A) shows that BDNF in the dorsal CA1 was lower in rats receiving drug treatment than those receiving vehicle injection prior to mTBI, and no differences between mifepristone and spironolactone treatments were observed (D). Representative image (B) of the cornu ammonis sub-region 3 (CA3) shows BDNF did not differ between groups receiving mTBI concurrent with social defeat stress and controls or between groups treated with vehicle, mifepristone, or spironolactone (E). Representative image (C) of the dentate gyrus (DG) showed that BDNF intensity was lower in rats receiving drug treatment than those receiving vehicle injection prior to mTBI concurrent with social defeat stress, and there were no differences between mifepristone and spironolactone treatments (F). Dashed line circles in A, B, and C represent adjacent regions quantified for background noise calculation. * = Significant difference ($P < 0.05$) compared to vehicle group, n = 5–6/group. Abbreviations: mild traumatic brain injury (mTBI), brain-derived neurotrophic factor (BDNF), neuronal nuclei (NeuN), cornu ammonis sub-region 1 (CA1), cornu ammonis sub-region 3 (CA3), dentate gyrus (DG). Arrows indicate BDNF labeling. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.3. Histology and immunohistochemistry

Three days following EPM testing, animals were anesthetized via sodium pentobarbital (100 mg/kg) and transcardially perfused with 0.05 M phosphate-buffered saline (PBS, pH 7.4, room temperature) followed by 4% paraformaldehyde (pH 7.4, 4 °C). The brains were then post-fixed in 4% paraformaldehyde (pH 7.4, 4 °C) for 20 h. Next, the brains were washed twice in PBS for 24 h each, before being transferred to 30% sucrose for 3–5 days. The perfused brains were flash-frozen in isopentane and stored in a −80 °C freezer until

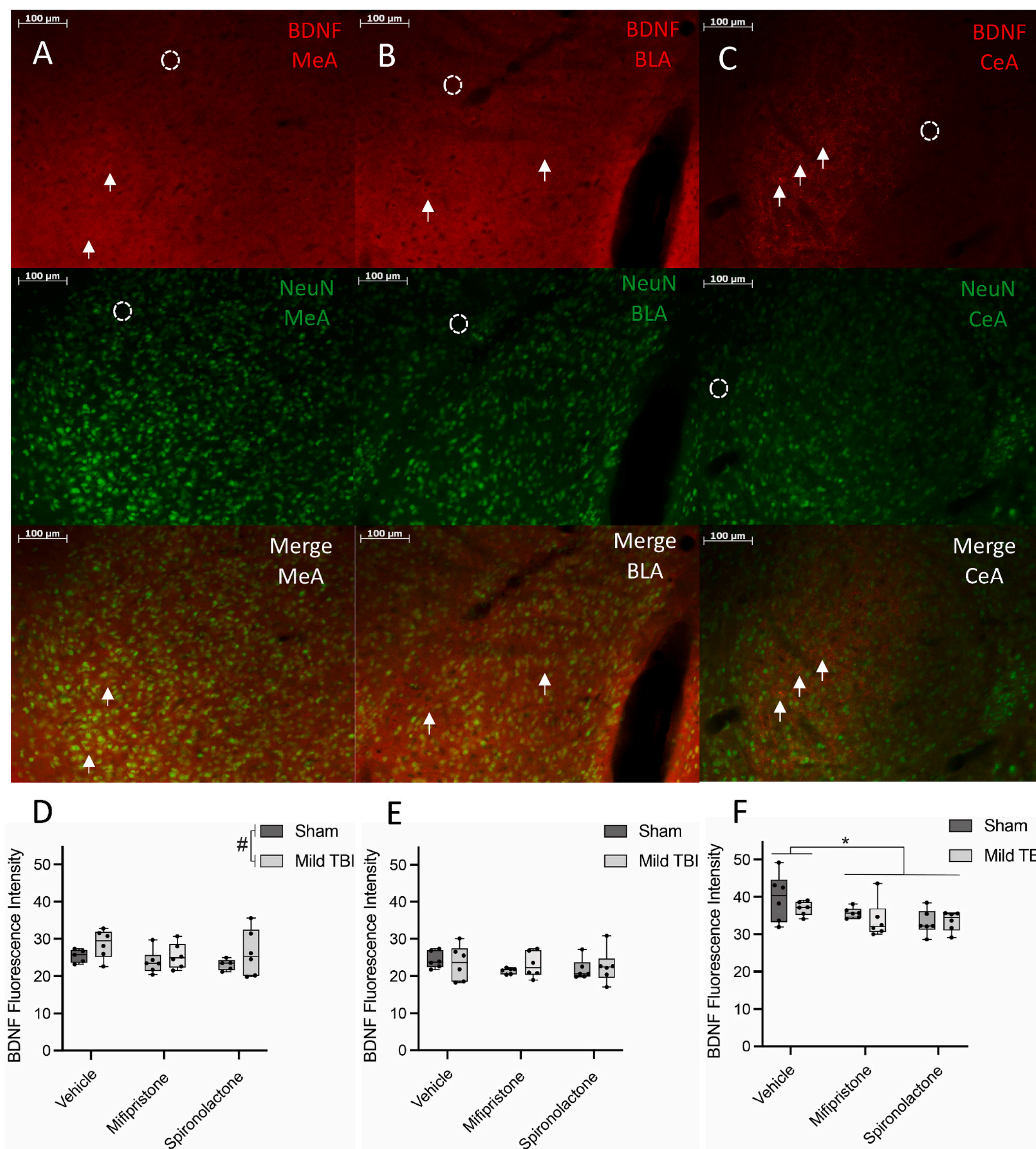

Fig. 3. Effects of mTBI and stress on BDNF expression in the Amygdala: Representative epifluorescent image (20x) of labeling of BDNF (red) and NeuN (green) in the medial nucleus (MeA) of the amygdala (A) shows that BDNF in the MeA was greater in rats receiving mTBI with social defeat stress than controls among the treatment groups (D). Representative image (B) of the basolateral amygdala (BLA) shows that BDNF did not differ between groups receiving mTBI with social defeat stress and controls or between groups treated with vehicle, mifepristone, or spironolactone (E). Representative image (C) of the central nucleus of the amygdala (CeA) showed that BDNF intensity was lower in rats receiving drug treatment than those receiving vehicle injection prior to mTBI with social defeat stress, and there were no differences between mifepristone and spironolactone treatments (F). Dashed line circles in A, B, and C represent adjacent regions quantified for background noise calculation. # = Significant difference ($P < 0.05$) compared to sham group, * = Significant difference ($P < 0.05$) compared to vehicle group, n = 5–6/group. Abbreviations: mild traumatic brain injury (mTBI), brain-derived neurotrophic factor (BDNF), neuronal nuclei (NeuN), medial nucleus of the amygdala (MeA), basolateral amygdala (BLA), central nucleus of the amygdala (CeA). Arrows indicate BDNF labeling. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

sectioning. Brains (n = 6/group) were sectioned in the coronal plane via sliding microtome at 40 μm thicknesses, anterior to posterior, saving every other section in the forebrain (bregma +4.20 to −1.60), every section in the diencephalon (bregma −1.60 to −6.0) and every fifth section in the mid- and hindbrain (bregma −6.0 to completion) [31]. Sections were stored in cryoprotectant (30% ethylene glycol, 20% glycerol) at −20 °C until processing.

Every fifth section in the diencephalon that encompassed the hippocampus and amygdala (bregma −1.60 to −3.80) was used for immunohistochemical processing (Fig. 1). Sections were processed for dual labeling of BDNF and of neuronal nuclei (NeuN), given our previous work showed mTBI resulted in regionally specific changes in neuronal cell count. Briefly, sections were washed three times for 5 min in room-temperature 0.5% PBS-based goat serum dilutant (#005-000-121, Jackson ImmunoResearch, West Grove, PA, USA), after which they were blocked for 1 h on a horizontal shaker in 5% PBS-based goat serum blocking solution to prevent nonspecific antibody binding. Next, sections were incubated at 4 °C for 22 ± 1 h in 1:750 mouse anti-NeuN (#MAB-377, Millipore Corporation, Temecula, CA, USA) [10,32] and 1:500 rabbit anti-BDNF (#AB1779SP, Millipore Corporation, Temecula, CA, USA) [33], followed by 1 h of shaking at room temperature. Sections were then washed three times in 0.05 M PBS. Next, sections were incubated at room temperature for 2 h in 1:200 Alexa Fluor goat anti-mouse NeuN secondary antibody (#115-545-146, Jackson ImmunoResearch, West Grove, PA, USA) and 1:400 Cy3 goat anti-rabbit BDNF secondary antibody (#111-165-144, Jackson ImmunoResearch, West Grove, PA, USA) [32]. Sections with only secondary antibodies applied to them were used as controls. Then, sections were washed three times for 5 min in room-temperature 0.05 M PBS. At the conclusion of the washes, sections remained in PBS and were transferred to a 4 °C fridge for 10–15 min before mounting.

Sections were mounted onto slides in 4 °C 0.05 M PBS and, after drying at room temperature, were coverslipped using Prolong Diamond Antifade Mountant (Life Technologies, Carlsbad, CA, USA). The dentate gyrus (DG), CA1, and CA3 of both dorsal hippocampi, as well as both central, medial, and basolateral nuclei of the amygdala of each section were imaged at a 20× magnification using a Zeiss Axio Imager 2 epifluorescence microscope (Carl Zeiss Microscopy GmbG, Jena, Germany). These regions were selected based on our previous work showing changes to neuronal cell counts and neurochemical activity in these particular areas following exposure to either mTBI alone [10] or SD + mTBI [8]. Imaging was done for each sub-region of each mounted section to determine presence of BDNF labeling, followed by separate imaging for NeuN labeling. ImageJ (National Institutes of Health, Rockville, MD, USA) was used to quantify fluorescence produced by each of the antibodies in the aforementioned sub-regions of the dorsal hippocampus and amygdala as a representation of the expression of BDNF and NeuN in each (Figs. 2 and 3). In order to quantify the proteins, composite images were separated into individual color channels in grayscale. Next, regions of interest were selected based upon the [31] atlas, and drawn in ImageJ via the freehand tool. The background signal for each color channel was determined by a second quantification of fluorescence intensity in tissue adjacent to the region of interest that lacked specific protein labeling (See dashed lines in Figs. 2 and 3), which was subtracted from the overall intensity to provide normalized values for each protein in each region of interest, and this method was repeated for each section. The normalized intensity values of either BDNF or NeuN from all sections from both hemispheres from an individual animal were averaged for statistical comparisons. All imaging and quantification were performed blind to treatment.

2.4. *Statistical methods*

The Grubb's outlier test was utilized to identify statistical outliers in BDNF and NeuN fluorescence intensity (corrected for background) for all hippocampal and amygdala sub-regions. This resulted in the removal of 7 of a possible 432 data points, all from BDNF quantification values – one from the SD + mTBI-mifepristone treatment group and one from the sham-spironolactone group for the BDNF dorsal CA1 and DG regions; one from the sham-vehicle group and one from the sham-spironolactone group for the MeA analysis, and one from the SD + mTBI-mifepristone group for the CeA analysis. There were no outliers for NeuN quantifications. To determine the effects of SD + mTBI and antagonist treatment on BDNF and NeuN expression, mean corrected fluorescence intensity from each sub-region was analyzed via separate two-way ANOVA, comparing surgery (sham vs. SD + mTBI) and drug treatment (vehicle vs. spironolactone vs. mifepristone) groups. Significant main-effects were further investigated by utilizing the Student-Newman-Keuls (SNK) *post hoc* method for multiple pairwise comparisons. $P \leq 0.05$ was used to determine significance, and analyses were conducted using SigmaPlot (Version 13.0, Systat Software, Inc., San Jose, CA), and GraphPad Prism 9.3.1.

3. Results

3.1. *Effects of glucocorticoid and mineralocorticoid receptor antagonism on brain-derived neurotrophic factor expression in dorsal hippocampal subregions*

Animals were given either a glucocorticoid receptor antagonist (mifepristone), a mineralocorticoid receptor antagonist (spironolactone), or a vehicle solution (propylene glycol) prior to SD followed by mTBI surgery or control handling. Within the dorsal hippocampus, there were region-specific differences in BDNF expression following drug treatment, with no apparent effect of SD + mTBI. Representative images of BDNF and NeuN labeling in each dorsal hippocampus sub-region can be found in Fig. 2.

Within the CA1 sub-region of the dorsal hippocampus (Fig. 2A), two-way ANOVA showed (Fig. 2D) no significant effect of SD + mTBI on BDNF expression ($F(1,27) = 0.137$, $P = 0.715$), but there was an effect of drug treatment ($F(2,27) = 7.555$, $P = 0.002$). There was also no significant interaction between SD + mTBI and drug treatment ($F(2,27) = 0.556$, $P = 0.580$). A *post hoc* analysis showed that rats receiving either the GR antagonist (SNK, $P = 0.003$) or the MR antagonist (SNK, $P = 0.010$) expressed less BDNF in the CA1 than those rats receiving vehicle solution; however, there was not a significant difference in BDNF expression between GR and MR

antagonist treatment (SNK, $P = 0.351$) (Fig. 2D).

Within the CA3 sub-region of the dorsal hippocampus (Fig. 2B), two-way ANOVA showed (Fig. 2E) no significant effect of SD + mTBI on BDNF expression ($F(1,30) = 0.004$ $P = 0.953$), no significant effect of drug treatment ($F(2,30) = 1.116$, $P = 0.341$), and no significant interaction between SD + mTBI and drug treatment ($F(2,30) = 0.870$, $P = 0.429$).

Within the DG sub-region of the dorsal hippocampus (Fig. 2C), two-way ANOVA showed (Fig. 2F) no significant effect of SD + mTBI on BDNF expression ($F(1,28) = 0.064$, $P = 0.802$), but there was a significant effect of drug treatment ($F(2,28) = 5.100$, $P = 0.013$). There was also no significant interaction between SD + mTBI and drug treatment ($F(2,28) = 0.002$, $P = 0.998$). A *post hoc* analysis showed that rats receiving either the GR antagonist (SNK, $P = 0.019$) or the MR antagonist (SNK, $P = 0.017$) expressed less BDNF in the dorsal DG than those rats receiving vehicle solution; however, there was not a significant difference between GR and MR antagonist treatment (SNK, $P = 0.715$) (Fig. 1F).

3.2. Effects of glucocorticoid and mineralocorticoid receptor antagonism on brain-derived neurotrophic factor expression in amygdalar subregions

Within the amygdala, there were region-specific differences in BDNF expression following both SD + mTBI and GR/MR antagonism. Representative images of BDNF and NeuN labeling in each amygdalar sub-region can be found in Fig. 3.

Within the MeA (Fig. 3A), two-way ANOVA showed (Fig. 3D) a significant effect of SD + mTBI on BDNF expression ($F(1,28) = 4.260$, $P = 0.048$) compared to sham controls, but no effect of drug treatment ($F(2,28) = 1.451$, $P = 0.251$), and no significant interaction between the SD + mTBI and drug treatment ($F(2,28) = 0.187$, $P = 0.830$).

Within the BLA/LA (Fig. 3B), two-way ANOVA showed (Fig. 3E) no significant effect of SD + mTBI on BDNF expression ($F(1,30) = 0.244$, $P = 0.625$), no significant effect of drug treatment ($F(2,30) = 0.999$, $P = 0.380$), and no significant interaction between mTBI and drug treatment ($F(2,30) = 0.449$, $P = 0.642$).

Within the central nucleus of the amygdala (CeA) (Fig. 3C), two-way ANOVA showed (Fig. 3F) a trend toward an effect of SD + mTBI on BDNF expression ($F(1,29) = 3.303$, $P = 0.079$). There was an effect of drug treatment ($F(2,29) = 7.729$, $P = 0.002$), but no significant interaction between SD + mTBI and drug treatment ($F(2,29) = 1.157$, $P = 0.329$). A *post hoc* analysis showed that animals treated with either the GR antagonist (SNK, $P = 0.004$) or the MR antagonist (SNK, $P = 0.003$) expressed BDNF less in the CeA than those rats receiving vehicle solution; however, there was no significant difference between GR and MR antagonists (SNK, $P = 0.736$) (Fig. 3F).

3.3. Effects of glucocorticoid and mineralocorticoid receptor antagonists and social defeat paired with mild traumatic brain injury on neuronal nuclei expression in the dorsal hippocampus and amygdala

Two-way ANOVA showed no significant effects of mTBI concurrent with SD stress or of antagonist treatment prior to mTBI surgery on NeuN expression within any sub-region examined following quantification and correction for background labeling ($P > 0.05$ for all sub-regions).

4. Discussion

As hypothesized, twelve days following mTBI concurrent with SD stress, rats had greater BDNF expression in the MeA than controls. However, in contrast with our predictions, BDNF in the dorsal CA1 and the BLA/LA were unaffected by mTBI, and instead, treatment of spironolactone or mifepristone prior to either mTBI concurrent with SD stress or control treatment resulted in lower BDNF expression in the CA1 and DG sub-regions of the dorsal hippocampus and the CeA, compared to rats receiving vehicle treatment. Also, in contrast to our predictions, there were no noted differences in BDNF expression in any region between spironolactone and mifepristone administrations. There were no interactions between drug treatment and SD + mTBI condition on BDNF expression, nor were there any differences in neuronal nuclei (NeuN) intensity among groups in any of the regions.

Here we report for the first time that animals receiving mTBI following SD had greater levels of BDNF in the MeA than those receiving sham treatment, which aligns with our previous findings that mTBI resulted in greater neuronal numbers and less apoptosis in this region [10]. However, antagonizing GR and MR prior to SD and mTBI induction did not affect BDNF expression in this sub-region, suggesting that the coup-contrecoup forces produced via weight drop [10,16] were responsible for the noted BDNF differences rather than stress-induced corticosterone. The MeA has many glutamatergic outputs, so an increase in BDNF following mTBI may suggest a greater amount of activation of and therefore excitatory output from the MeA to regions like the hypothalamus, ultimately resulting in greater glucocorticoid release and stress response [34,35], in line with our previous findings [8,9]. Interestingly, we did not see any differences in BDNF expression in the dorsal hippocampal CA1 or BLA/LA between SD + mTBI and sham groups, suggesting a more important role for other trophic factors in the cellular changes previously noted in those regions [10]. For example, several other factors which could be affecting the cellular outcomes in each region include nerve growth factor, which has critical roles in neural protection and cell recovery following such insults as ischemia, [36]; glial cell-derived neurotrophic factor, which has a high affinity for promoting the survival of dopaminergic cells and could similarly be affecting hippocampal and amygdala neurons [37]; and vascular endothelial growth factor, which promotes hippocampal adult neurogenesis [38]. Therefore, these other trophic factors could be explored following stress and mTBI as promoters of both the neural changes and anxiety behaviors noted with the injuries.

The current finding that mTBI induces an increase in MeA BDNF is in contrast to a previous study, which showed that mTBI in a rodent model led to significant methylation of the promoters for BDNF exons I, IV, and IX, resulting in the downregulation of mRNA for

BDNF exons IV and IXa, but not I, in the amygdala for 48 h and 30 days post-injury [25] which is suggestive of reduced BDNF protein expression in the amygdala following mTBI. One potential reason for these differences could be that the current study examined specific sub-regions of the amygdala, whereas [25] examined the amygdala as a whole, which could have been affected by BDNF changes in regions not examined in the current study. This warrants future examinations into the contributions of all components of the amygdala in BDNF expression following mTBI.

Similarly, the current finding that SD + mTBI does not affect dorsal hippocampal BDNF expression contrasts with other studies showing that moderate to severe TBI can alter BDNF expression in the hippocampus. For example, in a model of penetrating TBI using both protein and mRNA quantification, Rostami et al. [23] showed an increase in BDNF protein and mRNA in the CA3 and protein in the DG contralateral to the injury site. A decrease in BDNF was also noted in the damaged ipsilateral sub-regions, and the differences were noted up to two weeks and eight weeks post-injury, respectively [23]. Following cortical impact BDNF increases were found in dorsal hippocampal DG and CA1-4 BDNF mRNA ipsilaterally and contralaterally to the site of injury as soon as 1 h and persisting for at least 5 h following injury [24]. Further, Grundy et al. [21] noted an increase in BDNF mRNA in the DG 4 h following fluid percussion-induced TBI. Together, these studies suggest differences in hippocampal BDNF expression due to the severity of TBI, whereby mild TBI does not affect BDNF expression.

While stressors are not often utilized during TBI studies, the current study implemented a SD component to represent common mTBI induction more accurately in young adults. The current study presents surprising results, showing that independently antagonizing either GR or MR lowers BDNF expression in individual subfields of the dorsal hippocampus and amygdala, namely the CA1, DG, and CeA. Interestingly, there were no differences noted between MR or GR antagonism. While these results were not predicted, they do suggest a potential mechanism underlying the heightened anxiety following both social defeat and mTBI and the subsequent amelioration of that anxiety following antagonism of GR noted in our previous studies [8,9]. Here, the inhibition of the two receptor types responsible for corticosterone's effects immediately prior to an acutely stressful event lowered BDNF expression, which was persistent for twelve days. Release of BDNF is typically thought to be activity-dependent, and it functions to strengthen neural connections [17,18,39], so the decrease reported in BDNF in the DG, CA1, and CeA suggests that antagonism of either GR or MR weakens synaptic connections in these regions. Especially in the CeA, known for its roles in promoting fear behaviors [40], lower BDNF expression could result in fewer or weaker signals to and from the region, contributing to the previously noted effect of GR antagonism in reduction mTBI-induced anxiety [9]. The decreases in BDNF in the dorsal DG and CA1 suggest that these regions could have weaker connections to each other, the CA3, the entorhinal cortex, and other regions in the absence of glucocorticoids [41,42]. Notably, one target of the CA1, the subiculum, activates the BLA and the CeA [43], so weakening that connection could result in the inhibition of anxiety, seen previously [9]. While we previously showed that GR are more important than MR for the anxiety-like behaviors seen following SD and mTBI, there was also a trend toward significance for MR, and there were no significant differences noted between GR or MR on anxiety-like behaviors [9]. Overall, then, this suggests basal and acute levels of stress prime the CA1, DG, and CeA for injury by binding both MR and GR to promote BDNF production and synaptic strengthening with target areas. There could be other factors, such as the neurotrophic factors mentioned previously, in each of these areas that could be more important than BDNF for the anxiety-mediating differences between GR and MR, warranting further studies into which factors are more important for this difference.

Our results of decreased BDNF expression following GR or MR antagonism are contrary to some findings in the field regarding the regulation of BDNF by glucocorticoids. It has been reported that adrenalectomy (ADX), and therefore the removal of circulating glucocorticoids, promotes BDNF mRNA expression in the CA1 and CA3 sub-regions of the hippocampus, and administration of corticosterone either following ADX or in adrenal-intact animals decreases BDNF mRNA expression in the hippocampal CA1, CA3, and DG [26,28]. Another study also showed that the exon IV BDNF promoter was downregulated in all hippocampal sub-regions following corticosterone administration to ADX rats [27]. However, several studies have shown contrasting differences in BDNF expression in adrenal-intact animals in region-specific manners following different methods of stress induction. For example, Lakshminarasimhan and Chattarji [29] showed a lack of BDNF protein differences in the CA3 both one and ten days following acute stress but noted a decrease in BDNF protein therein one day following chronic stress. The same study also showed that the BLA had greater expression of BDNF both one and ten days following acute restraint stress, as well as both one and twenty-one days following chronic restraint stress compared to controls [29]. Overall this suggests that hippocampal BDNF alterations require greater GR activation than in the amygdala, and that their chronic activation reduces BDNF in specific hippocampal sub-regions, whereas the relatively fewer GR in the BLA were not activated differently by acute or chronic stress, and that their activation promoted BDNF expression [29]. The current findings support this, in that the acute stress of SD and mTBI was not sufficient to inhibit BDNF expression in any dorsal hippocampal region, though we also did note increases in BDNF in the amygdala due to stress alone. Smith et al. [30] also reported a decrease in BDNF mRNA in the hippocampal CA3 and DG following both acute and repeated restraint stress, although repeated restraint was necessary to lower BDNF mRNA in the CA1 and the BLA. They also noted an increase in BDNF mRNA in several other sub-regions, such as in the hypothalamus and pituitary gland, following both acute and repeated restraint stress [30]. Thus, BDNF may be altered differentially in subfield-specific manners, in part due to the amount of GR being activated and the persistence of that activation, though the current findings also suggest an important role for MR activation in BDNF expression.

Limitations to this study include other components of the hypothalamic-pituitary-adrenal axis, which have been shown to affect BDNF expression. For example, corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) are upregulated and bind to their respective receptors during chronic unpredictable mild stress events, which is coupled with a decrease in BDNF mRNA in the hippocampus [44]. This suggests a need to look further into the regulation of BDNF by these additional hormones and their respective receptors – MR and GR, CRH receptors, and type 2 melanocortin receptors– in both the hippocampus and amygdala with respect to anxiety states following SD and mTBI.

Additionally, whereas many of the previously mentioned studies utilized ADX to eliminate circulating glucocorticoids, the current study used receptor antagonists for both MR and GR to determine their respective roles in BDNF expression. ADX, however, entails removing the entirety of the adrenal glands in an animal [45] and is commonly used to eliminate circulating glucocorticoids [26–28]. The adrenal cortex and medulla also produce epinephrine, norepinephrine, and dehydroepiandrosterone (DHEA), among other hormones [46], and DHEA [47] and epinephrine [48] have been shown to increase BDNF in the hippocampus and in astrocyte cultures, respectively. Therefore, it is possible that performing ADX and thereby removing other factors that could be affecting BDNF expression here.

The current study utilized immunohistochemistry to quantify mature BDNF protein, however, many of the previously mentioned studies utilized BDNF mRNA quantifications [21,24–26,28,30]. It has been shown that mRNA translation accounts for roughly 40% of the variations in protein expressions of some mammalian cells [49], allowing for differences in progression to mature protein. This could mean that, while mRNA expressions increase following TBI, the enzymatic cleavages of the mRNA strand to the propeptide and subsequent mature protein forms [39] could each be a step preventing full translation into the mature protein form. As demonstrated by Rostami et al. [23]; an increase in BDNF protein was found in the hippocampal CA3 and DG contralaterally following penetrating TBI, but a change in the ratio of BDNF mRNA expression in the CA3 alone, with the contralateral side having greater mRNA expression than the ipsilateral side. This suggests that the mRNA differences likely influenced the protein expression in the CA3, but that other steps took place in the DG. Therefore, future studies should utilize multiple techniques to quantify both the mature BDNF protein and mRNA, regardless of TBI classification, to help bridge the disconnects presented here and by previous literature.

5. Conclusions

The current study shows two potential subfield-specific BDNF mechanisms relating to glucocorticoid activity, mTBI, and anxiety. Here, we have shown that BDNF in the MeA was upregulated by mTBI concurrent with SD, and that administration of either GR or MR antagonists led to decreases in BDNF in the dorsal CA1 and DG, as well as the CeA. Our TBI findings suggest a role for mTBI itself in promoting BDNF expression in the MeA, which supports our previous cellular findings in this region. The GR and MR antagonism findings suggest a potential mechanism for the inhibition of anxiety following SD and mTBI, whereby blocking GR and MR downregulates BDNF expression, resulting in weaker neural connections between the CA1, DG, CeA, and their inputs and outputs, ultimately reducing their abilities to promote anxiety-like behaviors. However, the lack of BDNF changes in the BLA and dorsal CA1 following mTBI suggest greater roles for other factors, such as nerve growth factor, glial cell-derived neurotrophic factor, and vascular endothelial growth factor, in the cellular changes noted previously in these regions. We did not see any changes in NeuN expression, and we noted a trend toward lower BDNF expression in the CeA following social defeat and mTBI, overall suggesting some insensitivity in the immunohistochemistry technique toward discrete changes, which should be addressed in future studies, as well. Future studies should also be conducted to examine the correlation between BDNF mRNA and mature protein differences following stress and mTBI to better understand the impact these two conditions might be having on the processes underlying the protein's functional translation. With these examinations, the molecular and cellular causes of anxiety following mTBI will be better understood, allowing for further studies into treatments for the head injuries.

Data availability statement

The datasets generated for this study are available upon request to all interested researchers. The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

BLA	Basolateral nuclei of the amygdala
BDNF	Brain-derived neurotrophic factor
CTE	Chronic traumatic encephalopathy
CA1	Cornu ammonis sub-region 1 of the hippocampus
CA3	Cornu ammonis sub-region 3 of the hippocampus
DG	Dentate gyrus
EPM	Elevated-plus maze
GR	Glucocorticoid receptor

LA	Lateral nuclei of the amygdala
MeA	Medial amygdala
mTBI	Mild traumatic brain injury
MR	Mineralocorticoid receptor
NeuN	Neuronal nuclei
SD	Social defeat
SNK	Student-Newman-Keuls

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