Aberrant hippocampal neurogenesis contributes to learning and memory deficits in a mouse model of repetitive mild traumatic brain injury

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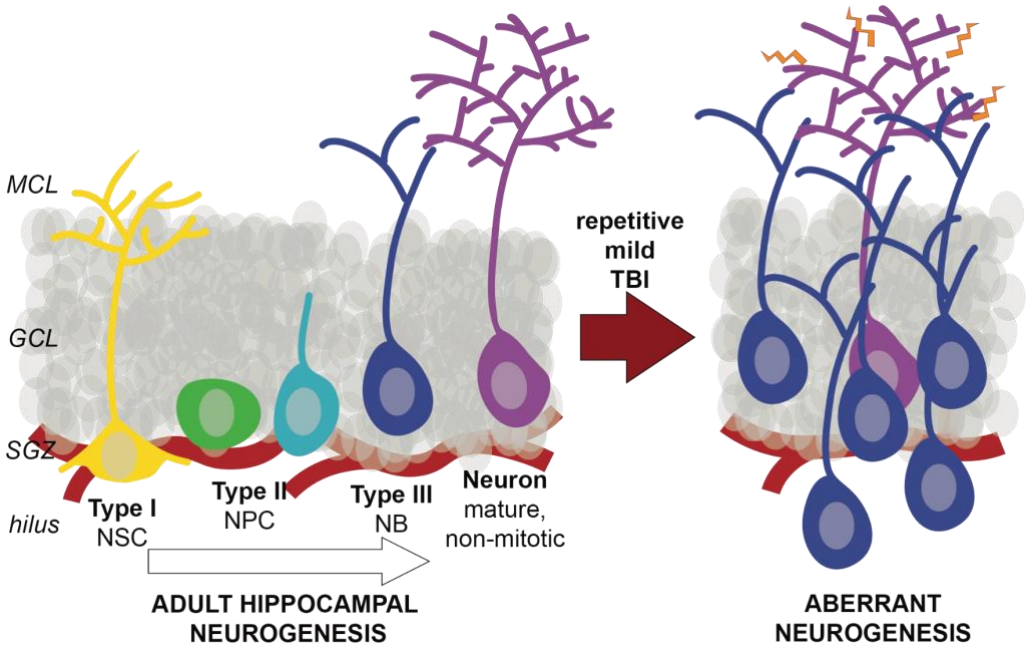
ABSTRACT



Adult hippocampal neurogenesis, or the process of creating new neurons in the dentate gyrus (DG) of the hippocampus, underlies learning and memory capacity. This cognitive ability is essential for humans to operate in their everyday lives, but cognitive disruption can occur in response to traumatic insult such as brain injury. Previous findings in rodent models have characterized the effect of moderate traumatic brain injury (TBI) on neurogenesis and found learning and memory shortfalls correlated with limited neurogenic capacity. While there are no substantial changes after one mild TBI, research has yet to determine if neurogenesis contributes to the worsened cognitive outcomes of repetitive mild TBI. Here, we examined the effect of neurogenesis on cognitive decline following repetitive mild TBI by utilizing AraC to limit the neurogenic capacity of the DG. Utilizing a BrdU fate-labeling strategy, we found a significant increase in the number of immature neurons that correlate learning and memory impairment. These changes were attenuated in AraC-treated animals. We further identified endothelial cell (EC)-specific EphA4 receptor as a key mediator of aberrant neurogenesis. Taken together, we conclude that increased aberrant neurogenesis contributes to learning and memory deficits after repetitive mild TBI.

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GENERAL AUDIENCE ABSTRACT

In the United States, millions of people experience mild traumatic brain injuries, or concussions, every year. Patients often have a lower ability to learn and recall new information, and those who go on to receive more concussions are at an increased risk of developing long-term memory-associated disorders such as dementia and chronic traumatic encephalopathy. Despite the high number of athletes and military personnel at risk for these disorders, the underlying cause of long-term learning and memory shortfalls associated with multiple concussions remains ill defined. In the brain, the hippocampus play an important role in learning and memory and is one of only two regions in the brain where new neurons are created from neural stem cells through the process of neurogenesis. Our study seeks to address the role of neurogenesis in learning and memory deficits in mice. These findings provide the foundation for future, long-term mechanistic experiments that uncover the aberrant or uncontrolled processes that derail neurogenesis after multiple concussions. In short, we found an increase in the number of newborn immature neurons that we classify as aberrant neurogenesis. Suppressing this process rescued the learning and memory problems in a rodent model of repeated concussion. These findings improve our understanding of the processes that contribute to the pathophysiology of TBI.

For my loving husband, I love the life we've built together…

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LIST OF ABBREVIATIONS

AAV	adeno-associated virus
AraC	cytarbine or cytosine b-Arabinofuranoside
BrdU	bromodeoxyuridine
c-Fos	cellular oncogene Fos
CA1-CA3	cornu ammonis fields 1-3
CCI	controlled cortical impact
CTE	chronic traumatic encephalopathy
Cx43	connexin-43
DCX	doublecortin
DG	dentate gyrus
DNA	deoxyribonucleic acid
EC	endothelial cell
Eph	erythropoietin-producing human hepatocellular receptors
EphA4-KO	EphA4$_{f/f}$/Tie2::Cre
GCL	granular cell layers
H.M.	Henry Molaison
HSV-TK	herpes simplex virus-thymidine kinase
mTBI	mild TBI
MWM	Morris water maze
NB	neuroblast
NeuN	Fox-3, Rbfox3, or Hexaribonucleotide Binding Protein-3
NOR	novel object recognition
NSPC	neural stem/progenitor
Prox1	prospero homeobox 1
RNA	ribonucleic acid
SGZ	subgranular zone
Sox2	sex determining region Y – box 2
SVZ	subventricular zone
TBI	traumatic brain injury
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
VEGF	vascular endothelial growth factor
WT	wildtype

Chapter 1

Introduction

Historical perspective on learning and memory

Our ability to store and recall information is a critical skill of human nature, and we've been trying to understand this process for thousands of years. Early philosophers from Socrates to Descartes had theories on how knowledge was stored, but it wasn't until centuries later that we began to fully understand the importance of the hippocampus in learning and memory [1].

In the early 1900s, Henry Molaison (H.M.) was injured in a bicycle accident that contributed to epilepsy. Over time, his seizures progressed, until in 1953, a neurosurgeon named William Scoville approached him to remove his medial temporal lobe to combat the seizures. The bilateral resection of the medial temporal lobe included the hippocampus, amygdala, and parts of the entorhinal cortex [2].

While the resection did help control H.M.'s epilepsy, Scoville noticed his memory was not returning to pre-surgery levels. Over time, researchers realized H.M.'s retrograde amnesia prevented him from developing any new memories from three years prior to his surgery. He often underestimated his age, apologized when he could not recall names, and could not focus on a topic to hold a conversation for more than a few minutes. Interestingly, he could develop skills related to dexterity that remained over time, but had no prior recollection of completing these tasks [3].

These findings helped to initiate a series of scientific discoveries that lead to the understanding that memory is a distinct function separate from cognitive abilities that is primarily associated with the hippocampus [4]. In the current century, we have a more thorough understanding of the cellular and molecular mechanisms of the hippocampus and how these contribute to learning and memory. A key part of the hippocampal firing is the flexibility of neuronal communication, or plasticity. This plasticity allows the hippocampus to form new connections a well as strengthen or weaken existing connections. The ability to create new neurons through the process of neurogenesis is a

unique element in hippocampal plasticity that helps promote the formation of new memories [5].

The structure and circuitry of the hippocampus

The hippocampus is made up of several sub regions of neurons that communicate with each other and the nearby entorhinal cortex to mediate higher brain functions. In brief, the original structure of the hippocampal was thought to have a lamellar organization [6, 7], but has since been modified to more accurately reflect the complex pathways. Projections from the entorhinal cortex connect with dentate gyrus granular neurons that are arranged in a lamellar structure, or in alternating layers. These lamellar features are also found in the cornu ammonis (CA) field 1 (CA1). Pathways from each layer of the dentate gyrus reach out to communicate with the CA3, which projects to the CA2, then to the CA1 before finally exiting the hippocampus with projections back to the subiculum and entorhinal cortex [8-10]. In the updated lamellar hypothesis, the cells in the entryway to the hippocampus in the DG are arranged in a similar lamellar structure to the cells in the exit population of the CA1, and appear to consistently transmit information to their specific targets. In addition, the updated hypothesis added to the importance of associational collaterals in the hippocampus, allowing the individual connections to contribute to the plasticity of the region [11].

Traumatic brain injury symptoms and shortfalls

Several disorders have been shown to affect hippocampal function and learning and memory in humans, including stroke [12], epilepsy [13], and traumatic brain injury (TBI) [14]. In 2013, approximately 2.8 million people visited the emergency department for a TBI, resulting in about 280,000 hospitalizations and 56,000 deaths [15]. A traumatic brain injury occurs when an outside force causes damage to the brain and disrupts normal function. TBIs can range from mild to severe TBI and are typically classified based on mental status, consciousness, and memory loss after the injury. Mild TBIs, or concussions, are the most common and are frequently seen in athletes [16]. According to the Centers for Disease Control and Prevention, most people with a concussion can recover from symptoms, but people who experiences multiple concussions over a lifetime

or who experience moderate or severe TBIs are likely to develop serious long-term consequences. Acute symptoms can include difficulty concentrating, difficulty remembering new information, headaches, dizziness, irregular sleep, and emotional changes. Long-term consequences can be debilitating for patients, and often cause them to leave their work environments leading to financial strain [17]. Of note among these consequences are severe issues with memory [18].

Classifications of memory

Memory is the ability to acquire new information, retain it, and recall it later. There are several different classifications of memory depending on the different types, stages, and functions. Memory can be broken down into three stages: sensory, short-term, and long-term. Sensory memory is received from stimuli the surrounding environments and is held in the brain until the stimuli has stopped, typically lasting less than one second. Short-term, or working memory contains the information we are currently thinking about and will only be kept for 20-45 seconds [19]. These short-term memories can be converted to long-term memory, which is classically what is considered as “memory” to the average person. Most commonly, long-term memory is classified into two main types: explicit and implicit.

Explicit memory (or declarative memory) contains information related to facts and events [20]. Within explicit knowledge, memories can be episodic (referring to firsthand experiences in your life) or semantic (knowledge of facts, concepts, names, etc.) [21]. Recognition memory is a type of episodic memory where people remember people, objects, and places that were previously encountered [22]. Explicit memory requires four distinct processes: encoding to process newly learned information, consolidation to alter newly stored information for long-term storage, storage to retain information over time, and retrieval to process information for recall. Importantly, short-term or working memory, is required to encode and retrieve explicit knowledge, and while short-term memory has a limited capacity, explicit long-term memory seems to have an almost unlimited storage capacity [23]. An important part of explicit, episodic memory is spatial context [24]. Spatial memory can encompass both working and reference memory, and relies heavily on the hippocampus and functional hippocampal place cells [25]. This memory type is

used frequently to test for learning and memory in rodent models. While most explicit memory relies heavily on the hippocampus, the associated cortical areas of the brain also play an important role. The sensory cortex is needed to process visual memory like faces, while factual, semantic knowledge is stored in a variety of places in the cortex. In addition, episodic knowledge and parts of working memory heavily involve the prefrontal cortex [21].

Unlike explicit memory, implicit memory does not depend on conscious processes. Implicit memory (or non-declarative memory) is acquired more slowly, through repetition over many trials as the unconscious processes are store like motor skills [20]. Since implicit knowledge is acquired through different forms of learning, it involves different brain regions. Fear conditioning requires the amygdala to process the emotional component; operant conditioning requires the striatum and cerebellum; classical conditioning, sensitization, and habituation all involve the sensory and motor systems.

Testing for memory capacity in rodent models

In rodent models, there are several types of tests created to identify deficits in specific types of memory.

Novel Object Recognition (NOR) is a novelty-preference paradigm in which a rat or mouse is more attracted to a novel object than a familiar one, suggesting they remember the physical characteristics of the old object [26]. The exploration of the novel object is likely to be motivated in part by an emotional activation that triggers an episodic-like memory formation in the frontal cortex[27]. In addition, the location of the familiar object requires the spatial memory of the hippocampus [28], and rodents will prefer objects they have explored less recently to those they have explored more recently which implies a higher, temporal-order for memory [29].

Richard Morris developed the Morris Water Maze (MWM) in 1981 [30]. In the test, groups of rats were required to escape from cloudy water onto a platform that was just above or just below the surface of water. As the test advanced, researchers switched to a clear platform in clear water and added visual cues to the surrounding area of the MWM. Over time, the MWM has proven to be a reliable test that is strongly correlated with hippocampal function and spatial memory [31].

The Barnes Maze was developed in 1979 by Carol Barnes as a way to test for spatial learning and memory [32]. As a dry land maze, the Barnes Maze induces less stress relative to water-based mazes like the Morris Water Maze [33]. The design of the maze is relatively simple, a circular table is placed directly under a set of bright lights. The lights induce mild anxiety in mice and they are motivated to remember the location of an escape box located under one of 12 or 20 holes located around the table's edge [23]. While spatial memory is located primarily in the hippocampus, the Barnes Maze also requires working memory which has been linked to other cortical areas of the brain [34].

Hippocampal plasticity and adult hippocampal neurogenesis

Researchers have correlated the creation of newborn neurons through neurogenesis to learning and memory in both homeostatic and injured environments. Increases in neurogenesis are correlated with better spatial memory [35] and enhanced long-term memory in adult rodents [36] in normal conditions. Conversely, ablating neurogenesis impairs fear conditioning [37] and interferes with hippocampal-dependent memory function [38]. Together, these results suggest an important regulatory role of neurogenesis in learning and memory. The discovery of neurogenesis in the adult human brain overturned the notion that people are born with all the neurons they will ever have. However, due to limited tissue availability, most findings related to neurogenesis have been discovered in animal models, such as the mouse.

The process of neurogenesis is highly coordinated and heavily relies on adult neural stem/progenitor cells. Derived from the ectoderm during development, neural stem cells are responsible for turning into neurons that create firing patterns in the brain. In adulthood, most NSPCs have differentiated, leaving a small amount in select niches within the developed brain. Neurogenesis occurs in two regions of the mouse brain: the subventricular zone (SVZ) of the lateral ventricles and dentate gyrus (DG) of the hippocampus (Figure 1). In both regions, neurogenesis is characterized by 4 highly coordinated but separate processes: proliferation, migration, differentiation, and survival/maturation. In the SVZ, neural stem/progenitor cells proliferate prior to migrating

along the rostral migratory stream to the olfactory bulb where they differentiate into interneurons [39].

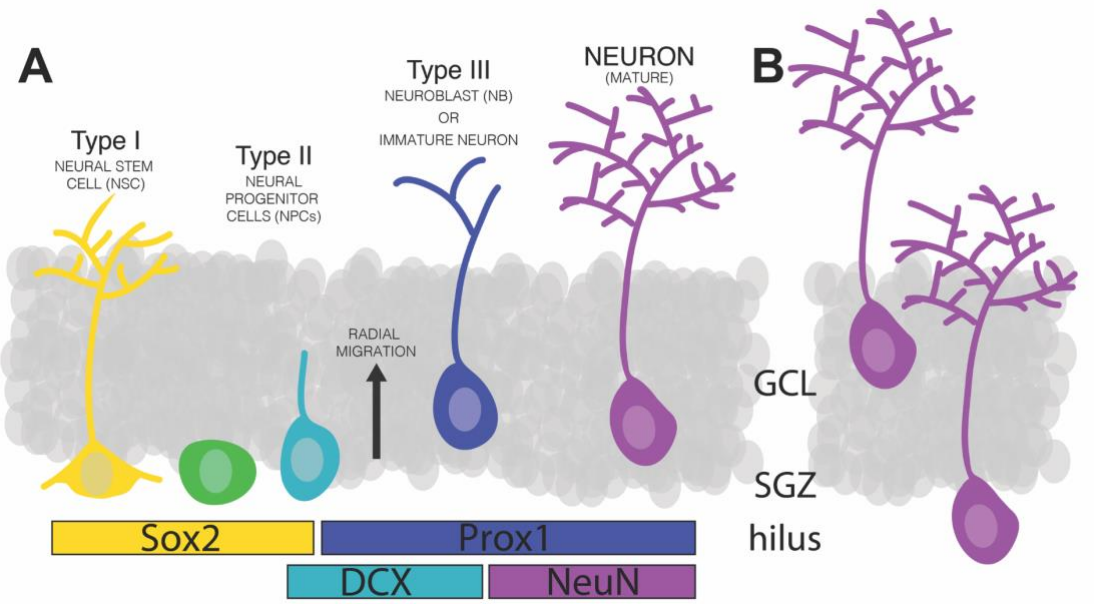

Figure 1. Adult hippocampal neurogenesis. (A) The process of adult hippocampal neurogenesis begins in the dentate gyrus when Sox2-expressing Type I and Type II neural stem/progenitor cells (NSPCs) differentiate into Prox1/DCX-positive neuroblasts before their final maturation into non-mitotic NeuN-expressing dentate gyrus granular neurons. (B) In aberrant neurogenesis, too many new neurons can be created or they can migrate too high up through the granular cell layers (GCLs) or stay too low in the subgranular zone (SGZ) and hilus.

In the hippocampus, stem cells are found in the dentate gyrus. Quiescent neural stem cells are activated and proliferate in the subgranular zone (SGZ) where they migrate radially through the granule cell layers (GCL) before their final differentiation into dentate granule neurons [40]. In both niches, the newborn neurons undergo a final survival step where they continue to mature into fully functional cells that are incorporated into the neuronal network [41]. One prime distinction between these two regions is the involvement in olfactory bulb neurogenesis in turnover, while the new neurons in the DG are more additive [42, 43].

In the DG, NSPCs remain predominantly quiescent in their Type I phase where they remain in close contain with endothelial cells and reach long, small processes up through the granular cell layer to sense their environment. When activated cells are classified as Type II, and are characterized by their separation from the vasculature in the

DG and retraction of processes. Type II cells have a much higher proliferation rate, typically diving asymmetrically to create one Type II cell and one Type III, neuroblast (NB), during the process of neurogenesis. NBs can continue to proliferate or can differentiate further in immature neurons.

Interestingly, these NSPCs can communicate closely with cells in their surrounding niche, including neurons. In fact, this communication is necessary for controlled neurogenesis to occur, as many new neurons will die within 4 weeks after birth [44]. However, immature neurons that survive this period have input-dependent and cell-specific neurotransmission from neurons [45].

Initially, neuroblasts and newborn neurons show increased excitability through reduced GABA inhibition and enhanced plasticity [46], but limited spiking activity due to reduced glutamatergic innervation and functional synaptic inhibition [47, 48]. As these cells continue to mature, they maintain high GABA inhibition [41] and hyperpolarized resting potentials that contribute to sparse activity [49, 50]. Over time, they form glutamatergic synapses with other hippocampal neurons [41], and the introduction of their unique firing patterns in the hippocampal network can contribute to learning and memory [51]

Influences of the niche on neurogenesis and learning and memory

Numerous neurodegenerative disorders are associated with decreased learning and memory outcomes that reflect hippocampal dysfunction, and in part dysfunctional neurogenesis [52, 53]. These negative cognitive outcomes can in part be prevented by increasing physical exercise, which in turn enhances neurogenesis in the hippocampus [54-56]. However, there are several brain impairments that result in debilitated neurogenesis and negative learning and memory outcomes [57-61]. Notably, moderate traumatic brain injury (TBI) causes selective cell death of the Type III, neuroblast (NB) population in the hippocampus that correlates with significant learning and memory deficits [62]. These NBs are replaced by a robust proliferative response from the Type II neural stem/progenitor cells [63]. These proliferative cells differentiate to repopulate lost NBs in addition to fulfilling the lost neuronal population in this niche. Therefore, the

neurogenic response is an excellent target for repair and regeneration following brain injury [64].

Many studies have identified factors to enhance neurogenesis in the hippocampus that correlated with better functional recovery following injury. Growth factors such as bFGF [65], EGF [66], and VEGF [67-70] have been linked to increases in proliferation and enhanced neurogenesis [71, 72]. Additional studies have linked infusion of S100b to enhanced neurogenesis in the hippocampus [73, 74]. Several drugs have been tested in clinical trials to enhance neurogenesis and cognitive function with limited success [75, 76]. In addition, promising research has identified non-invasive strategies to enhance hippocampal neurogenesis, such as hypothermia [77-79] and environmental enrichment [80, 81].

Role of Eph/ephrin signaling in neurogenesis

Several mechanisms underlie the niche effects on neurogenesis[82, 83], but the Eph/ephrin pathway is in the distinctive position to influence behavior by direct cell-cell contact [84] and by release of soluble factors [85]. Making up the largest tyrosine kinase family, Eph receptors and their membrane-bound ephrin ligands are well-known regulators of cell adhesion [86-88] and have been implicated in a wide variety of developmental [89] and pathological processes [90]. Unlike most receptor/ligand pairs, both Eph receptors and their ephrin ligands can stimulate intracellular signaling cascades through forward (ligand activates receptor) and reverse (receptor activates ligand) signaling (Figure 2) [91-95]. Eph receptors and ephrins are classified as either A or B based on their preferred binding affinity in each class [85]. While most Eph receptors typically only bind to those of their class, EphA4 can bind to both ephrin-As and ephrin-Bs [96], and its expression on blood vessels in the DG has been linked to the regulation of neurogenesis. Several studies have contributed to our understanding of the Eph/ephrin signaling pathway in adult neurogenesis, specifically EphA4 [97, 98]. In the dentate gyrus, ephrin-A5 mediates proliferation of NPSCs and promotes the survival of newborn neurons where EphA4 on blood vessels was shown to play a role [99, 100], [101]. Additionally, [102] injecting ephrinA5-Fc into the hippocampus lead to reduced microvessel density and fewer neuroblasts in the DG of animals with temporal lobe

epilepsy [103]. Given these infusion studies can affect both forward and reverse signaling, it remains unclear which cell type and directionality are responsible. Our current study will expand our understanding of how EphA4 expression on endothelial cells within the DG niche influences adult hippocampal neurogenic process in response to rmTBI using conditional KO mice.

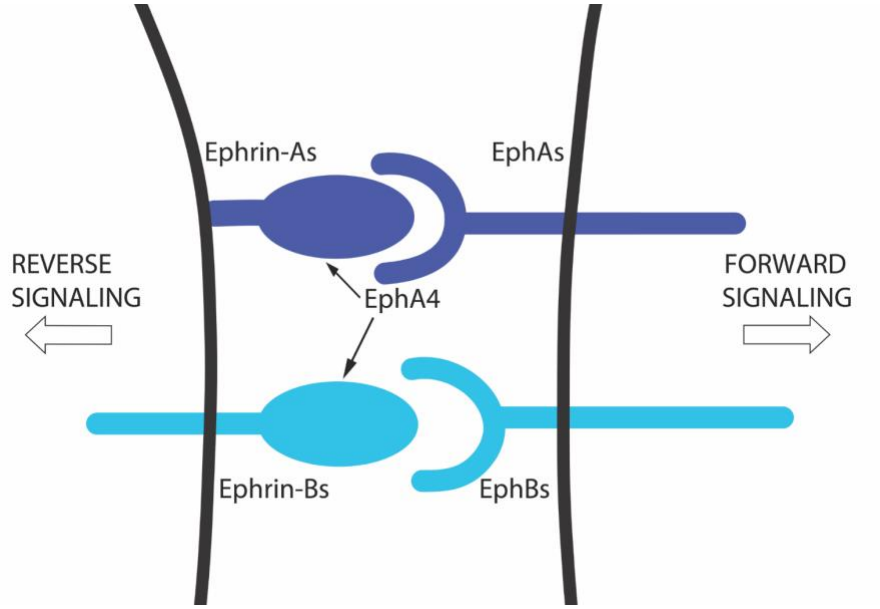

Figure 2. Eph/ephrin communication. Eph receptors and their membrane-bound ephrin ligands are labeled according to their binding affinity for either the A or the B class. EphA4 is an exception and can communicate with both ephrin-As and ephrin-Bs. Eph/ephrin communication is unique in that the direct cell-cell communication can stimulate both forward and reverse signaling, triggering signaling cascades within both cell types that can lead to release of various extracellular release factors.

Effects of insult on the hippocampal neurogenic niche

Since adult hippocampal neurogenesis is a more additive process than a turnover process, it requires a fine balance between newborn neurons and the maximum level of hippocampal firing [104]. When neurogenesis is uncontrolled, or aberrant, it often contributes to negative outcomes. Aberrant neurogenesis can occur when newborn neurons migrate to the incorrect place in the hippocampus, when neurogenesis occurs at an unchecked rate without cell death, or when newly formed neurons take on the incorrect morphology.

Of the many neurological disorders associated with poor learning and memory, epilepsy is most readily associated with aberrant neurogenesis. Epileptic seizures lead to aberrant hippocampal neurogenesis, including increased proliferation of neural progenitors, production of ectopic granule cells (EGCs), mossy fiber sprouting (MFS), neuronal hypertrophy and persistence of hilar basal dendrites on adult-generated granule neurons [105-107]. Investigators have shown that ablating adult neurogenesis prior to acute seizures can suppress chronic seizure frequency and improve seizure-related memory impairment [108]. Taken together, these findings suggest a key role of neurogenesis in epileptic seizures and their associated cognitive function.

Recently, TBI has been linked to increased risk of developing epilepsy [109-112], and studied have found an increase in aberrant migration following moderate TBI in mice. These newly proliferative cells should have migrated radially no more than ⅓ the way through the GCL, but after injury they end up in the outer portions of the GCL closer to the MCL. As the granular neurons mature, they develop less branches, have less total dendrite length, and shorter dendrite averages when compared to sham animals [113].

In addition, TBI has a huge effect on other processes of adult hippocampal neurogenesis, including stimulating proliferation [114], survival [115], and differentiation [114, 116]. Interestingly, moderate TBI induce cell-specific death in doublecortin-expressing neuroblasts in the DG prior to inducing proliferation in Nestin-expressing progenitors to reconstitute the niche and help alleviate cognitive deficits [62]. Other types of brain injury, such as stroke, are also known to significantly stimulate adult hippocampal neurogenesis [58].

NSPC ablation techniques

Genetic Ablation

Targeting NSPC populations in the SVZ and DG can be achieved by using a variety of genetic manipulation techniques [117]. Most of these manipulations are driven under genes expressed in both neurogenic niches (Figure 1), and can be used to target NSPCs at different phases of the differentiation timeline. Nestin and DCX are regularly used to target early NSPCs and late neural progenitors, respectively.

Herpes Simplex Virus Thymidine Kinase (HSV-TK)

The herpes simplex virus-thymidine kinase (HSV-TK) system allows researchers to selectively deplete cells that express HSV-TK after treatment with ganciclovir (GCV). In this system, ganciclovir induces HSV-TK to produce toxic metabolites that disrupt DNA synthesis and result in the death of proliferating cells [117]. This model has been utilized to identify the importance of early Nestin-expressing progenitors in TBI-induced hippocampal neurogenesis [62]. Further, HSV-TK mice under the control of DCX promoter demonstrate the contribution of injury-induced neurogenesis to cognitive recovery from stroke [118]. However, studies that use the GFAP-driven HSV-TK ablation technique can be challenging to interpret because ablation may not be specific to NSPC populations and can lead to neuronal degeneration and overall blood-brain-barrier degradation [119]. Therefore, care must be taken when selecting the correct gene to drive TK expression in mice. Regardless, Nestin-delta-HSV-TK transgenic mice have been successfully used to conditionally ablate neurogenesis in a variety of models. After stroke, conditional ablation impedes recovery of cognitive function and reduces synaptic connectivity in the performant pathway [120]. In addition to injured environments, depletion of adult neurogenesis also exacerbates cognitive deficits in a mouse model of Alzheimer's disease [121].

CreER

Site-specific recombinases (SSRs) can be used to genetically manipulate cell populations by inducing the deletion, insertion, translocations, or inversions at specific sites of DNA sequences (nagy, 2000; wu et al 2007). The most commonly used SSR in animal models is Cre recombinase, where the enzyme and the original Lox sites are derived from bacteriophage P1. In this system, the Cre recombinase recognizes two specific loxP sites and catalyzes the recombination of DNA to remove the genetic sequence located within them. To target a specific cell population, researchers simply add the DNA sequence for Cre recombinase after the promoter for a gene found in that population. The promoter drives expression of Cre recombinase in these cells and allows the enzyme to be produced and edit DNA sequences found within the two loxP sites [122].

While the Cre/loxP recombination system can be used to temporally edit genes expressed later in development, use on its own is limited in NSPC populations that are required embryonically to develop the neuronal network. For this reason, researchers have developed several iterations of a tamoxifen-dependent Cre recombinase (CreER) where Cre is activated by administration of tamoxifen to the animal [123]. These CreER mice are more commonly used to label and trace NSPCs [124], REFs) since the Cre recombinase lacks the specificity of HSV-TK mice [125]. Regardless, researchers have applied this ablation technique to show that ablating diving NSPCs during early life leads to a reduction in adult hippocampal neurogenesis [126]. In addition, conditional reduction of adult born DCX-positive neurons reversibly impairs anxiety-related behaviors [127].

Viral Delivery

While there are several viral vector delivery systems commonly used to deliver genes to cell populations, vectors based on adeno-associated virus (AAV) present several advantages.

AAVs are small, non-enveloped virus that can contain a single-stranded piece of DNA [128]. There are 12 human serotypes and over 100 nonhuman primate serotypes of AAVs, and each can be employed to target specific cell populations. Recently, researchers have utilized engineered novel AAV variants that are capable of efficient transduction of NSPCs in vivo [129]. In addition, AAVs can be used to mediate peptide delivery to NSPCs in the hippocampal niche [130]. These results suggest a novel approach for manipulating the neurogenic niche, and may have important implications for the use of viral delivery systems in targeting NSPCs *in vivo*. AAVs are particularly useful to target NSPCs because they can incorporate into both mitotic and non-mitotic cells without inducing cytotoxicity or cellular immune response [131]. In addition, AAVs can be used to knock-out important proteins and channels in the NSPC population that lead to reduced survival of newborn neurons [132] or deliver short hairpin RNA (shRNA) to mediate knockdown of Nox1 in NSPCs to increase functional recovery and the number of new born neurons after stroke [133].

Drugs and their delivery strategies

Common drugs for NPSC ablation target proliferative capacity, and thus, many researchers have adapted chemotherapeutics for use in the brain. Of note, antimitotic cytosine b-Arabinofuranoside (AraC) can actively eliminate dividing cells by competing with dCTP nucleotides to inhibit DNA synthesis [134]. Several studies have utilized this depletion strategy to target NSPCs in the SVZ and SGZ [135], REFs). However, the effect of AraC is not specific to the NSPC population, and it will have significant systemic effects on highly proliferative cell populations [136]. Still, AraC can be easily delivered to the brain through mini-osmotic pump infusion [137], and additional specificity can be achieved in the SVZ by directly infusing it into the lateral ventricles [138]. Unlike genetic manipulations, AraC has the added advantage of being adaptable to a variety of animal models and has been used to promote neurogenesis-related vestibular compensation in the adult cat [139] and rats [140]. In rodent models, AraC has successfully linked downregulated neurogenesis to reversibly diminished persistent pain [141] and to decreased social recognition memory [142].

Irradiation

Cranial radiation is commonly used to control tumor growth in the brain, but can also be employed to investigate the role of NSPCs in injury and disease. In 2008, McGinn et. al demonstrated the ability to eliminate NSPCs specifically from neurogenic regions in the rat brain by placing a lead shield over the left hemisphere and performing irradiation. Their results showed no significant change in the number of NPSCs in the shielded half, but significant reduction in the number of NSPCs in the irradiated half [143]. Further, NSPC depletion in the SGZ leads to chronic neurogenic deficits whereas the SVZ appears to recover with time after treatment with irradiation [144]. Interestingly, autophagy can prevent irradiation-induced NSPC death in the juvenile mouse brain [145]. However, irradiation also induces p53-dependent apoptosis in neurons with double-strand breaks in their DNA, implying a non-specific effect on the neurogenic niche [146]. Researchers have linked irradiation-induced NSPC ablation to impaired contextual fear conditioning [37], improvement in hippocampal-dependent working memory [147], decreased stress-induced social avoidance [148], and exercise-dependent reversal of depleted adult hippocampal neurogenesis [149].

Selecting an appropriate ablation technique

Several important, and often limiting, factors should be considered when selecting an appropriate ablation technique: cost, time, equipment availability, and reproducibility. Cost is typically the first factor that will play a role in whether a technique is chosen; techniques that involve breeding and maintaining several different lines of mice may have additional costs in comparison with techniques that allow hippocampal manipulation of mice in an already existing colony. Genetic manipulation techniques such as the Nestin-TK mice also have an increased cost to test efficiency of ablation using the ganciclovir. However, with increased cost often comes a balance in time where it may be worth paying more for a solution that can happen more rapidly. After a Nestin-TK colony is established and the efficiency of the ablation has been proven, researchers can easily pull mice from a colony and immediately begin ablation. Even faster would be an injection solution such as AraC or retroviral delivery, and faster still would be irradiation since these techniques can be used with any mice in an already established mouse colony. However, equipment availability can often limit technique choice since irradiation machines are not always readily available in research environments. Further, space in colonies may prevent some researchers from having several different mouse lines. Finally, reproducibility is an important factor that should be considered when selecting an appropriate ablation technique. Injection or infusion techniques can have off-target effects and be hard to accurately and effectively deliver to the deeper layers of the brain such as a hippocampus.

For the presented research, we decided on a 2% AraC solution infused via mini-osmotic pump. This technique gives us a cost-effective way to limit the neurogenic potential in the brain without having to maintain mice with several genetic manipulations. Further, it does not require any equipment that cannot be easily purchased and shipped to the facility as needed. While this technique has been shown to cause significant cell death in the hippocampal niche, it will also have off-target effects on other highly proliferative areas, such as the gut microbiome. However, since the main conclusions of this study are on learning and memory, we believe AraC provides an excellent tool to manipulate the hippocampus and has been previously used to specifically manipulate spatial learning and memory [150].

Pathophysiology of mild traumatic brain injury

Traumatic brain injury (TBI) accounts for about 2 million emergency room cases in the United States each year with about 70-80% of these cases classified as mild TBI (mTBI) or concussion [15]. Common symptoms include headache, confusion, dizziness, and nausea, but typically resolve within 2 weeks of injury. If symptoms persist longer than 3 months, patients are diagnosed with post-concussive syndrome [151]. While a single mTBI may not always result in long-term cognitive deficits, receiving another mTBI can exacerbate cognitive impairments and contribute to the development of neurodegenerative diseases such as chronic traumatic encephalopathy (CTE) [152]. Better understanding the potential mechanisms of damage after repetitive mTBI is critical to identifying techniques to combat the progression of negative behavioral outcomes.

Mild traumatic brain injuries are a result of external force that subject the brain to rapid acceleration and deceleration. During these physical forces or primary injury, the brain stretches and deforms causing microlesions in cell bodies that may result in death [153]. Smaller, unmyelinated neuronal axons are especially susceptible to stretch injuries as they often extend long distances from the neuronal cell bodies and can be injured without loss of neuronal bodies [154]. In addition to physical damage, there is also a neurometabolic cascade caused by a rapid exchange of small ions and molecules in the brain [155]. This secondary injury hyper-metabolism occurs concurrently with reduced cerebral blood flow that increases the disparity between required glucose and energy demand from repairing cells [156].

Imaging studies reveal non-detectable traumatic axonal injury (TAI) following mTBI with computed tomography (CT) scan and magnetic resonance imaging (MRI) [157]. However, diffusion tensor imaging (DTI) appears to show microlesions in white matter that correlate with severity of symptoms [158, 159]. In addition, alterations in brain activation from blood oxygen level-dependent (BOLD) signals and functional MRI (fMRI) may also be useful in diagnosing non-resolving symptoms of post-concussive symptoms [160-162].

Multiple studies have identified mild TBI as a risk factor for late-life neurodegenerations [163], including dementias such as Alzheimer's Disease (AD) [164]. Patients who accrue multiple concussions over a lifetime are at an increased risk of

developing chronic traumatic encephalopathy (CTE) [165]. Symptoms of these neurodegenerative disorders can range from emotional to physical [165], and are debilitating for patients. Of note, neurodegenerative disorders are across the board associated with learning and memory problems. Many patients present with spatial learning and memory deficits, working memory deficits, and that are correlated with changes in the hippocampus and cortex [162]. To better characterize the effects of multiple concussions on brain structure and function, researchers have developed a variety of animal models.

Animal models of repetitive mild TBI

One key distinguishing feature of mTBI when compared to moderate-severe TBI is the lack of a lesion in patients. Therefore, open-head animal models that damage directly to cortical tissues are more translatable to the more severe injuries that better reflect a focal injury [166]. However, mTBIs can be created in open-head animal models using two main techniques: lateral fluid percussion injury (LFP or FPI) and controlled cortical impact (CCI). The fluid percussion injury device utilizes fast injection of fluid into the epidural space to create a focal lesion while the CCI model utilizes an electromagnetic driven piston to hit directly on the cortical surface at a fixed velocity and depth [167]. Both models are easily reproducible, but mimic focal injuries rather than diffuse injuries typically seen in concussion.

In contrast to the open-head injury models, a closed head injury can mimic the effects of concussion without creating a focal lesion. There are two main techniques to create diffuse injuries: the weight drop model and the controlled cortical impact (CCI) model. The CCI model still utilizes an electromagnetic driven pistol, but in contrast to an open-head injury, the skull is still intact and the piston does not hit directly on the cortical surface. The weight drop model [168] has becoming increasingly popular over the years, since it does not require the expensive equipment needed for the CCI model. In the Marmarou weight drop model, a weight of fixed size is dropped a specific distance onto a stainless-steel disk that is mounted directly onto the skull without securing the head to a stereotaxic frame [169].

Both closed-head injuries have distinct benefits and downfalls. In the Marmarou weight drop model, mice are placed onto a foam bed, allowing the head to move slightly and mimic the forward/backward motion found in humans [170]. However, while this model is easy to perform, it has a high rate of variability in injury severity that is associated with high mortality rates. Counter to the weight drop model, the CCI model is easily reproducible in injury severity and allows researchers to efficiently manipulate injury parameters such as velocity, depth, and dwell time of the piston. However, by fixing the skull to the stereotaxic frame you limit the natural rotational and linear acceleration motions of the head [171]. Notably, the CCI model has been successfully adapted to identify significant spatial learning and memory deficits starting about one month after injury [172] that continue to compound up to 24 months [173, 174].

Aberrant neurogenesis and negative cognitive outcomes

What we know about changes to the neurogenic niche in response to TBI is limited to single mild, moderate, and severe injuries. In a single mild TBI, there appears to be little to no difference in proliferation and the birth of new neurons in the hippocampus [116]. However, following moderate TBI, there is a rapid induction in cell death of immature neurons in the hippocampus [175] that is followed by sharp increases in NSPC proliferation [62]. This selective cell death and neurogenic remodeling correlates with cognitive deficits. NSPC ablation studies revealed Nestin-expressing early progenitors are necessary for this TBI-induced remodeling to occur [62]. Further ablation studies revealed a unique function for NSPCs in stabilizing the perilesional cortex after injury, stating their presence increases neuronal survival and glial cell expansion after TBI that is lacking in NSPC-ablated mice. These ablated mice also have worse motor outcomes [176]. Mild traumatic brain injury shows no changes to the neurogenic niche [116]. However, little is known about the effect of repeated mild TBI on the neurogenic niche.

In conclusion, NSPCs serve in a variety of functions including learning and memory processing in homeostasis and are necessary for TBI-induced cognitive and functional

recovery. While the most significant effect on the hippocampal neurogenic niche is found in moderate and severe TBI, the effect of the most prevalent form of TBI (repeated mild TBI) on the neurogenic environment and whether these changes contribute to cognitive dysfunction is severely understudied. The present study evaluates the effect of repetitive mild TBI on the hippocampal neurogenic niche and its role in mediating learning and memory dysfunction. This research will provide crucial information regarding the mechanism regulating the highly-controlled process of neurogenesis during aberrant changes which contribute to learning and memory deficits following repeated mild TBI.

Chapter 2

Suppressing aberrant neurogenesis ameliorates cognitive deficits induced by repetitive mild traumatic brain injury

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Abstract

Traumatic brain injuries (TBIs) effect millions of people in the United States each year, and are characterized by cognitive impairments such as learning and memory deficits that are underwritten by significant changes to adult hippocampal neurogenesis. In moderate TBI, neuroblasts selectively apoptosis and NSPCs proliferate and differentiate to replace these damaged and lost cells, and animals without NSPCs are never able to recover their cognitive capacity. However, following repetitive mild TBI, no one has yet established a link between learning and memory deficits and changes to adult hippocampal neurogenesis. The following study delivers 2% AraC to limit the neurogenic potential before 5 mild TBI injuries, one every other day for a total of 5 hits. We went on to conduct Barnes Maze testing about two weeks after the final injury and identified learning and memory shortfalls that were linked to the neurogenic niche and absent in mice treated with 2% AraC. We further characterized significantly more cells in the neuronal lineage after injury in control mice by quantifying the number of Prox1-positive cells co-labeled with the number of BrdU-positive cells in the DG, a sign of aberrant neurogenesis. Interestingly, the repetitively mild injury-induced aberrant migration of Prox1-positive cells into the hilus was independent of AraC treatment, indicating that aberrant migration into the hilus is likely not contributing to learning and memory shortfalls at this time point. We also found increased numbers of c-Fos-positive cells in the DG, a sign of hyperactivity that directly correlated with performance on the Barnes Maze. These results are seen two weeks after the final injury, and are the starting point to describing how aberrant neurogenesis contributes to the learning and memory shortfalls associated with repetitive mild TBI. Taken together, these outcomes imply a significant role for the neurogenic niche in regulating learning and memory shortfalls following repetitive mild TBI.

Introduction

Traumatic brain injury is a leading cause of disability and death in the United States, with mild traumatic brain injuries (TBIs), or concussions, making up more than 70% of TBI-related emergency department visits [15]. Studies in human patients have identified a correlation between multiple concussions over a lifetime and the development of chronic neurodegenerative diseases [162, 163], with expanding awareness of inflating rates of chronic traumatic encephalopathy (CTE) in football players each year [16]. Over time, these patients exhibit several classical aging-related cognitive impairments, including chronic learning and memory deficits [163, 164, 177], as early as 25 years old with no viable treatment options [178]. However, the mechanisms underlying the development of these learning and memory deficits have only recently been investigated in animal models, with few efforts focusing on the contribution of acute and sub-chronic effects to the long-term cognitive sequela following repetitive mild TBI [179-181].

Neurogenesis, specifically in the hippocampus, has been identified as a fundamental, supportive process of learning and memory. Adult hippocampal neurogenesis occurs in the dentate gyrus (DG) of the hippocampus, where Nestin- and Sox2-expressing neural stem/progenitor cells (NSPCs) differentiate into doublecortin- (DCX) and Prox1-positive neuroblasts and immature neurons before developing into non-mitotic NeuN-positive mature neurons. This highly-coordinated process involves the proliferation, differentiation, migration, and survival of NSPCs which is influenced by environmental cues in the surrounding niche during maintenance of homeostasis and after injury [182].

Neurogenic inhibition through pharmacological and transgenic approaches have demonstrated a correlative effect on the suppression of hippocampal-dependent spatial learning and memory in naïve mice [35, 38]. Additional findings demonstrate a single, moderate cortical contusion impact injury results in cell-selective death of immature DCX-positive neurons in the DG which initiates the proliferation and differentiation of the early NSPC pool and directly correlates with cognitive recovery [175, 183]; withholding this neurogenic response by TK-mediated ablation of Nestin-expressing NSPCs attenuates learning and memory recovery [62]. While the neurogenic contribution to learning and

memory deficits in a single, moderate cortical contusion is well characterized [64, 113, 184, 185], little research has linked neurogenesis to the cognitive deficits associated with repetitive mild TBI.

To address whether the NSPC niche contributes to learning and memory deficits following repetitive mild TBI, we utilized a known chemotherapy medication and antimitotic agent, cytarabine (also known as cytosine arabinoside, or AraC). AraC is the combination of a cytosine base with an arabinose sugar and acts as a nucleotide base when it is converted into cytosine arabinoside triphosphate. This compound damages DNA during the Synthesis phase of the cell cycle, and therefore, has the greatest effect on rapidly proliferating cells that require DNA replication for mitosis and forces them to undergo apoptosis rather than cell division [134]. Previous studies have applied the AraC agent to investigate the contribution of the NSPCs to a variety of mouse model diseases. Several of these studies focus on infusing AraC into the lateral ventricles to influence the SVZ niche [184], but researchers have also utilized AraC to inhibit hippocampal neurogenesis and prevent recognition memory [186]. Further, previous findings characterized the effect of AraC-induced neurogenic inhibition to diminished cognitive recovery following moderate TBI [187, 188].

Here, we demonstrate the novel contribution of aberrant adult hippocampal neurogenesis in learning and memory deficits following repetitive mild TBI. We also correlate injury-induced learning and memory deficits to increased numbers of immature neurons or neuroblasts alongside hyperactivity in the dentate gyrus. Our findings provide evidence that atypical neurogenesis contributes to learning and memory deficits after repetitive mild TBI. We characterized the effect of repeated injury on the proliferation, differentiation, migration, and survival of the NSPCs in the hippocampal DG niche after repetitive mild TBI.

Materials and Methods

Animals

For the following experiments, male mice were kept in an AALAC accredited, virus/specific antigen-free facility with a 12-hour light-dark cycle. They were given access

to food and water *ad libtum*. We purchased CD1 mice from Charles River and reared them until age P60-P90 prior to beginning experiments, which were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were conducted under the approval of the Virginia Tech Institutional Animal Care and Use Committee (IACUC; #17-093) and the Virginia-Maryland Regional College of Veterinary Medicine.

AraC Infusion

Alzet® Mini-Osmotic Pumps Model 1007D (Catalog # 0000290, DURECT Corporation, Cupertino, CA) were used to provide continuous systematic delivery of the saline or 2% AraC (Sigma, #C1768, St. Louis, MO, USA). For 7 days. During the pilot study, animals were also subcutaneously injected with 1.5mg BrdU for 5-days, starting the day after the pump was installed and ending the day before it was removed.

Repetitive Mild Traumatic Brain Injury Protocol

Prior to placement in a stereotatic frame, experimental mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) via subcutaneous injection. We monitored the body temperature with a rectal probe to maintain temperature at 37˚C with a controlled heating pad set. After making a small incision through the skin of the skull, we utilized a programmed controlled cortical impactor connected to an eCCI-6.3 device (Custom Design & Fabrication, LLC) to deliver a single impact with a velocity of 5.0 m/s, depth of 1.0mm and 200ms using a 5mm flat tip. The tip was placed to hit directly on the skull on midline between bregma and lambda. Sham animals did not receive an impact, but were still given an incision. After impact, the incision was closed using Vetbond tissue adhesive (3M, St. Paul, MN, USA) and mice were placed into a warm cage and monitored every 20 minutes until fully recovered from anesthesia. Repeated impacts for TBI and sham were conducted once every 48 hours for a total of 5 surgeries. Mice were also given daily subcutaneous injections of 1.5mg BrdU for 7 total days beginning immediately after their first injury or sham surgery. Injury model was adapted from previously published reports [172, 174, 189, 190].

Barnes Maze Evaluation

To test for learning and memory deficits, we utilized the Barnes Maze (Maze Engineers, Boston, MA USA) and the Ethovision XT software (Noldus, Leesburg, VA USA). We started by pre-training mice 20-days following their initial injury by allowing them to freely explore the maze for 5-minutes, during which time they were prompted to enter the escape box 2 times: once immediately after being placed on the maze and again at the very end of the 5-minutes if they were not already in the escape box. No analyses were gathered during the pre-training phase. Following the pre-training phase, we began the training or acquisition phase. During this phase, mice were placed on the maze for a maximum of 180seconds/trial each day for 3 trials/day to make a total of 9 trials. For days 21-23, we collected the total number of mistakes they made prior to finding the escape box (total errors), the time they took to find the escape box (primary latency), and the distance they traveled to find the escape box (distance). If the mice did not find the escape box during the 180-seconds, they were prompted to enter the box. Regardless of whether they entered the box on their own, mice were required to remain in the box for 30-seconds before returning to their home cage. On day 24, mice were run through a single probe trial in which they were placed on the maze for 90-seconds with no escape box. The same analyses were conducted as well as the number of times each mouse returned to the hole the escape box was previously under (target revisits) and the relative amount of time they spent in each quadrant of the maze.

Stereological analysis

Total cell counts for the granular cell layers (GCL) and hilus of the dentate gyrus (DG) (mm3) were assessed by a blinded investigator using Optical Fractionator probe from MBF StereoInvestigator software (MicroBrightField, Williston, VT, USA) and an upright Olympus BX51TRF motorized microscope (Olympus America, Center Valley, PA, USA). Each contour consisted of either the dentate gyrus of the hippocampus or the hilus of the dentate gyrus. Grid size was set at 150x150mm with a 75x75mm counting frame, counting was performed used exhaustive analysis. Five serial coronal sections were used to analyze both hemispheres of the brain. Total estimated number for each hemisphere was combined and graphed as estimated total number.

Results

AraC reduces neurogenic potential in the DG by decreasing the number of proliferating cells

In a pilot study, we tested the capability of AraC to inhibit hippocampal neurogenic potential by infusing a 2% concentration via mini-osmotic pump for 7 days. To identify the effect on proliferating cells, we injected 2-bromodeoxyuridine (BrdU) for 5 days (Figure 3A) and found decreases in the number of BrdU-positive cells remaining in the dentate gyrus (DG) (Figure 3B-C) and subventricular zone (SVZ) (Figure 3D-E) after infusion of AraC (Figure 3C,E) when compared to control animals (Figure 3B,D). In addition, we qualified decreases in the amount of Sox2-positive NSPCs and DCX-positive neuroblasts after AraC infusion when compared to control animals (data not shown).

Effect of neurogenic ablation on learning and memory deficits

Previous have characterized learning and memory deficits following repetitive mild TBI [172, 174, 190] that we adapted to create our own surgery protocol after 7-days of 2% AraC infusion via mini-osmotic pump (Figure 4A). To confirm these results in our mice, we developed a shortened Barnes Maze protocol (Figure 4B) to test for deficits after injury. We performed repetitive mild TBI and sham surgeries following removal of AraC or vehicle treatments and found mice pre-infused with PBS, vehicle control display trending learning and memory deficits using Barnes Maze analysis at 24d post-onset of repetitive mild TBI which was reduced in mice receiving 2% AraC (Figure 4C). This implies a significant role of NSPCs in learning and memory deficits following repetitive mild TBI.

Effect of repetitive mild TBI and NSPC ablation on the DG niche

Next, we evaluated the effects of repetitive mild TBI on changes in hippocampal neurogenesis and whether these changes mediated cognitive dysfunction. To test for changes in proliferative capacity, we injected mice with 7-days of BrdU starting immediately following their first injury (Figure 4A). About two weeks following their final

injury, we identified significant differences in BrdU-positive cells in the DG of injured control animals when compared to sham, but no differences between AraC-treated groups (Figure 5A). Evaluation of neurogenesis showed that repetitive mild TBI induced a significant increase in the number of neuroblasts and/or new born neurons compared to vehicle-treated sham or AraC-treated mice by quantification of co-labeled BrdU+/Prox1+ cells, in the subgranular/granular layers of the DG (Figure 5B). However, we did not identify significant changes in the percent of BrdU-positive cells in the Prox1-positive lineage after injury in either experimental group, and instead found significantly increased percent of mature, non-mitotic NeuN-positive cells in AraC Sham animals (Figure 5C). Interestingly, the changes correlated with an increase in the number of c-Fos-positive cells in the DG of vehicle-treated repetitive mild TBI mice, indicative of hyperactivity in the neuronal niche that was not present in AraC-treated mice (Figure 5D). We also find a direct correlation between the number of errors on the Barnes Maze and the estimated number of c-Fos-positive cells in the DG of vehicle-treated mice that was not present in AraC-treated mice (Figure 5F). The presence Prox1-positive cells in the hilus was also increased following repetitive mild TBI, which was not significantly different between treatment groups (Figure 5E), implying a specific role for newborn neurons generated in the DG may mediate the observed learning and memory deficits Taken together, these data imply that the hippocampal neurogenic response contributes to learning and memory deficits following repetitive mild TBI.

Discussion

The process of neurogenesis has been linked to learning and memory capacity before and after TBI. Aberrant, or uncontrolled, neurogenesis occurs when the creation of new neurons goes awry. This can develop when mature neurons migrate to the incorrect place within the dentate gyrus, whether that is too high within the granular cell layers [113] or too low into the hilus [191]. In addition, aberrant neurogenesis is also the result of too many newborn neurons without enough cell death, resulting in an increase in synaptic potential of the niche [105, 108, 192]. Our study identifies aberrant neurogenesis following repetitive mild TBI, to be induced in two ways: by the deviant

migration of Prox1-positive dentate granular neurons into the hilus and by the creation of new neurons in the DG. By limiting the neurogenic response, we can conclude aberrant neurogenesis contributes to cognitive dysfunction following repetitive mild TBI.

Through our pilot study, we identified AraC, a known chemotherapeutic, that can be utilized to dampen the neurogenic potential of the hippocampal niche by limiting the number of dividing NSPCs [184]. AraC delivered via mini-osmotic pump at a 2% concentration for one week efficiently reduced the number of proliferating cells in the DG (Figure 3B-C) and subventricular zone (SVZ) (Figure 3D-E) without causing any adverse health-related side effects in the treated mice. The 4% concentration also efficiently reduced the NSPC population, but caused significant weight loss in mice (data not shown). In addition, we saw a decreased NSPC population and decreased number of DCX-positive neuroblasts in the DG (data not shown). While we see a significant effect in both the DG and SVZ neurogenic niches, the cells in the SVZ migrate become incorporated into the olfactory bulb and have little direct effect on hippocampal-dependent learning and memory function. Taken together, this pilot study proved a 2% infusion of AraC capably reduced the neurogenic potential of the hippocampal niche. For the succeeding experiments, mice were treated with 2% AraC or PBS-control for one week prior to undergoing repetitive mild TBI or sham surgeries (Figure 4A).

To confirm previously published learning and memory deficits after repetitive mild TBI [172], we created a novel, shortened Barnes Maze protocol (Figure 4B). Following repetitive mild TBI, PBS-treated animals were unable to recover their learning and memory capacity and made more mistakes prior to finding the goal box (Figure 4C). Notably, after treatment with AraC, there was no distinction between injured or sham animals. This implies a significant role for the neurogenic niche in injury-induced cognitive decline and recovery that needs to be further characterized. Interestingly, repetitive mild TBI induces aberrant migration of Prox1-positive cells to the hilus. This effect is not reversed by AraC-treatment, indicating it does not contribute to learning and memory deficits at this time-point.

Repetitive mild TBI induces substantial changes in the process of neurogenesis. PBS-treated mice have significantly more BrdU-positive cells in the dentate gyrus (DG) following injury when compared to sham animals. This increase in proliferative potential

is dampened in mice treated with AraC, with no significant differences between injured and sham groups (Figure 5A). While the effect could be due to cells in the DG undergoing DNA repair, the lack of increase in the AraC-treated injured animals suggests an increase in proliferation after injury.

After repetitive mild TBI, there are trending increases in the number of cells that have taken on a neuronal lineage and are co-labeled with Prox1 or NeuN and BrdU in PBS-treated animals (Figure 5B). This trend is not present in AraC-treated animals. Interestingly, the number of mature, NeuN-positive newborn neurons is still increased in AraC sham animals in comparison to AraC injured animals. The expression of Prox1 begins when NSPCs commit to the neuronal lineage as neuroblasts and remains expressed in dentate granular neurons as the differentiate into mature NeuN-positive neurons. Therefore, we can identify the number of mitotic neuroblasts by taking the number of newborn, mature NeuN-positive cells out of the total number of co-labeled Prox1-positive cells. This will give us the DCX-positive population of cells. The number of neuroblasts appears to be slightly increased following injury regardless or treatment, implying a significant amount of the cells have likely taken on a non-mitotic immature neuron or mature neuron state.

To determine the effect of injury on the cell fate of BrdU-positive cells, we took the number of cells co-labeled with each marker and normalized it over the total number of BrdU-positive cells. Interestingly, when presented as a percent, the final cell fate does not appear to increase along the neuronal lineage following repetitive mild TBI. Interestingly, AraC sham mice appear to have a significant amount of newborn mature neurons (Figure 5C). This could be due to an increased rate of differentiation after AraC treatment that is dampened after injury. Overall, the limited number of BrdU-positive cells in AraC-treated mice restricts neurogenic potential, and the number of cells added to the population may provide more insight to the neurogenic behavior within the niche. .

Notably, there is a slight increase in hyperactive, c-Fos expressing cells in the DG following PBS-treated injured animals that is not present in AraC-treated mice (Figure 5D). NSPCs, immature neurons, and mature neurons can all communicate with surrounding cells and contribute to this hyperactivity with increased numbers of newborn cells in the niche. Further, in PBS-treated animals this hyperactivity directly correlates

with learning in memory deficits (Figure 5F). However, after NSPC ablation using AraC, the hyperactivity no longer correlates with cognitive deficits. This implies that the NSPC population is playing a crucial role in the neuronal behavior of the hippocampal niche.

Finally, we identified an injury-dependent surge in the number of Prox1-positive cells in the hilus of both PBS- and AraC-treated animals (Figure 5E). Since this increase occurs after repetitive mild TBI regardless of treatment, we can conclude it is likely not contributing to learning and memory deficits during this time point. In long-term experiments, it is likely these cells survive, incorporate into the hippocampal synaptic network, and add to hippocampal dysfunction, but this is independent of NSPCs and neurogenesis.

In summary, these findings link the NSPC response to short-term behavioral deficits following repetitive mild TBI. However, the characterization of this response as part of chronic learning and memory deficits remains unknown. The neurogenic response could continue to be awry, or the injured mice may demonstrate a delay in cognitive recovery. Further, a more specific NSPC ablation technique will more directly link the direct contribution of these cells to memory shortfalls without the off-target effects on proliferating cells in the periphery, such as the highly proliferative microbiome. Our research has highlighted a key gap in hippocampal neurogenesis-related knowledge following repetitive mild TBI. Taken together, these findings provide evidence that aberrant neurogenesis contributes to cognitive decline following repetitive mild TBI, and should continue to be investigated. Given the importance of endogenous neurogenesis in learning and memory recovery from TBI, augmenting this activity could be a favorable opportunity for future treatments.

Author Contributions

Kisha Greer and Michelle Theus, PhD conceived and designed the experiments. Kisha Greer collected and analyzed the data. Xia Wang bred and maintained animals for testing. Kisha Greer compiled the information and wrote this paper.

Figures

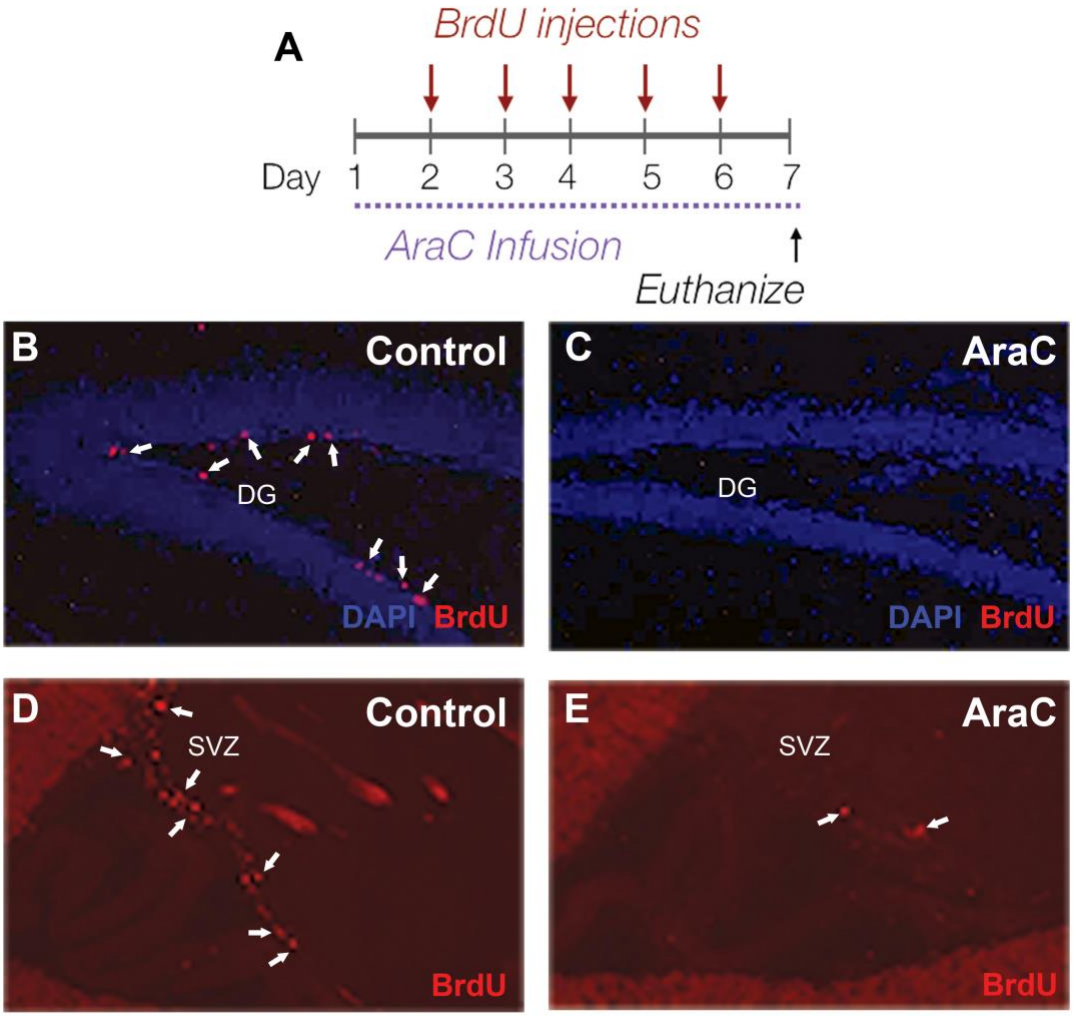

Figure 3. AraC Pilot Study. (A) After 7-days of 2% AraC infusion, (B,D) there are significantly more proliferating cells in the dentate gyrus (DG) and subventricular zone (SVZ) of control animals (C,E) when compared to animals treated with 2% AraC. BrdU-positive cells are depicted with white arrows.

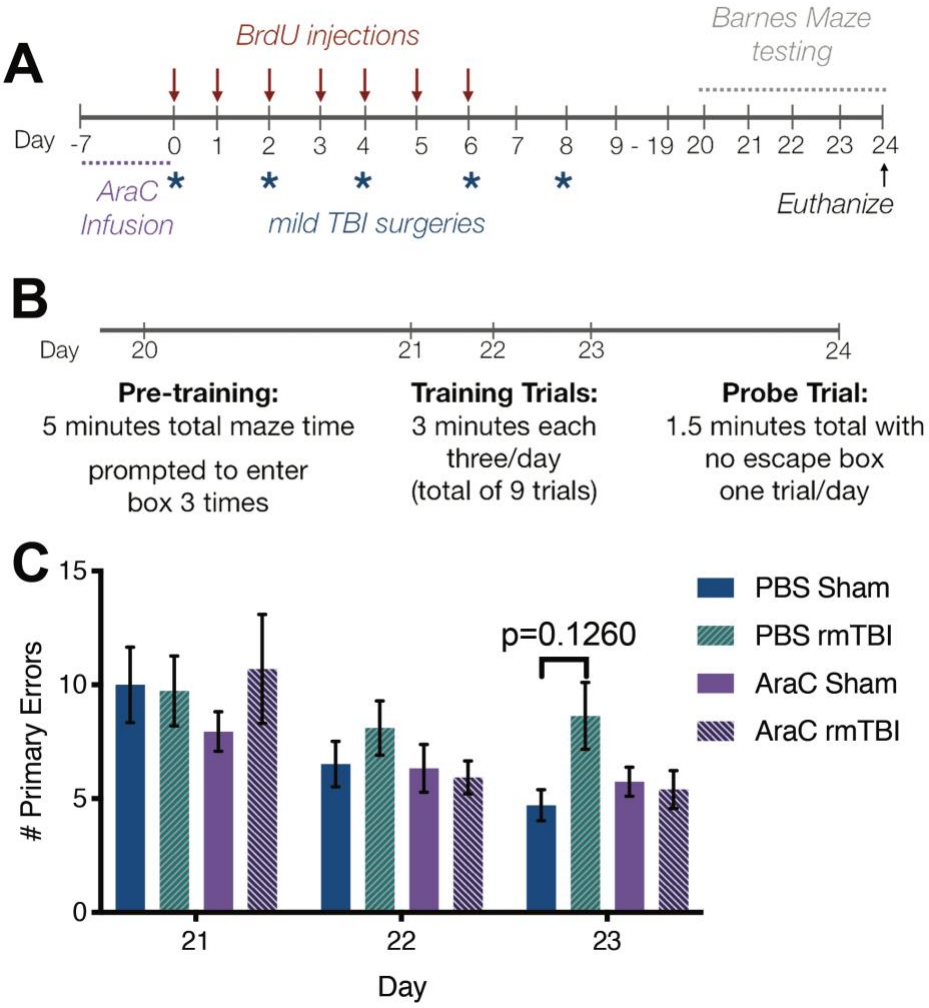

Figure 4. AraC treatment alleviates cognitive decline. (A) Mice were treated with AraC or PBS control via mini osmotic pump for 7-days prior to repetitive mild TBI surgeries every other day during which BrdU was injected daily for the first 7-days. (B) Barnes Maze testing consisted of pre-training, training, and probe trials where collected data showed (C) an increased number of primary errors between PBS-treated animals but not AraC treated during the Training Trials.

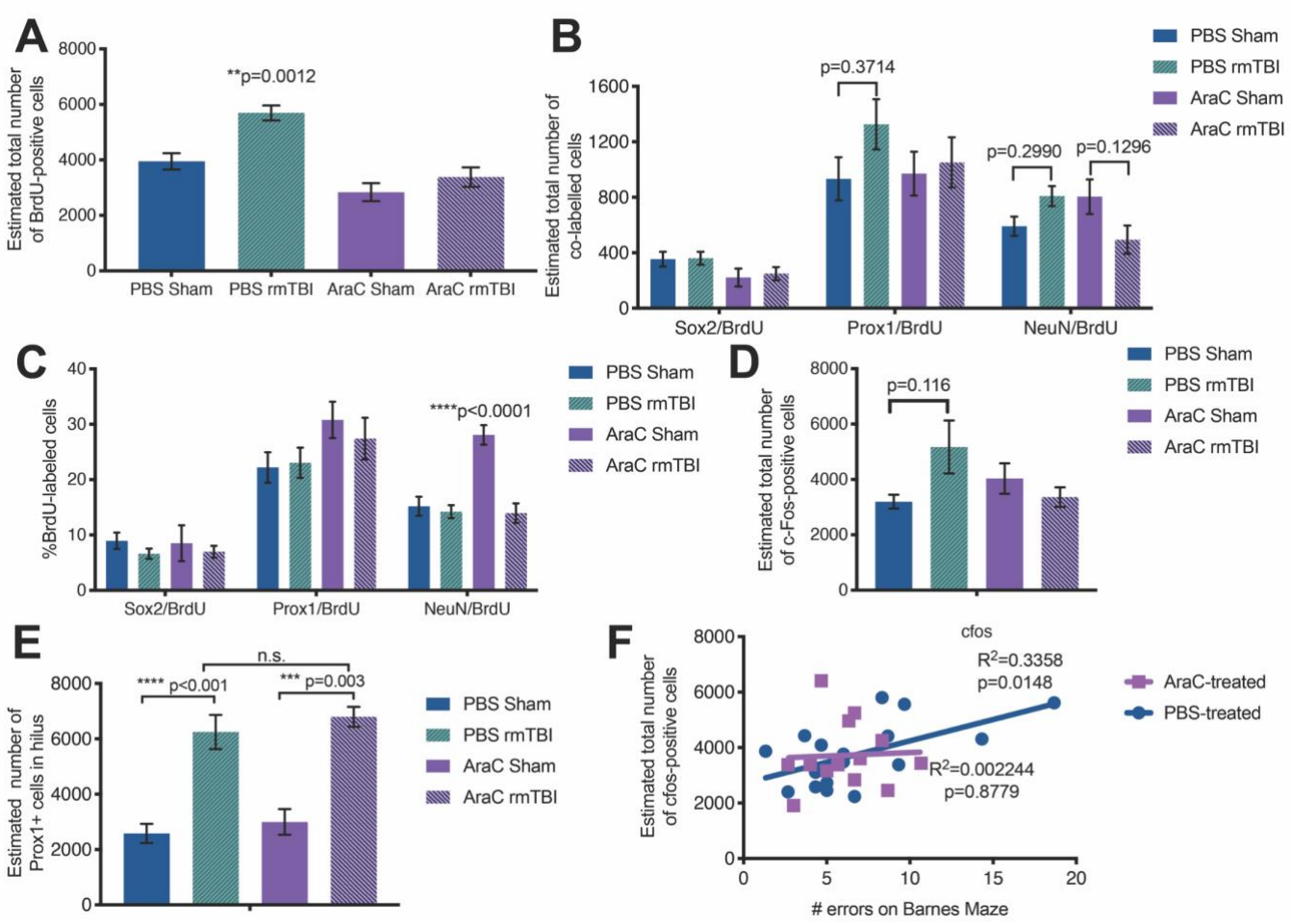

Figure 5. AraC treatment significantly affected the neurogenic response following repetitive mild TBI. (A) There are significant increases in the number of BrdU-positive cells following repetitive mild TBI in PBS-treated animals, but not in AraC-treated animals. (B)Of these BrdU-positive cells, more are Prox1- and NeuN-positive after injury in the PBS animals but not in the AraC animals. However, when (C) the percent of co-labeled cells is taken over the total number of BrdU-positive cells, there are no significant increases in the neuronal population after injury in either group. These results compare to (C) increased number of c-Fos positive cells after injury in control groups with (F) a direct correlation between the number of c-Fos cells counted in the dentate gyrus to the number of errors on the Barnes Maze. This effect was seen in control mice, but there was no correlation after AraC treatment. In addition, (E) treatment with AraC did not prevent injury-induced migration of Prox1-positive cells into the hilus.

Chapter 3

Aberrant neurogenesis accompanies learning and memory deficits following repetitive mild traumatic brain injury

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Abstract

Every year, millions of patients will go to the emergency room to be treated for a concussion, or mild traumatic brain injury (TBI), with military personnel and athletes often being treated for their second injury. Unlike in more severe types of TBI, the effects of repetitive mild TBI on adult hippocampal neurogenesis have not yet been investigated. Our research has characterized a unique amplified neurogenic response following TBI that is highlighted through an increased number of newborn neurons in the dentate gyrus (DG) of the hippocampus and indicative of aberrant neurogenesis. Further, we correlated aberrant adult hippocampal neurogenesis with learning and memory deficits and increased hyperactivity as seen via c-Fos activation after injury. Interestingly, this response takes place starting about two weeks following the final injury, rather than just the few days it takes to see neurogenic changes in more severe TBI models. In addition, we identified more Prox1-positive cells aberrantly migrated in the hilus of injured animals. This research continues to characterize the distinct reaction of the neuroblast population in the DG to TBI and specifically identifies the delayed role of aberrant adult hippocampal neurogenesis in the negative learning and memory outcomes of repetitive mild TBI .

Introduction

More than 5 million people will go to the hospital to be treated for a traumatic brain injury (TBI) in the United States each year. When these patients present to the clinic, their symptoms help classify their injuries into severe, moderate, or mild TBIs, with more than 70% of TBI-related emergency room visits being classified as mild, or concussions [15]. Patients presenting with mild TBIs show a variety of physical and emotional symptoms that typically resolve within two weeks and do not develop into chronic conditions [193]. However, even after symptoms have resolved, patients are still susceptible to further damage as those that go on to receive subsequent concussions will develop long-term neurological deficits [163], such as chronic traumatic encephalopathy (CTE) [194, 195]. The mechanisms underlying the delayed development of chronic

neurodegenerative diseases following repetitive mild TBIs are under substantial investigation, but researchers have yet to determine whether the adult hippocampal neurogenic niche contributes to these cognitive losses.

Adult hippocampal neurogenesis is the creation of new neurons from neural stem/progenitor cells (NSPCs) in the dentate gyrus (DG) of the hippocampus. During this multi-faceted process, a Type I neural stem cell is activated to divide or proliferate prior to beginning their differentiation into a Type II neural progenitor cell. These neural progenitors continue to divide and differentiation into Type III neuroblasts that can migrate radially through the granular cell layers (GCL) of the DG [182]. Over time, the mitotic neuroblasts can develop small synaptic connections with surrounding neurons and continue to mature into young, non-mitotic neurons that further develop synaptic maturity until they are fully incorporated into the hippocampal network as established, mature neurons [196]. Under normal conditions, it can take 4-5 weeks for a newborn neuron to be created from a neural stem cell, but in injured environments this mechanism can be accelerated or delayed [183, 197].

The process of neurogenesis has been largely linked to learning and memory capacity [198]. In homeostasis, limiting the process of adult hippocampal neurogenesis by an array of NSPC ablation techniques leads to decreased learning and memory capacity [37, 38]. Conversely, expanding the neurogenic potential by boosting the NSPC proliferative capacity drives increased learning and memory capacity [51]. The contribution of the surrounding cells in the hippocampus has also been investigated, with a significant number of cells influencing the process of neurogenesis through cellular release factors or direct cell-cell contact [199, 200]. If the surrounding environment is significantly changed, it can lead to uncontrolled, or aberrant neurogenesis. Increases in aberrant neurogenesis negatively contribute to learning and memory shortfalls, while decreasing this aberrant response has been shown to alleviate cognitive deficits [108]. Previous findings have also linked injured environments to neurogenic reductions and rises and found subsequent decreases and increases in learning and memory deficits, respectively [74].

Following a single hit in moderate traumatic brain injury (TBI), neuroblasts undergo an immediate selective cell death. To fill the gap in the niche, the NSPCs are activated

to proliferate and differentiate to create new neuroblasts, therefore significantly increasing the process of neurogenesis in just a matter of days [62]. This injury-induced cell death followed by a substantial expansion of the neurogenic niche correlates with learning and memory deficits and cognitive recovery, respectively [184]. Further, by limiting this neurogenic response through NSPC ablation, previous studies have promoted injury-related learning and memory deficits and delayed cognitive recovery [62, 184]. While the effect on single hit mild is not as pronounced [116], no one has investigated the influence of multiple mild traumatic brain injuries on the process of neurogenesis.

The following study will discern the effects of repetitive mild traumatic brain injury (TBI) on the immediate and delayed response of adult hippocampal neurogenesis using a previously published mouse model. We classify specific increases in differentiation of NSPCs along the neuronal lineage that present immediately following injury and are still present about two weeks later. We further correlate injury-induced dentate gyrus hyperactivity with learning and memory deficits about two weeks but not immediately following the final injury. These delayed aberrant neurogenic responses are unique to repetitive mild TBI, and our findings continue to fill the gap in knowledge regarding the specific response of the NSPC population to repetitive mild traumas.

Materials and Methods

Repetitive Mild Traumatic Brain Injury Protocol.

Male mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) subcutaneous injection and positioned in a stereotaxic frame. Body temperature was monitored with a rectal probe and maintained at 37˚C with a controlled heating pad set. Injury was induced by a program controlled cortical impactor (Φ=5-mm flat tip) connected to an eCCI-6.3 device (Custom Design & Fabrication, LLC) at a velocity of 5.0 m/s, depth of 1.0 mm, and 200 ms impact duration. Following injury, the incision was closed using Vetbond tissue adhesive (3M, St. Paul, MN, USA) and the animals were placed into a heated cage and monitored every 20 minutes until fully recovered from anesthesia. Repeated surgeries for TBI and sham were conducted once every 48 hours for a total of 5 surgeries [172, 190].

Barnes Maze Evaluation.

Mice were evaluated for learning and memory utilizing the Barnes Maze (Maze Engineers, Boston, MA USA) and the Ethovision XT software (Noldus, Leesburg, VA USA). Mice were first pre-trained to enter the escape box by allowing them to freely explore the maze for 5-minutes total with no anxiety-inducing lights. During this training phase, mice were prompted to enter the hole 3 times, once at the very beginning and once at the very end of the 5-minute trial if they had not already entered the escape box. No analyses were collected during the training phase. The following three days (days 21-23 post primary-injury), mice were evaluated for distance, time, and the number of errors to find the escape box each day for 180seconds/trial in 3 trials/day for a total of 9 trials. During each of these acquisition trials, if the mice did not enter the escape box, they were prompted to enter and remain for 30seconds before returning to their home cage. On the final day (24 days post-primary injury), mice were placed on the maze for 90seconds with no escape box. During this probe trial, the same analyses were recorded as the acquisition phase and included the number of times the mouse returned to the target hole (target revisits) and the relative amount of

Tissue Processing and Staining.

For each histology group, brains were harvested at either 9-days or 24-days post-primary injury and immediately cryo-preserved using dry ice. Whole brains were processed on a cryostat (Cryostar, Fisher Scientific, Pittsburgh, PA, USA) into five 30□m coronal sections spaced ten sections apart and placed in the -80C freezer until use. Slides from the -80C freezer were placed on a slide warmer for 15-30m prior to fixation with 10% formalin (Fisher Scientific, Pittsburgh, PA, USA) for 10m followed by three 5-minutes 1X PBS washes. For BrdU staining, slides were placed into a 4% Triton-X solution in PBS for 5-minutes and washed three times with 1X PBS. Sections were then placed into room temperature (RT) or slightly warmed 2N HCl acid and placed in a 37C incubator for 30-45 minutes then transferred into 0.1M sodium borate buffer for 10minutes, washed with 1X PBS then placed in 2% fish gelatin (Sigma, #G7041, St. Louis, MO, USA) for 2-4 hours at RT. Primary antibodies were applied at the appropriate dilution in 2% fish gelatin and left overnight in the 4°C. The following day, sections were

washed with 1X PBS before placing into a 1X PBS solution with secondary antibodies for 1.5-2 hours (1:250 donkey anti-rabbit Alexa-Fluora 488 and 1:500 donkey anti-rat Alexa-Fluora 594). The sections were washed with 1X PBS before mounting in DAPI-G Fluormount (Southern Biotech, Birmingham, AL, USA). For sections without BrdU processing, slides were fixed in 10% formalin for 10 minutes, washed with 1x PBS then placed in blocking buffer for 1-2 hours prior to adding primary antibody in blocking solution.

Results

Repetitive mild TBI induces learning and memory deficits via Barnes Maze analysis

To explore the contribution of neurogenesis to learning and memory deficits following repetitive mild TBI, we utilized a previously established mouse model (Figure 6A) [172, 190]. We subjected mice to repetitive mild TBIs or shams and demonstrate an increased expression in GFAP immunoreactivity (Figure 6F) in the cortex (Figure 6E3-F3), entorhinal cortex (Figure 6E1-F1), and hippocampus (Figure 6E2-F2) of repetitive mild TBI mice (Figure 6D-F) compared to repetitive sham-injured mice (Figure 6B-D). In addition, we developed a new, shortened protocol to test for learning and memory deficits using the Barnes Maze (Figure 7A). Through this shortened protocol, we identified significant learning and memory deficits in the Barnes Maze on the last day of the Acquisition Phase through primary errors (Figure 7B) at 24-days post-onset of repetitive injury compared to sham mice. Furthermore, mice developed a preference for escape in the probe trial as shown by the number of target revisits (Figure 7C). These findings further confirm that repetitive mild trauma to the murine brain elicits significant learning and memory deficits in the sub-acute phase of recovery.

Injury does not stimulate proliferation, but increases neuronal lineage of NSPCs

Next, we wanted to examine the effect of repetitive mild traumatic brain injury on the process of neurogenesis. Five days of BrdU injections does not stimulate proliferation (Figure 8A), and there are no significant changes in the number of dying, TUNEL-positive cells in the DG (Figure 8B). We did identify a rise in the total number of DCX-positive

neuroblasts in the DG two weeks (24d) following repetitive mild TBI (Figure 8C) that correlated with an increase in the number of Prox1-positive cells co-labeled with BrdU (Figure 8D), a sign of newborn neurons. This neuronal lineage increase was also seen in cell fate analysis of both Prox1-positive and NeuN-positive cells two weeks after last surgery, but injury only induced significant increases in Prox1-positive cells (Figure 8E). Little changes happen to the NSPC population and there are no significant changes in number (Figure 8D) or percent (Figure 8E) of co-labeled Type I and Type II NSPCs that express Sox2. These results indicate a neuroblast-specific response as part of the aberrant neurogenic process underlying learning and memory shortfalls associated with repetitive mild TBI.

Prox1-positive cells aberrantly migrate after injury

After characterizing the effect of repetitive trauma on neurogenesis, we wanted to examine the effect on the migration of neuronal cells. After repetitive mild TBI, it takes about two weeks for there to be a significant increase in the number of Prox1-positive cells found aberrantly migrated into the hilus of the DG (Figure 8F). These cells do not appear to be co-labeled with BrdU (data not shown).

Increased number of newborn neurons contributes to hyperactivity in the DG

Finally, we began to examine the effect of repetitive injury on firing potential in the dentate gyrus. We found significant increases in hyperactivity within the DG via c-Fos activation after repetitive mild TBI (Figure 8G). Further, this quantified increase in hyperactivity correlates with learning and memory deficits as shown via Barnes Maze analysis as the number of Target Revisits (Figure 8H). Taken together, this implies a significant role for hyperactivity in learning and memory deficits following repetitive mild traumatic brain injury.

Discussion

The process of adult hippocampal neurogenesis is highly regulated, and any minor changes can have meaningful impacts on hippocampal circuitry and cognitive

performance. Aberrant, or uncontrolled, neurogenesis underlies many cognitive-related issues caused be disorders such as epilepsy and traumatic brain injury (TBI) [105, 108, 192, 201]. In these previous findings, the neurogenic process is disrupted by uncontrolled proliferation, migration, differentiation, or survival of NSPCs in the hippocampal niche and contributes to cognitive losses [113, 202]. In the present study, our lab has also categorized these uncharacteristic neurogenic responses following repetitive mild traumatic brain injury and demonstrated aberrant neurogenesis by two mechanisms: the incorrect migration of neuronal cells into the hilus and the creation of new cells in the DG niche independent of cell death. The process of repetitive trauma-induced aberrant neurogenesis is characterized as soon as two weeks after the final injury.

The proliferative response of NSPCs in the hippocampal niche is critical to cognitive recovery following a single hit, moderate TBI [62]. However, with this experimental design, we do not see significant injury-related increases in the number of BrdU-positive cells remaining 9-days or 24-days after repetitive moderate TBI (Figure 8A). We did see a decrease in the number of BrdU cells remaining over time, which implies these cells are either not surviving to the 24-day time point or are migrating outside of the granular cell layers within the DG. Since there are no increases in dying, TUNEL-positive cells immediately following the final injury or two weeks later (Figure 8B), we can conclude the cells are migrating outside the GCLs.

For this study, we utilized four nuclear markers to distinguish between three distinctive stages of neurogenic differentiation. The first marker, Sox2 is expressed in both Type I and Type II NSPCs, and these cells are still able to undergo glial lineage. The second marker, doublecortin (DCX) is expressed in Type III neuroblasts. Our third marker, Prox1, is also expressed as cells enter the early neuronal lineage as Type III neuroblasts stage and remains expressed as the cells reach their full neuronal maturity. Finally, to discriminate between the neuroblasts and mature neurons, we utilized NeuN, which is only expressed in mature neurons that are no longer mitotically active and able to proliferate [182]. We first quantified the number of DCX-positive cells after repetitive mild TBI and found significant increases about two weeks after injury that were not present the day after the final injury (Figure 8C). To evaluate the differentiation status of the BrdU-positive cells, we quantified both the total number and the cell fate of the co-

labeled cells for each of the three markers. To calculate cell fate, we divided the number of co-labeled cells for each marker over the total number of BrdU-positive cells in the DG. Unlike total number, this allows us to visualize the overall population of cells remaining after injury. Immediately following injury, there are no significant changes in the differentiation status of cells between the injured and sham groups. (Figure 8D-E). About two weeks later, we found significantly more cells in the neuronal lineage (Figure 8D-E) as Prox1-positive neuroblasts and NeuN-positive mature neurons. Whether this is at the expense of the glial lineage is under investigation.

The total number of co-labeled cells implies an increase in the number of cells within a population. Immediately after repetitive injury, there are significant increases in the number of Prox1-positive cells that have incorporated BrdU, indicating an activation of the mitotic neuroblast population. Interestingly, this same population still has an increased number of Prox1-positive cells two weeks later, with no changes in the number of NeuN-positive mature neurons. Further, we quantified the number of DCX-positive neuroblasts two weeks after injury and found increases within the granular cell layers (GCLs) of the hippocampus. Since there are no significant changes in cell death in injured animals at either time-point (Figure 8B), these results imply a stoppage in the differentiation of neuroblasts that contributes to a delayed aberrant neurogenic response.

The migration of neuronal cells into the hilus can lead to uncontrolled firing within the hippocampal niche, and could underlie learning and memory deficits [184]. Immediately following the final repetitive mild injury, there is no difference in the number of aberrantly migrated Prox1-positive cells into the hilus (Figure 8F). Nonetheless, there are significantly more Prox1-positive cells in the hilus two weeks after the final injury when compared to sham animals at the same time point. However, our previous reports have found no link between learning and memory capacity and the number of cells in the hilus (Figure 5E). While these Prox1-positive cells are not adding to learning and memory deficits two weeks after the final repetitive hit, if they continue to survive and mature in the hilus, their synaptic connections could trigger irregular hippocampal circuitry during chronic time points. Interestingly, none of these cells were co-labeled with BrdU, implying injury is likely leading to the aberrant migration of neuroblasts into the hilus independent of the proliferative reaction.

Further, we identified significant increases in the hyperactivity of the DG with increased c-Fos expression about two weeks that are not present directly following injury (Figure 8G). The hyperactivity directly correlates with learning and memory deficits (Figure 8H), implying the increased c-Fos activation underlies cognitive shortfalls at the two-week time point. The birth of new neurons without cell death in the existing circuitry could produce aberrant neurogenesis and contribute to the hyperactivity that underlies learning and memory deficits.

Our findings have identified a unique neurogenic response to TBI. Unlike after more severe TBIs, changes in neurogenesis following repetitive mild TBI do not manifest until about two weeks after the final trauma has occurred. Notably, we have further characterized a group of cells that are inclined to respond following TBI. After a single hit, moderate TBI, the neuroblasts selectively apoptosis immediately following injury and lead to learning and memory deficits. However, after repetitive mild TBI, the increased differentiation and survival of cells in the neuroblast cell state is negatively contributing to learning and memory deficits. Taken together, these findings have established an aberrant neurogenic response at a sub-acute time following repetitive mild TBI that should continue to be investigated at chronic time points. Other future directions will identify niche-related factors underlying this aberrant neurogenic response.

Author Contributions

Kisha Greer and Michelle Theus, PhD conceived and designed the experiments. Kisha Greer collected and analyzed the data. Xia Wang bred and maintained animals for testing. Alison Cash assisted in perfusion of animals for DCX analysis. Kisha Greer compiled the information and wrote this paper.

Figures

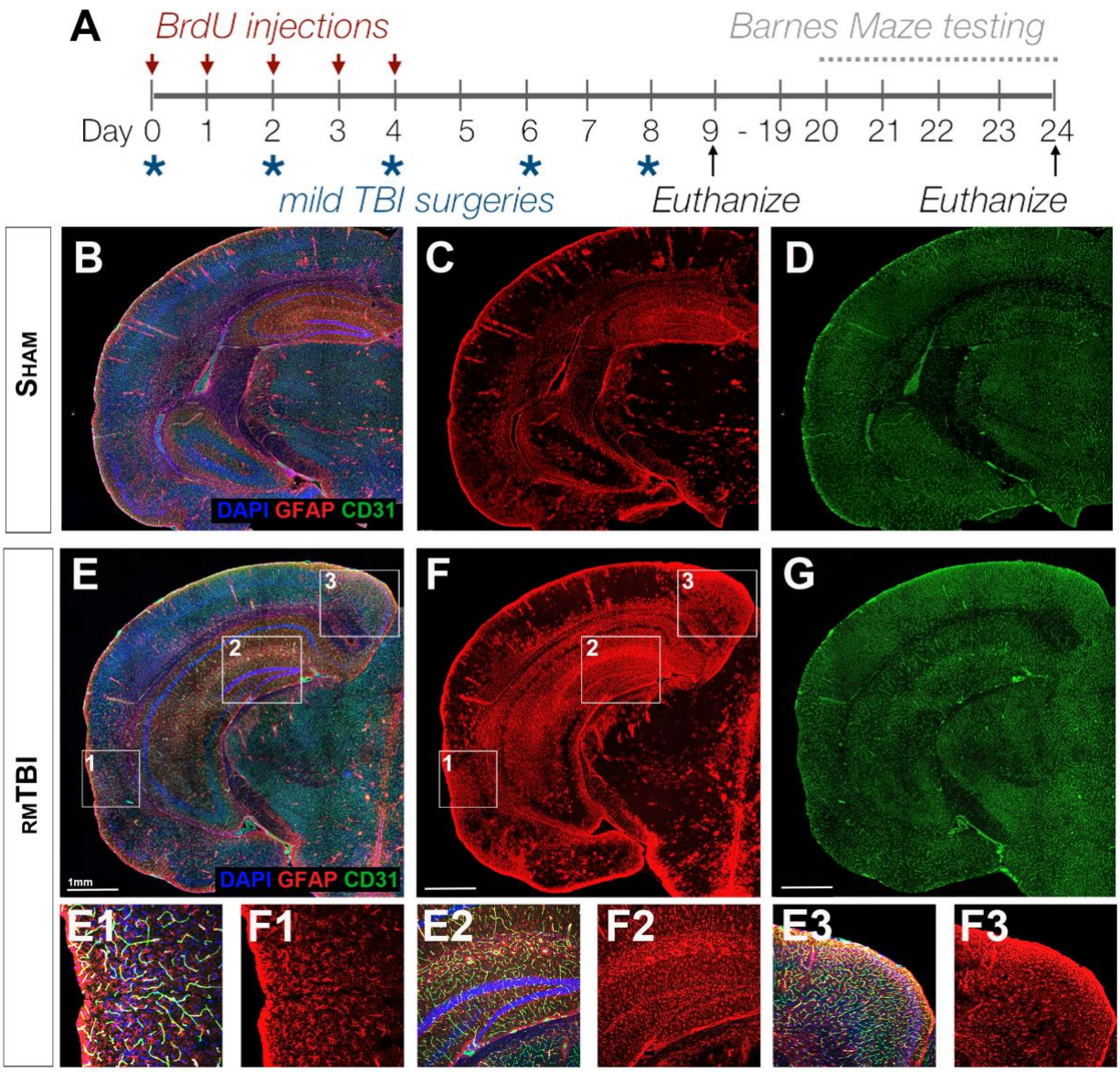

Figure 1. Increased astrogliosis following repetitive mild TBI. (A) About two weeks following injury (24-days post-primary injury), (E-G) there appears to be an increased expression of astrogliosis (GFAP, red) when compared to (B-E) sham animals that show limited astrocyte activation. Specifically, increases are seen in the (E1-F1, E3-F3) cortical areas and (E2-F2).

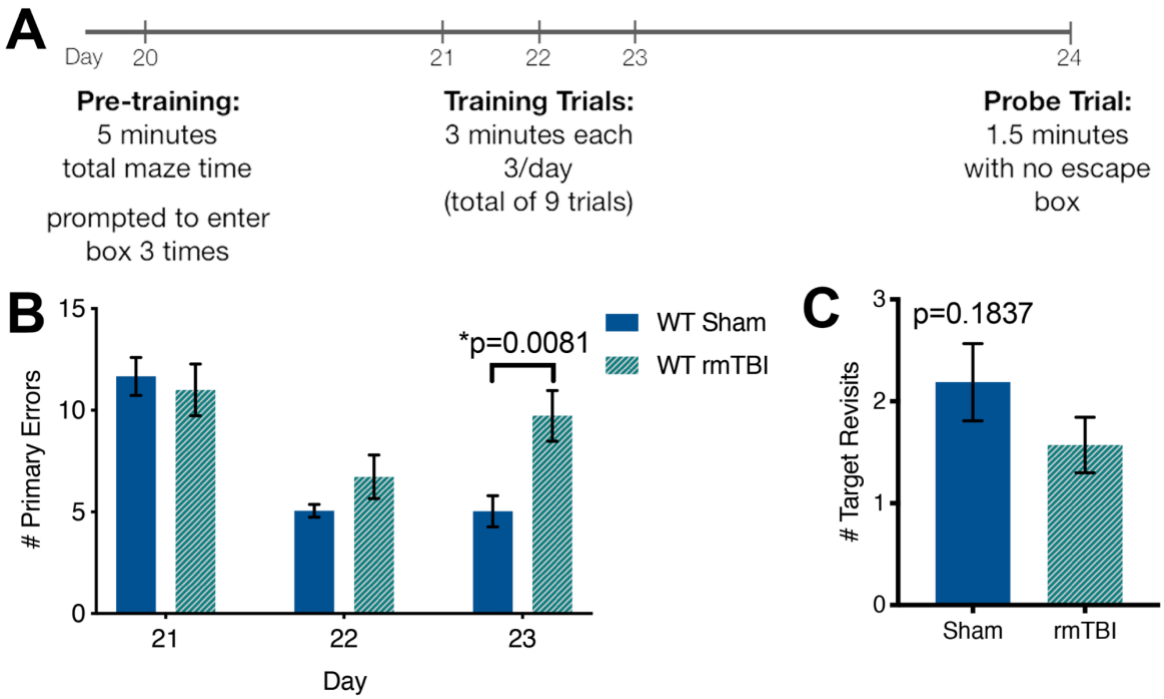

Figure 2. Repetitive mild TBI induces learning and memory deficits. (A) Experimental Timeline shows data from Training Trials, about two weeks after the final injury. (B) Injured mice make more errors before finding the hole on the last day of the Training Trials, and (C) do not revisit the target hole as often as Sham animals during the Probe Trial.

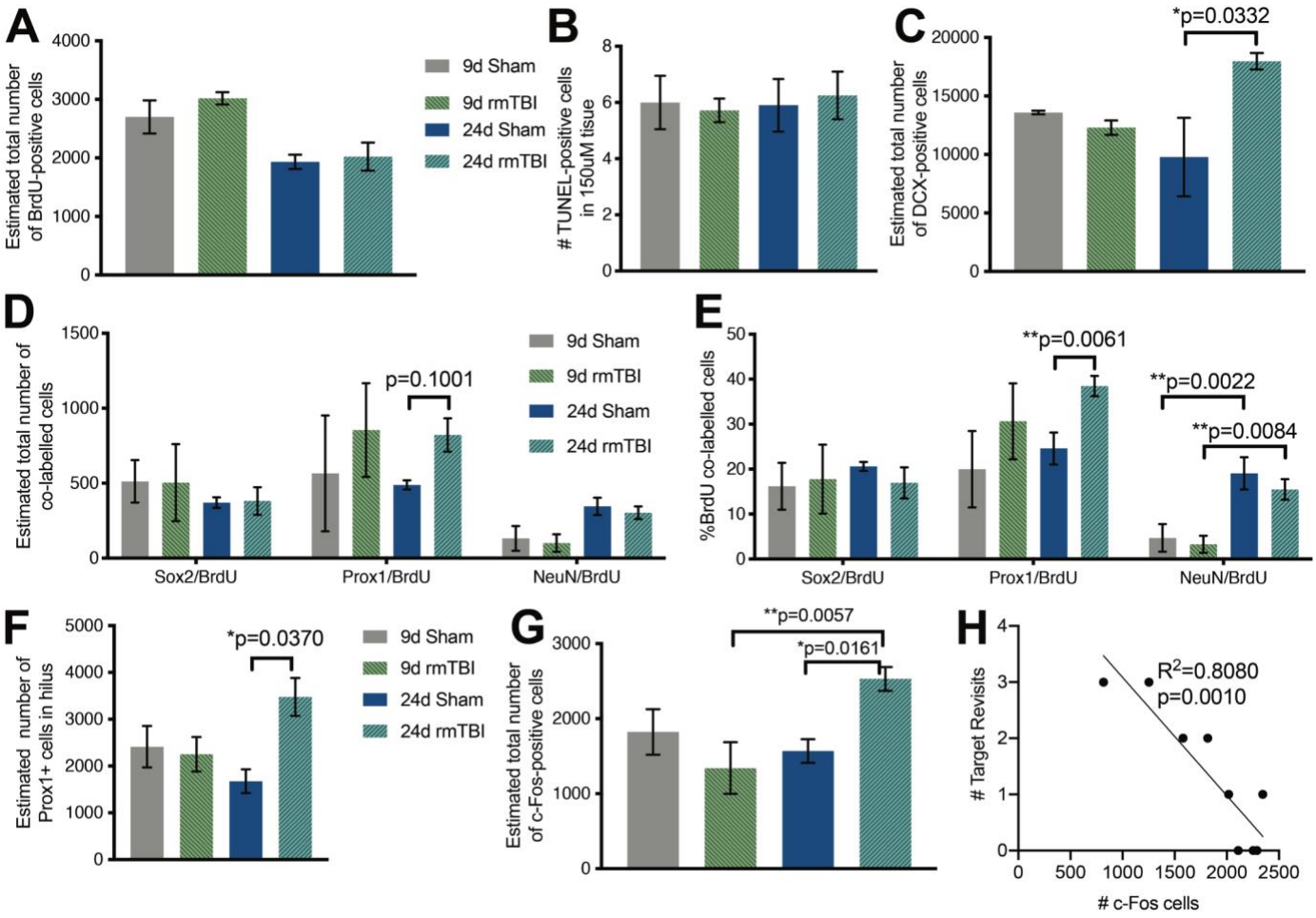

Figure 3. rmTBI induces changes in neurogenesis at 24d post-initial impact. (A) There is no proliferative response following injury at either time point, nor (B) is there an increase in cell death. (C) There are significantly more DCX-positive cells about two weeks after injury when compared to sham animals. (D)Of the BrdU-positive cells, there are more co-labeled with Prox1 about two weeks after injury and (E) also when taken as a total percent of BrdU-positive cells. (F) There is a significant increase in the number of Prox1-positive cells in the hilus two weeks after injury. (G) c-Fos activity is increased two weeks after injury, but not one day after injury. (H) The number of c-Fos-positive cells directly correlated with the number of Target Revisits on the Barnes Maze.

Chapter 4

Endothelial-specific EphA4 plays a role in aberrant neurogenesis and cognitive deficits following repetitive mild TBI

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Abstract

Traumatic brain injury (TBI) is a leading cause of cognitive disability in the United States each year, with more than 70% of diagnosed patients experiencing a concussion or mild TBI. While previous findings have characterized the neurogenic contribution to cognitive deficits in more severe TBIs, little is known regarding adult hippocampal neurogenesis in learning and memory outcomes following repetitive mild TBI. We previously demonstrated these negative outcomes underlie aberrant neurogenesis as evidenced by increased numbers of neuroblasts in the dentate gyrus (DG) and hilus fo the hippocampus. The changes also correlated with increased c-Fos immunoreactivity implying hyperactivity. In the following study, we evaluated the role of EphA4 in the hippocampal neurogenic response. Utilizing endothelial cell (EC)-specific EphA4 knockout mice (EphA4-KO), we characterized a unique vascular contribution to aberrant neurogenesis and learning and memory deficits after repetitive mild TBI. These findings uncover an important role for the niche vasculature in the injury-induced neurogenic responses in the hippocampus.

Introduction

The ability to precisely store and retrieve memories in the brain is an essential component of daily life. Adult hippocampal neurogenesis guides this process, with stimulations in neurogenesis enhancing learning and memory capacity [74, 203, 204] and inhibitions in neurogenesis dampening learning and memory capacity [38]. The process of adult hippocampal neurogenesis is highly coordinated mechanism in which neural stem/progenitor cells (NSPCs) are stimulated to proliferate, migration, differentiation, and survive to become new neurons, and is regulated by a variety of factors that come from surrounding cells in the niche. Interestingly, the endothelial cells (ECs) that make up the vascular system are in a unique position to contribute both direct cell-cell contact and cellular release factors that can stimulate or dampen different parts of the neurogenic process [100, 205].

As the process of neurogenesis begins in the hippocampus, quiescent Type I neural stem cells (NSCs) are in the subgranular zone (SGZ) of the dentate gyrus (DG) and extend processes up through the granular cell layers to wrap around endothelial cells and communicate via direct cell-cell contact. When the NSC is activated to proliferate, it losses these extended processes and takes on a ball-like morphology, gathering in small groups near blood vessels as they take on the Type II structure. As they continue to differentiate, Type III neuroblasts remain in close contact with ECs, migrate to their destination a few cells up the granular cell layer, and extend processes to form synaptic connections with surrounding neurons [182]. These neuroblasts continue to differentiate and mature into dentate granular neurons that have fully developed dendritic arborizations and axonal projections that are essential to be incorporated into the hippocampal neuronal network. (Figure 1). Throughout the course of neurogenesis, the endothelial cells can regulate proliferation [100, 205], migration [206, 207], differentiation [103, 205], and survival of NSPCs via direct cell-cell contact and by secreting a wide variety of cellular factors.

Previous findings stimulated cerebral blood flow in the dentate gyrus of the hippocampus with exercise and found significant stimulation of adult hippocampal neurogenesis [208]. Further, preceding research has characterized the EC-specific secretion of vascular endothelial growth factor (VEGF) to drive both EC proliferation and NSPC proliferation in the DG [209-212]. ECs also secrete brain-derived neutrophic factor (BDNF) to promote survival and differentiation of neuronal precursors [213]. In addition, endothelial cells can directly contact NSPCs and influence their behavior during the process of neurogenesis [100, 214, 215]. Interestingly, research has identified the Eph/ephrin signaling pathway, a family of receptor tyrosine kinases and their ligands, as key regulators of the neurovascular unit that influence surrounding cells through direct cell-cell contact and by secreting factors involved in neurogenesis [216, 217].

Eph receptors and ephrins are divided into two classes: A and B, with each receptor typically binding to ligands from their respective class. Uniquely, Eph receptors and their ligands are both cell-membrane bound and able to initiate intracellular signaling cascades as part of bi-directional, forward/reverse signaling. Eph/ephrin signaling is well-characterized for its role in cell repulsion and migration, a key part of cell migration during

neurodevelopment. However, recent studies have implicated the Eph/ephrin pathway in a variety of adult neurological mechanisms, including neurogenesis and learning and memory [99]. Of note, researchers identified a critical role for the ephrin-A5/EphA4 complex in mediating proliferation and survival in the DG and spontaneous recurrent seizures in temporal lobe epilepsy [103].

Prior studies identified the Eph/ephrin signaling pathway as crucial to induce cellular alterations in the hippocampus to regulate learning and memory [218]. Interestingly, a range of studies have also focused on EphA4 as a target for learning and memory capacity. Unlike other Eph receptors, EphA4 can bind to many ephrins from both the A class and the B class, making it an excellent target to address the general influence of Eph/ephrin signaling. Past findings have characterized short-term spatial recognition deficits in EphA4 knockout mice [219, 220], and EphA4 knockdown *in vitro* influenced NSPC differentiation [101]. To date, few studies have sought to understand the cell-specific influences of vascular-specific EphA4 signaling on neurogenesis and learning and memory, after traumatic brain injury.

While most TBI-related animal studies of EphA4 have characterized overall responses within the hippocampal niche, our research seeks to identify the specific vascular influence on neurogenesis after repetitive injury. Our study will utilize a Tie2 driven, endothelial cell (EC)-specific knockout of EphA4 (EphA4$_{f/f}$/Tie2::Cre)[221] to characterize the contribution of the vascular system to neurogenic-specific learning and memory shortfalls following repetitive mild TBI. We find significant cognitive deficits that correlate with aberrant neurogenesis after injury in wild type (WT) animals that is rescued in (EC)-specific EphA4 knockout (EphA4-KO) animals. These findings continue to further depict the neurogenic contribution to injury-induced behavioral deficits by characterizing the vascular effect through the EphA4 pathway.

Materials and Methods

Animals

Procedures dealing with animals were approved under the guidelines of the Virginia Tech Institutional Animal Care and Use Committee (IACUC, protocol #17-093). All mice were

bred and housed in an AAALAC approved facility with a 12-hour light/dark cycle without the presence of viruses and antigens and given access to food and water *ad libitum*. We employed a well-characterized mouse strain where EphA4 is flanked (floxed) by LoxP (Jackson Labs, Epha4tm1.1Bzh/J). To specifically target endothelial cells, these EphA4flox.flox mice were bred with another transgenic mouse strain where Cre recombinase is driven under the direction of tyrosine kinase Tek (Tie2) promoter (Tie2::Cretg/+) (Jackson Labs, B6.Cg-Tg(Tek-cre)12Flv/J). Tie2 is expressed in endothelial and hematopoeitic cells during both embryogenesis and adulthood. To generate controls and experimental mouse stains, we bred EphA4flox/flox/Tie2::Cre male mice with EphA4flox/flox female mice to produce both EphA4flox/flox (wildtype, WT) and EphA4flox/flox/Tie2::Cre (knockout, KO) pups within the same litter. We identified WT and EphA4-KO by genotyping tail samples from newborn pups as previously described [222].

Repetitive Mild TBI Surgery Model

Mice were given repetitive mild traumatic brain injuries based on a previously published report [172]. In sum, experimental mice were anesthetized with ketamine (100mg/kg) and xylazine (10mg/kg) prior to being placed in a stereotaxic frame. Prior to injury induction, a small incision was made along midline of the brain and the skin was peeled to the side to reveal the skull. Injuries were induced using a program controlled cortical impactor (Φ=5-mm flat tip) connected to an eCCI-6.3 device (Custom Design & Fabrication, LLC) at a velocity of 5.0 m/s, depth of 1.0 mm, and 200ms impact duration. After the injury, the skin was brought back together to close the incision and held in place using Vetbond tissue adhesive (3M, St. Paul, MN, USA). A controlled heating pad and rectal probe were used to maintain the body temperature of each mouse at 37˚C throughout the surgery and monitored every 20 minutes following injury until fully recovered. Sham animals were induced in the same manner, except for the injury with the CCI device. Repeated surgeries for TBI and sham were conducted once every 48 hours for a total of 5 injuries. To label proliferating cells, 0.15mg bromodeoxyuridine (BrdU) was injected daily for five days beginning immediately after the first injury (Figure 9A).

Barnes Maze Testing

To characterize learning and memory deficits following repetitive mild TBI, we developed a novel, shortened Barnes Maze protocol. The Barnes Maze (Maze Engineers, Boston, MA, USA) consists of 20 holes placed around a 100cm circle where a bright light is shined down on the center of the maze to induce mild anxiety and encourage mice to enter a target box. 20-days after the final injury, mice were pre-training on the Barnes Maze for a total of 5-minutes, during which they were prompted to enter the box a total of three times: two times immediately after being placed on the maze and one final time at the end of their 5-minutes if they were not already in the target box. For the following three days, mice went through the Acquisition Phase where they were placed on the maze for 3-minutes each trial, 3 trials/day for a total of 9 trials. During the Acquisition Phase, the amount of time it takes to find the goal box (primary latency) and the number of wrong holes each mouse visited before finding the goal box (primary errors) was recorded. On the final day, each mouse was placed on the maze with no goal box for 1.5-minutes as part of their Probe Trial. During the Probe Trial, we conducted the same analyses as the Acquisition Phase and included the number of times the mouse revisited the goal box (target revisits).

Histology and Stereology

Cell counts were conducted utilizing the StereoInvestigator software (MicroBrightField, Williston, VT, USA) and an upright Olympus BX51TRF motorized microscope (Olympus America, Center Valley, PA, USA). Grid size was set at 150x150 with a 75x75 and 4 serial sections were counted for each animal. Cell count analysis was conducted on whole, cryo-preserved brains that were frozen 24-days after the initial injury and were serial sectioned on a cryostat (Cryostar, Fisher Scientific, Pittsburgh, PA, USA) into five 30micron coronal sections spaced ten sections apart. Each slide was placed in the -80C freezer until use. To identify cells co-labeled with BrdU and a cell-specific marker, slides from the -80C were warmed on a slide warmer for 30-minutes prior to a 10-minute fixation in 10% formalin. After three, 5-minute washes with 1X PBS, the slides were transferred to 4% Triton-X (in PBS) for 5-minutes before being placed into 2N HCl that was pre-warmed to 37C. For the next 45-minutes, slides were left in a 37C incubator before

returning to room temperature (RT) for 15-minutes of sodium borate treatment. The slides were washed twice with 1X PBS, then placed in blocking solution of 2% fish gelatin with 0.1% Triton-X in PBS for 2-4 hours at RT. Each slide was then placed in primary antibody in 2% fish gelatin and left in the 4C overnight. The next day, slides were washed six times for 10-minutes/wash with 1X PBS before secondary solution was added for 1-2 hours. The slides were washed with 1X PBS two times for 10-minutes/wash before mounting in DAPI-G Fluoromount (Southern Biotech, Birmingham, AL, USA).

Statistical analysis

Data was analyzed and graphed using GraphPad Prism, version 8 (GraphPad Software, Inc. San Diego, CA). Two experimental groups were compared using Student's two-tailed t-test. Three or more experimental groups were compared using one-way and two-way ANOVA where appropriate followed by Tukey or Bonferroni test for multiple comparisons. Changes were identified as significant if their p-value was less than 0.05, and mean values were reported together with the standard error of mean (SEM) for each graphical result.

Results

Loss of EC-specific EphA4 attenuates learning and memory deficits following repetitive mild TBI

To investigate the learning and memory response following repetitive mild TBI, we injured animals (Figure 9A) and developed a new, shortened Barnes Maze protocol (Figure 9B). 24-days after the initial injury, there are significant differences in the number of mistakes mice make on the maze in the WT group, but not between the sham and injured EphA4-KO groups (Figure 9C). In addition, WT injured mice take longer to find the escape box when compared to WT shams, but this effect is attenuated in EphA4-KO mice (Figure D). These findings characterize a neuroprotective effect in EphA4-KO mice.

Aberrant migration into the hilus does not contribute to learning and memory deficits

First, we wanted to characterize the aberrant migration of cells into the hilus, a known contributor to learning and memory losses in epilepsy. Following repetitive mild TBI, control (WT) animals have an increase in the number of aberrantly migrated Prox1-positive cells into the hilus when compared to sham animals (Figure 10A). However, EphA4-KO injured and sham animals display an increase when compared to WT Sham, but no differences within treatment groups. Taken together, this implies the aberrant migration to the hilus is not contributing to the learning and memory deficits associated with this repetitive mild TBI model.

Loss of EC-specific EphA4 prevents aberrant neurogenesis after rmTBI

Next, we wanted to continue to link neurogenesis to learning and memory shortfalls following repetitive mild TBI. To determine whether adult hippocampal neurogenesis underlies repetitive injury-induced learning and memory deficits, we BrdU-labeled proliferating cells during the first five days of injuries and tracked their fate until 24-days after the initial injury. Following repetitive mild TBI, there were no changes in the number of BrdU-positive cells compared to sham animals from the WT background. However, there was a significant increase in the number of BrdU-positive cells after injury in animals from the EphA4-KO background (Figure 10B).

Of these BrdU-labeled cells, significantly less of them are Type I and Type II, Sox2-positive NSPCs in EphA4-KO animals, but there are no significant differences between injured and sham animals within each group (Figure 10C). Further, there are no significant differences in the number of newborn mature neurons 24-days following injury between any of the experimental groups. Finally, we see increases in the number of Prox1-postive neuroblasts after injury in WT animals when compared to sham, but no differences in EphA4-KO animals.

To further classify cell identity, we conducted cell fate analysis by taking the number of cells co-labeled with each marker over the total number of BrdU-positive cells for the animal. Again, we find significantly less cells left in the Type I or Type II NSPC fate in the EphA4-KO animals when compared to WT animals, but no differences between injured and sham mice of each group (Figure 10D). We also find no increased cell fate

of mature neurons at this time-point. Finally, we see significant increases of Prox1-positive neuroblasts after injury in WT animals when compared to sham animals that are alleviated in EphA4-KO animals.

Generally, these results demonstrate a neuroblast-specific response to repetitive mild TBI that could be classified as aberrant neurogenesis.

Hyperactivity in the DG is ameliorated in EphA4-KO mice

In addition to classifying the neurogenic response, we wanted to determine if aberrant neurogenesis contributed to any firing changes within the DG. We found increased hyperactivity via c-Fos activation in WT injured animals when compared to sham animals, but decreases in EphA4-KO injured animals when compared to shams (Figure 10E). This hyperactivity could be a result of the increased neuronal population and can contribute to the learning and memory deficits seen above.

Discussion

Adult hippocampal neurogenesis supports learning and memory in homeostasis and is critical for cognitive recovery in some models of brain injury. This highly coordinated process is heavily influenced by surrounding cells and factors. The endothelial cells that make up the vascular system are uniquely positioned to impact neurogenesis through direct cell-cell contact with NSPCs and by releasing a wide variety of cellular factors. Previous findings have identified the ephrin-A5/EphA4 complex as a critical player involved in temporal lobe epilepsy [103].

In the present study, our lab has combined BrdU labeling with cell-specific immunostaining to evaluate the influence of the vascular system on neurogenesis following repetitive mild TBI. We utilized a Tie2-driven Cre:lox knockout system to specifically eliminate EphA4 on endothelial cells and conducted cell fate analysis to determine the differentiation status of NSPCs 24-days following the initial injury. Tie2 is also expressed on peripheral immune cells, but the blood-brain-barrier remains intact in this injury model and will not allow these immune cells to enter the brain.

Following repetitive mild TBI, there was a significant increase in the number of BrdU-positive cells in the EphA4-KO animals when compared to EphA4-KO shams that was absent in WT animals (Figure 10B). At two weeks following repetitive mild TBI, we characterized the cell populations co-labeled with BrdU. Interestingly, we found a significant decrease in the number of Sox2-positive NSPCs in the EphA4-KO group when compared to WT animals but no differences between injury and sham groups within each genotype (Figure 10C). We also conducted cell fate analysis by taking the total number of each co-labeled cell over the total number of BrdU-positive cells and found comparable results to the total number of cells (Figure D). While the lack of NSPC response after injury does cross genotypes, the increase in EphA4-KO sham animals compared to WT sham animals could be due to a difference in the number of NSPCs already existing in the niche or a more proliferative potential of these cells. The expansion in proliferative potential of these early cells would jumpstart the neurogenic process and contribute to the cognitive protection seen after injury. However, since Sox2 is also expressed in astrocytes, the increased numbers in EphA4-KO animals could also be due to a heightened glial proliferation rate. The rise in total number of BrdU-positive cells in the EphA4-KO mice after injury does not align with an increase in numbers of Sox2, Prox1, or NeuN co-labeled cells. Since the rise is not due to an increase in the neuronal lineage, these cells may be in the glial lineage.

The lack of Prox1-positive cells co-labeled with BrdU (Figure 10C) after injury in EphA4-KO animals could be due to two mechanisms. First, the Prox1-positive neuroblasts may be activated to differentiate by the EphA4 present in the WT animals. Second, these cells may simply be activated to incorporate BrdU and surviving in WT animals, but remaining inactive in EphA4-KO animals. In addition, we found a significant increase in the total number of neuroblasts found in the DG of injured WT animals when compared to WT shams (Figure 8C). Previous reports have demonstrated ephrinA5/EphA4, present on neuroblasts and endothelial cells respectively, positively regulates proliferation and survival in the DG. After repetitive mild TBI, the lack of EphA4 on ECs could prevent expansion of Prox1-positive neuroblasts compared to WT-injured animals. Further, there is no significant increase in the number of dying cells found in WT

animals (Figure 8B). This would imply the results are due to a lack of proliferative response in the neuroblast population.

The aberrant, significant increase in the neuronal lineage in the WT repetitively injured animals, as shown via co-labeled Prox1-positive with BrdU-positive cells, could also contribute to the increase in c-Fos activity indicative of hyperactivity in the DG (Figure 10E). The number of c-Fos positive cells in the DG directly correlates with learning and memory deficits on the Barnes Maze (Figure 10F). Further, we identified an increased number of Prox1-positive cells in the hilus of WT injured animals that were comparable to both sham and injured EphA4-KO animals. Interestingly, the EphA4-KO animals do not show a difference before or after injury, but have a heightened number of c-Fos positive cells in the DG when compared to WT sham animals (Figure 10A). These results imply Prox1-positive cells in the hilus may be contributing to c-Fos activation in the EphA4-KO animals. Taken together, this implies the injury-induced increased number of cells in another, potentially glial, lineage is helping to inhibit cognitive decline. Regardless, these findings are not contributing to learning and memory deficits found in this injury model at this time point.

Finally, we wanted to evaluate molecular changes that may be associated with the divergent response in the DG of WT and EphA4-KO animals. We micro-dissected the DG 9-days post-initial impact and isolated RNA to utilize qPCR. We found a decrease in *Mcp-1*, *Tgfβ*, Connexin-43 (*Cx43*) following injury in WT animals via qPCR analysis that is not present in EphA4-KO animals. As NSPCs differentiate into neuroblasts, Cx43 expression is reduced, which could in part explain why we see a decrease in Cx43 correlate with an increase in the number of newborn (or surviving) Prox1-positive cells in the DG. This contributes to our hypothesis that the neuroblast population is susceptible to injury models and needs to be further investigated to better characterize the mechanisms underlying these significant responses to TBI.

Taken together, our findings have implicated EphA4 function on microvessels in the DG contribute to the aberrant neurogenic response following repetitive mild traumatic brain injury. While the absence of EphA4 on vascular cells rescued cognitive deficits, and normalize the neurogenic response, more research is required to determine whether forward or reverse signaling is affected. For example, infusion of clustered EphA4-Fc

molecules into the DG will restore reverse ephrin signaling and determine if this prevents the protective effect found in EphA4-KO animals. In addition, more studies are required to determine whether these effects are mediated by direct cell-to-cell communicate or release of soluble factors.

Author Contributions

Kisha Greer and Michelle Theus, PhD conceived and designed the experiments. Kisha Greer collected and analyzed the data. Xia Wang bred and maintained animals for testing and performed qPCR analysis. Kisha Greer compiled the information and wrote this paper.

Figures

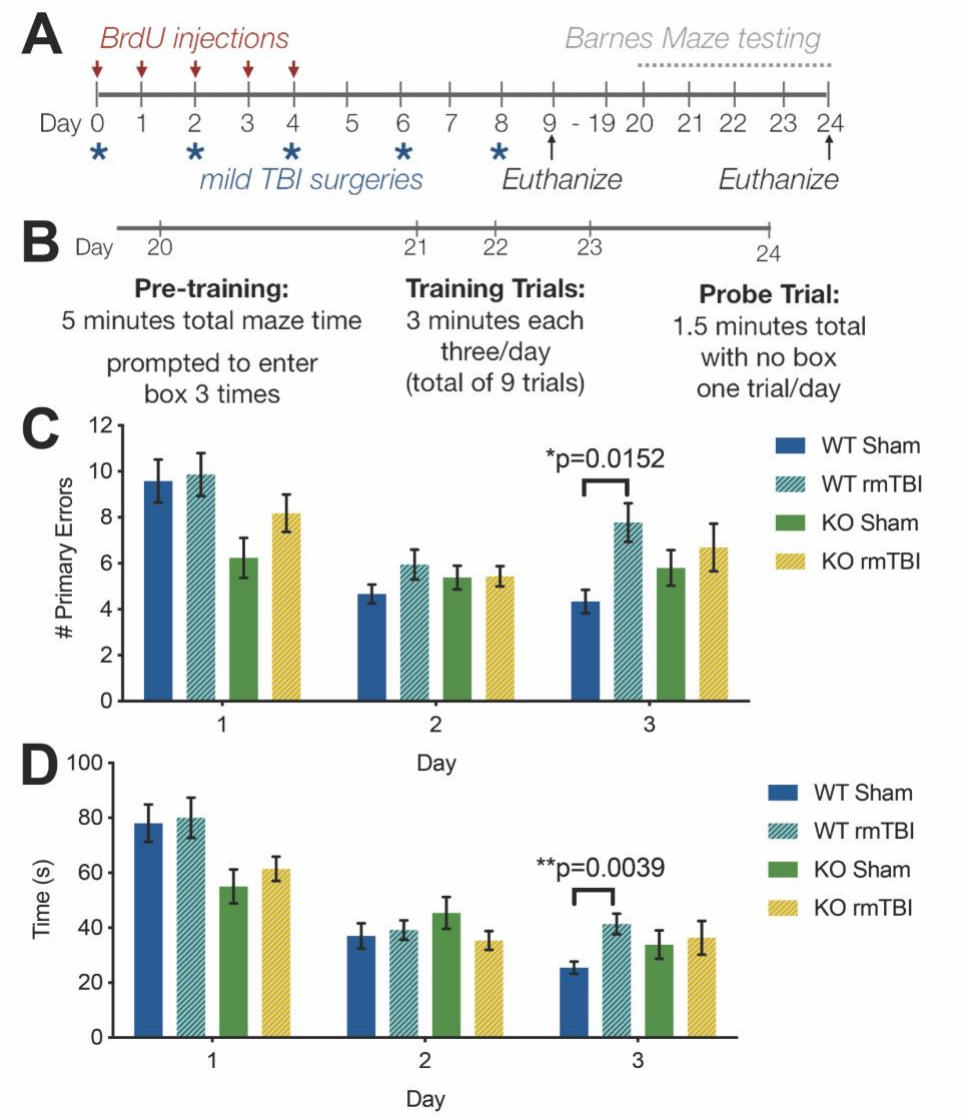

Figure 4. Loss of EC-specific EphA4 rescues behavioral deficits following reptitive mild TBI. (A-B) In our expeirmental design, Barnes Maze testing takes place starting 20-days after the first injury. (C-D) EphA4-KO mice show attenuated learning and memory deficits on the Barnes Maze by (C) making less errors and (D) taking less time to find the escape box than WT mice after injury.

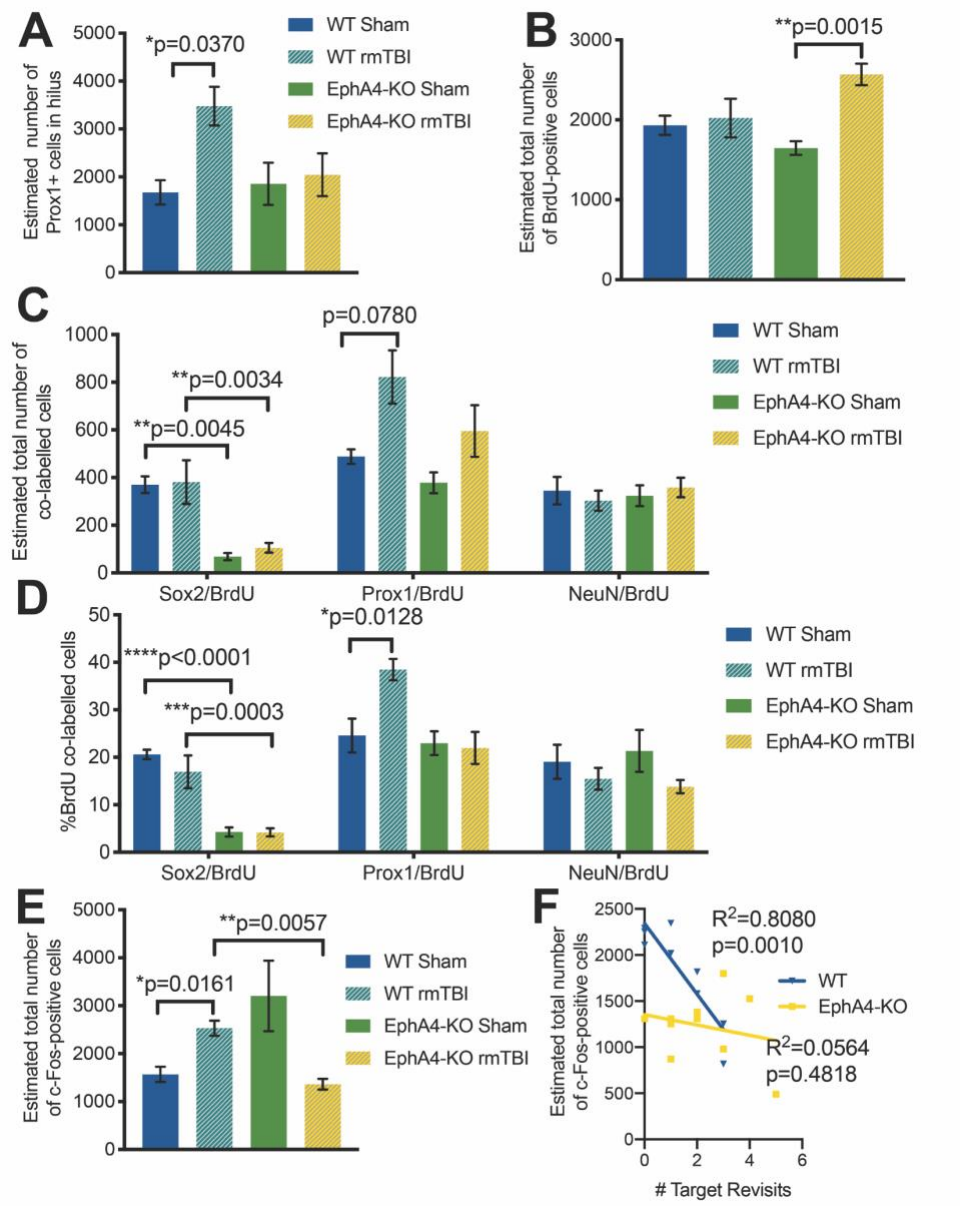

Figure 5. Injury-induced neurogenic changes are ameliorated in EC-specific EphA4-KO mice at 24d post-initial impact. (A) After injury, WT mice have increased numbers of Prox1-positive cells in the hilus, but this effect is not present in EphA4-KO animals. (B) While there are no significant increases in BrdU-positive cells in the dentate gyrus (DG) of WT mice, we found significant differences in EphA4-KO animals after repetitive mild TBI. (C-D). Of these BrdU-positive cells, significantly less of them are co-labeled with Sox2 in both the injured and sham EphA4-KO groups when compared to WT animals. In addition, we identified significantly more cells co-labeled with Prox1 in WT injured animals when compared to WT sham, but not in EphA4-KO injured, an indicator of increased neuronal lineage. (E). We also characterized an increase in c-Fos activation after injury in WT animals that was absent in EphA4-KO animals. (F) This hyperactivity via c-Fos directly correlated with behavioral deficits (# Target Revisits) in WT mice, with no direct correlation in EphA4-KO animals.

Chapter 5

General Discussion

Learning and memory

One main symptom of mild TBIs, or concussions, is learning and memory loss, with patients sustaining multiple mild TBIs developing chronic memory losses as part of neurodegenerative diseases such as chronic traumatic encephalopathy (CTE) [163, 194, 223]. Previous findings in animal models of repetitive mild TBI characterized significant learning and memory deficits as early as two weeks after injury and progressing up to two years [172, 174, 190, 224]. Our studies have contributed to this knowledge by identifying two separate conditions that ameliorate these short-term deficits: dampening neurogenic potential with AraC treatment and limiting vascular input by knocking out EphA4 on endothelial cells (ECs).

The antimitotic cytosine b-Arabinofuranoside (AraC) can specifically eradicate proliferating cells by competing with dCTP nucleotides to inhibit DNA synthesis [134]. In the described studies, we utilized AraC to force all the proliferating NSPCs within the DG to die, limiting the neurogenic potential of the niche. We delivered 2% AraC for 7 days prior to the first mild hit and found mice performed better on the Barnes Maze, a learning and memory test after injury than those who were given to the control treatment. It is important to note, AraC will affect any proliferating cell in the body, and therefore, has many off-target effects. However, our study is only the first step to linking hippocampal neurogenesis to behavioral outcomes after repetitive mild TBI. We also found a direct correlation between hyperactivity in the niche via c-Fos activation and cognitive deficits on the maze in control mice, but not in AraC treated mice. By inhibiting the neurogenic potential with AraC prior to surgeries, the rescued effect on learning and memory implies a significant role for the neurogenic niche in injury-induced cognitive decline and recovery that needs to be further characterized.

Previous studies have linked the Eph/ephrin pathway to learning and memory capacity in a variety of animal models, specifically activating the (EC)-EphA4 in the hippocampus with ephrin-A5 leads to a better outcomes of temporal lobe epilepsy [103]. Our lab utilized an EC-specific EphA4 knockout (EphA4-KO) and found significantly better

learning and memory capacity after repetitively injured animals when compared to injured control wild-type (WT) animals. This is the one of the first studies to link vascular-dependent signaling in the dentate gyrus to significantly better cognitive outcomes following repetitive mild TBI.

Proliferation

The process of neurogenesis begins when Type I quiescent neural stem cells are activated to Type II and begin to proliferate [182]. To understand the effect of repetitive mild TBI on proliferation within the adult hippocampal neurogenic niche, we utilized cell fate analysis with bromodeoxyuridine (BrdU) injections. BrdU incorporates into cells as they undergo the synthesis phase of cell division and remains part of the cell's DNA as they continue to divide and differentiate, making it an excellent tool to track the final cellular state. Days after moderate and severe traumatic brain injury, NSPCs are activated to proliferate and incorporate BrdU to replace the immediate neuroblast-specific cell death. By inhibiting this proliferative response with NSPC ablation techniques, previous studies have demonstrated a lack of cognitive recovery chronically. However, no one has yet characterized a potential proliferative response after repetitive mild TBI.

The endothelial cells (ECs) that makeup the vascular system have a specific role in proliferation of NSPCs [100, 205, 225]. However, in our experiments, eliminating EphA4 on endothelial cells lead to an increase in the number of proliferative cells after injury when compared to EphA4-KO sham animals. We did not see any increased in total BrdU numbers of WT animals immediately after injury or two weeks after the final injury. This implies the input from EphA4 is not required to stimulate proliferation as part of neurogenesis in the hippocampal niche.

Interestingly, we did find substantial increases when we increased the number of BrdU injections from the first 5-days to the first 7-days. Since BrdU remains in progeny cells as they continue to proliferate, we would expect to see increases in BrdU-labeled cells regardless of injection timeline. This significant difference could be the activation of a quiescent cell population during the later injuries that was not picked up in the first 5 days. However, the differences in experimental design could also contribute for these differences. For example, the mice treated with BrdU for 7-days as a part of the AraC

experiments were also handled for an extra week, which has been linked to increased neurogenesis [226].

Differentiation

Neurogenesis, or the creation of new neurons, is typically characterized by co-labeling BrdU-positive cells with markers for neuroblasts and mature neurons [227]. To determine the final fate of cells in the neurogenic after repetitive mild TBI, we co-labeled BrdU-positive cells with Sox2, Prox1, and NeuN to label for NSPCs, neuroblasts, and mature neurons, respectively. While Prox1 is also expressed in mature dentate gyrus granular neurons, we can easily quantify the neuroblast population by subtracting the total number of mature neurons co-labeled with NeuN from the total number of co-labeled Prox1 cells to leave the number of neuroblasts or immature neurons. The remaining number will leave us with the doublecortin (DCX)-positive population.

Following repetitive mild TBI, we have identified a specific increase in the number of co-labeled cells in the neuronal lineage (Prox1-positive) in control, WT animals about two weeks after the final injury, but not one day after the final injury. Uniquely, this population shows a delayed proliferative or survival response after repetitive mild TBI whereas these cells are more likely to die after more severe TBI models. Treatment with AraC rescues the aberrant number of newborn neurons after injury as shown with Prox1 and NeuN expression. Further, the EC-specific EphA4-KO animals did not show signs of aberrant neurogenesis. Taken together with the total increase in DCX-positive cells found in the DG of injured WT animals, the lack of neuroblast response in these two treatments implies a lack of neuroblast-specific proliferative response. The increased numbers of neuroblasts could contribute to the increase in c-Fos activation seen after injury. Dampening the neurogenic response with AraC also ameliorates the hyperactivity, implying the NSPC niche is playing an important part of injury-induced hyperactivity.

Taken together, these experiments confirm the neuroblast population is especially susceptible to respond after various traumatic brain injuries. Moving forward, long-term experiments will determine if these results are temporary or will contribute to chronic cognitive shortfalls seen in repetitive mild TBI.

Migration

Migration is an essential part of the neurogenic process because it places NSPCs and newborn neurons in the proper place to receive input from cells in the surrounding niche [5, 192]. Neuroblasts can also migrate a few layers radially through the granular cells to ensure synaptic projections are created in the correct direction and location as they differentiate and mature into neurons that are fully incorporated into the hippocampal network. However, previous studies have linked aberrant migration of cells within the DG with negative cognitive outcomes as moderate TBI forces neuroblasts cells to migrate to the outer portions of the granular cell layers and the inner portion of the hilus where they never fully develop synaptic maturity [113, 191]. Still, research has yet to characterize the effects of repetitive mild TBI on the location of newborn neurons within the hippocampus.

Our research has identified aberrant migration of Prox1-positive cells into the hilus of the DG following repetitive mild TBI about two weeks after the final injury. Interestingly, this response is independent of NSPC behavior as it is still present in AraC-treated mice where the neurogenic potential is dampened. In addition, sham EphA4-KO animals have a significant increase in Prox1-positive cells in the hilus when compared to sham WT animals. Since the EphA4-KO injured animals show no difference when compared to their sham counterparts, the vascular system must be playing an important role in the migration of Prox1-positive cells during development. Generally, these aberrant migrated cells are not contributing to learning and memory shortfalls. However, the long-term survival and fate of these cells is still under investigation.

Survival

A necessary factor of neurogenesis is the ability of cells to survive within the niche. In fact, most newborn neurons will not survive to be fully integrated into the hippocampal network [228]. After a single, moderate TBI the neuroblasts undergo cell-specific apoptosis, forcing the Type I and Type II NSPCs to proliferate and differentiate to replace the lost cell population [62, 63]. However, after repetitive mild TBI, the neuroblasts do not apoptosis; instead they survive and divide to continue the neuronal lineage. In both the AraC-treated and EphA4-KO animals, this injury-induced effect correlates with normal c-

Fos activation and better learning and memory outcomes, implying the slight increase in firing capacity after injury may further contribute to hippocampal hyperactivity, an underlying factor of cognitive dysfunction. If these neuroblasts underwent cell-specific apoptosis, as seen in the moderate TBI model, the learning and memory shortfalls may improve. This continues to characterize a neuroblast population that is specifically sensitive to respond after traumatic brain injuries.

Chapter 6

Summary and Future Perspectives

This thesis has contributed to the knowledge related to the adult hippocampal neurogenic influence and learning and memory deficits following repetitive mild TBI. However, we are only scratching the surface of the vast number of questions and experiments left to be answered to determine the contribution of neurogenesis to chronic neurodegenerative disease, such as chronic traumatic encephalopathy (CTE). Our lab identified a unique neurogenic reaction as early as two weeks after the final injury that is characterized by an increase in the neuronal population after injury and correlates with learning and memory deficits. The uncontrolled increase in the neuroblast population is indicative of aberrant neurogenesis and contrasts the typical neuroblast-specific cell death seen after moderate and severe TBI. These findings confirm the hippocampal neuroblast population is especially susceptible to respond after TBI. Still, the neurogenic response during more chronic time points still requires heavy investigation.

Long-term, chronic experiments should focus not only on the pathology to classify diseases such as CTE, but should also consider the mechanisms underlying the symptoms to better develop targeted treatments. Our lab determined the vascular system is particularly important for the process of neurogenesis by knocking out endothelial cell (EC)-specific EphA4. These (EC)-EphA4-KO animals appear to have a rescued effect on learning and memory deficits that correlates with no aberrant neurogenesis. More research is required to better characterize the role of vascular-derived Eph/ephrins in neurogenesis, and can be applied not only to repetitive mild TBI but to other injury models that result in cognitive decline. Future experiments should also focus on determining whether it is direct cell-cell contact from an EC to NSPCs or whether the ECs from EphA4-KO mice are excreting different cellular factors that are influencing how NSPCs behave

Taken together, these findings will influence the way researchers approach their experimental designs for repetitive mild TBI. The field needs to continue to grow and develop these experiments to answer a wide array of questions at both short-term and chronic time points that will help identify ways to prevent the cognitive deficits associated with repeated traumatic events to the brain.

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