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Award Number: W81XWH-18-1-0598

TITLE: Adult-Born Neurons and the Development of Posttraumatic Epilepsy

PRINCIPAL INVESTIGATOR: Eric Schnell

CONTRACTING ORGANIZATION: OREGON HEALTH & SCIENCE UNIVERSITY
PORTLAND OR 97239-3011

REPORT DATE: Sept 2020

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE Sept 2020		2. REPORT TYPE Annual		3. DATES COVERED 9/1/2019-8/31/2020	
4. TITLE AND SUBTITLE Adult-Born Neurons and the Development of Posttraumatic Epilepsy				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-18-1-0598	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Eric Schnell E-Mail: schnelel@ohsu.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) OREGON HEALTH & SCIENCE UNIVERSITY 3181 SW SAM JACKSON PARK RD PORTLAND OR 97239-3011				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Post-traumatic epilepsy (PTE) occurs in over 50% of veterans with a history of penetrating head injury, and individuals with severe head injuries have a seventeen-fold increased risk of developing epilepsy later in life. Although PTE is a heterogeneous condition with multiple etiologies, clinical data suggest that it often involves seizure foci in the temporal lobe, and in particular from within the hippocampus. The hippocampus manifests numerous anatomical changes in epilepsy, which are thought to mediate its contribution to neuronal hyperexcitability and seizures. Although the neuroanatomical changes associated with epilepsy are well described, how these abnormalities lead to the development of epilepsy are unknown. This CDMRP project is analyzing whether newly generated neurons in the hippocampus contribute to the formation of hyperexcitable circuits in a mouse model of posttraumatic epilepsy (PTE). The project's specific aims are (1) to assess adult-born hippocampal granule cell contributions to the formation of recurrent excitatory brain circuits after traumatic brain injury (TBI); (2) to characterize network properties of circuits involving adult-born granule cells after TBI; and (3) to assess whether modulation of posttraumatic neurogenesis by diazepam prevents hippocampal hyperexcitability and posttraumatic epilepsy. If successful, we will not only substantially increase our understanding of how hippocampal function has changed after TBI, but we will also investigate the potential of pharmacologic modulation of the re-wiring process to reduce the subsequent development of epilepsy. This would have great translational relevance to future human clinical trials, which is a major focus of our work.					
15. SUBJECT TERMS Traumatic Brain Injury, Epileptogenesis, Hippocampus, Diazepam, Post-traumatic Epilepsy					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			USAMRMC
			UU	49	19b. TELEPHONE NUMBER (include area code)

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1. INTRODUCTION

Post-traumatic epilepsy (PTE) occurs in over 50% of veterans with a history of penetrating head injury, and individuals with severe head injuries have a seventeen-fold increased risk of developing epilepsy later in life. Although PTE is a heterogeneous condition with multiple etiologies, clinical data suggest that it often involves seizure foci in the temporal lobe, and in particular from within the hippocampus. The hippocampus manifests numerous anatomical changes in epilepsy, which are thought to mediate its contribution to neuronal hyperexcitability and seizures. Although the neuroanatomical changes associated with epilepsy are well described, how these abnormalities lead to the development of epilepsy are unknown. This CDMRP project is analyzing whether newly generated neurons in the hippocampus contribute to the formation of hyperexcitable circuits in a mouse model of posttraumatic epilepsy (PTE). The project's specific aims are (1) to assess adult-born hippocampal granule cell contributions to the formation of recurrent excitatory brain circuits after traumatic brain injury (TBI); (2) to characterize network properties of circuits involving adult-born granule cells after TBI; and (3) to assess whether modulation of posttraumatic neurogenesis by diazepam prevents hippocampal hyperexcitability and posttraumatic epilepsy. If successful, we will not only substantially increase our understanding of how hippocampal function has changed after TBI, but we will also investigate the potential of pharmacologic modulation of the re-wiring process to reduce the subsequent development of epilepsy. This would have great translational relevance to future human clinical trials, which is a major focus of our work.

2. KEYWORDS

Traumatic Brain Injury, Epileptogenesis, Hippocampus, Diazepam, Post-traumatic Epilepsy

3. ACCOMPLISHMENTS

What were the major goals of the project?

The goal of this proposal is to identify how epilepsy develops after traumatic brain injury, and to identify a strategy to prevent PTE. The development of epilepsy after head injury is often delayed by months or years, which suggests that neuronal circuit functions change slowly over time. Recent work suggests that newly-born hippocampal neurons contribute to hippocampal circuit changes after TBI, so our hypotheses were that (1) newly born neurons contributed to circuit changes after TBI, (2) that these changes contributed to circuit changes after TBI, and (3) that we could modulate neurogenesis after TBI and reduce circuit rewiring and post-traumatic epilepsy. We are investigating these hypotheses using the following specific aims:

Aim 1: Assess adult-born hippocampal granule cell contributions to the formation of recurrent excitatory brain circuits after TBI. Milestone: Identification of functionally aberrant neuronal inputs and outputs formed by adult-born cells after TBI. Anticipated completion: 14 months. Percent complete: 65%

Aim 2: Assess adult-born granule cell contribution to hippocampal hyperexcitability after TBI. Milestone: Identification and characterization of circuits formed by neurons born

during post-traumatic neurogenesis. Anticipated completion: 30 months. Percent complete: 25%

Aim 3: Assess whether modulation of post-traumatic neurogenesis by diazepam prevents hippocampal hyperexcitability and post-traumatic epilepsy (PTE). Milestone 1: Identification of a method to normalize neurogenesis after TBI. Anticipated completion: 36 months. Percent complete: 100%. Milestone 2: Understanding the role of aberrantly developed adult-born neurons in the pathogenesis of epilepsy, and pre-clinical investigation of a strategy to prevent PTE. Anticipated completion: 36 months. Percent complete: 15%.

What was accomplished under these goals?

Major activities: The primary activities in the second year of this proposal involved ongoing analysis of circuit rewiring by adult-born hippocampal neurons after TBI and preparations to begin EEG studies. We hired and trained a new post-doctoral scholar in single cell electrophysiology on acutely prepared mouse brain slices, continued breeding and translational modeling, and implemented a new video-EEG recording room for the translational analysis of mice after TBI.

Specific objectives: Throughout this year, we continued to obtain data on the circuit integration of neurons born after TBI, although laboratory closures significantly impacted experimental progress (see below). Physiology and imaging experiments are soon to restart, as we have been working aggressively to re-expand our colony of genetically modified mice for analysis, implementing regular brain injury modeling, and preparing to begin EEG studies.

Significant results: We not only have replicated prior results demonstrating aberrant post-traumatic neurogenesis after TBI in our transgenic mouse model, but now have identified a specific functional defect in neurons born after TBI that appear to drive their hyperexcitability. Our most recent results suggest that loss of a certain hippocampal cell type (the hilar mossy cell) after TBI may be an integral step in the development of aberrant neurogenesis after TBI, and we are actively following this line of investigation.

Other Achievements: In addition to our manuscript published in the *Journal of Neurotrauma* (Villasana et al., 2019; Appendix 1), we have presented newer findings on some of the physiological consequences of post-traumatic circuit rearrangement at an international neuroscience conference (Chen and Schnell, 2020, please see Appendix 2), which was presented with an award. We have also published another related study on the signaling involved in driving hippocampal rewiring after brain injury, which has significant relevance to the current proposal as it relates to the process of post-seizure neurogenesis (Gupta and Schnell, 2019, please see Appendix 3).

What opportunities for training and professional development has the project provided?

Although this project was not explicitly intended to provide training and professional development opportunities, two junior scientists and one senior scientist have obtained significant scientific development through the performance of this work. The contributions of a graduate student (Beeson) and a post-baccalaureate researcher

(Wilson) have both had significant training benefits. In the first case, Ms. Beeson continues to gain experience working with mouse translational models as well as through the mentorship and training of both a recently graduated undergraduate, Ms. Wilson, as well as a new post-doctoral scholar in the lab, Dr. Chen, who has never before performed brain slice recordings. In Ms. Wilson's case, she has been able to learn advanced neuroscience research techniques and obtained insight into future career possibilities, and in Dr. Chen's case, she was able to broaden her research expertise in preparation for a career in translational neuroscience.

How were the results disseminated to communities of interest?

Our primary result dissemination to the wider scientific community is via publication of manuscripts and conference presentations. Recent results from our work have been presented at an international medical/scientific meeting (Society for Neuroscience in Anesthesia and Critical Care), with the intent of raising awareness amongst perioperative clinicians on the mechanisms and impacts of post-traumatic neurogenesis and how circuit rearrangements involving new neurons might drive brain hyperexcitability. This work won the "Best of Basic Science" presentation award and was given a platform presentation live-streamed across the globe (Chen, M. and Schnell, E., 2020, see below).

What do you plan to do during the next reporting period to accomplish the goals?

We will continue to expand and maintain our mouse colony and generate mice for the current work. Staff are performing regular TBI modeling experiments, and soon will be able to re-start physiologic recordings. New staff have been trained in osmotic pump placement, and we are coordinating with our collaborator Dr. Miranda Lim to perform video-EEG recordings from mice after TBI to assess how post-traumatic epilepsy is modulated by diazepam. We feel that this aspect of our project has the most translational relevance to the future management of patients with severe head injury, and we have been accelerating preparations to pursue this work.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

Our prior work demonstrated that adult-born hippocampal neurons contribute potently to recurrent circuit formation in epilepsy and that these recurrent circuits potently drive epileptiform activity (Hendricks et al., 2019; citation below). Together, these data strongly suggest that the aberrant neurogenesis leads to circuit hyperexcitability, and may directly contribute to epileptic seizures. Although the notion that post-seizure neurogenesis might contribute to increased brain hyperexcitability has long been considered in the temporal lobe epilepsy field, the TBI field has until recently viewed post-traumatic neurogenesis as a beneficial response of the brain that contributes to healing and recovery. Although our work does not yet clearly delineate the positive and negative aspects of post-TBI neurogenesis, it is clear that this may be a double-edged sword, which (in part through our work) is becoming more widely appreciated.

Our discovery that diazepam can potently modulate (and normalize) post-injury neurogenesis allows for a direct examination of whether this manipulation can reduce post-injury epilepsy. If our mouse EEG recordings validate this hypothesis, this could directly impact future clinical research, including the development of clinical trials and

potential therapeutic management approaches after TBI which include short-term administration of diazepam to normalize early post-traumatic neuronal circuit remodeling, with the goal of reducing the incidence of epilepsy after head injury.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

5. CHANGES/PROBLEMS

Changes in approach and reasons for change

Early on, we deviated from our expected timeline by accelerating the most translational aim (Aim 3), which was completed well ahead of our original schedule. This was done intentionally, to allow us to proceed with our EEG experiments in epileptic mice. Otherwise, we are continuing with our experiments as originally proposed, with the only minor change comprising of the addition of some immunohistochemical experiments to help further characterize some additional morphologic circuit changes that we have noticed after TBI, which necessitated an addition to our SOW (submitted/approved last year). This additional insight has focused our analysis on the proximal synaptic input layer in the dentate gyrus, as this is the site of both granule cell axon sprouting and mossy cell axon loss.

Actual or anticipated problems or delays and actions or plans to resolve them

As mentioned previously in our quarterly reports, our initial data collection was delayed due to the need to breed and age the appropriate experimental animals (experiments are begun in mice at 2 months of age, and analyzed an additional 2 months later; thus a guaranteed 5 months before the first data were acquired). We were making steady progress over the latter half of the 1st year and the beginning of the 2nd year of this award. Unfortunately, the lab member whose primary project was the performance of these experiments (Wilson) had to leave the lab due to personal/health-related issues in December 2019. Fortunately, I was already engaged in the process of hiring a new senior post-doctoral scholar (Chen) during this time. She was able to immediately pick up this project, and almost no experimental progress was lost.

As you are likely aware, COVID-19 precautions shut down labs across the country (including OHSU and the VA), in accordance with institutional and local guidance, and all experiments ceased. Sadly, this included mandatory mouse colony culling, which was a substantial set-back for this project, as experimental consistency requires TBI modeling to occur at 2 mo of age, and recordings/analysis to happen 2 months later. Thus, the 3 month shut-down caused us to lose all experimental animals for approximately 4 months

prior to the shut-down (they had not yet reached analysis age, and had to be culled before experiments), and breeding was only allowed to resume after the shutdown ended (end June 2020). Thus, these mice are still too young for experimental analysis, although many have now undergone TBI modeling. Fortunately, however, the culling of our colony and minimal use of reagents/services during the lockdown significantly reduced expenditures, which will allow for these funds to still be used for experiments via an anticipated no-cost extension.

Currently the health of lab members and our community remains the top priority of our institution, so although laboratories recently re-opened, modified operations continue and laboratories are working at a reduced capacity. However, over the past year, I was able to operationalize a second laboratory site, so laboratory personnel could be spread out amongst sites more efficiently and allow nearly unrestricted lab access (less densely concentrated staff workstations).

Moving forward, in addition to the evolving pandemic, the PI is always aware of the potential for staff turnover, breeding colony issues, and equipment failure, and thus is constantly working to acquire spare staff and equipment capacity, to prevent these potential issues from negatively impacting experimental progress.

Changes that had a significant impact on expenditures

Due in part to the delayed start to data acquisition, and then again due to colony loss and experimental pauses during the shutdown, we remain below budget. However, we expect our overall budget for these experiments to remain the same.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

6. PRODUCTS

One manuscript directly related to the Scope of Work in this proposal was published last year (see Appendix 1):

Villasana, L. E., Peters, A. J., McCallum, R., Liu, C., and Schnell, E. (2019) Diazepam inhibits post-traumatic neurogenesis and blocks aberrant dendritic development. *Journal of Neurotrauma*, 36: 2454-2467.

More recent data from this project were presented at an international scientific meeting, as mentioned above, and also published in the *Journal of Neurosurgical Anesthesia* (Appendix 2). Of note, this abstract won the “Best of Basic Science” presentation award for this meeting, delivered as a platform presentation:

Chen, M. and Schnell, E. (2020) Imbalanced excitatory and inhibitory inputs onto hippocampal dentate granule cells born after traumatic brain injury. *Journal of Neurosurgical Anesthesia*, 32(4): 396. SNACC 48th Annual Meeting, September 11-13, 2020, Virtual

Indirectly related to this project, during the past year of this award, one additional manuscript was published from our lab, which investigated the signalling mechanisms underlying the control of circuit plasticity during post-seizure neurogenesis in the hippocampal dentate gyrus. Although not part of the current proposal, we believe this work has direct relevance to post-traumatic circuit rewiring that we have reported after TBI, and it was based in part on intellectual contributions to the lab resulting from the current work, and thus it is included as Appendix 3.

Gupta, K. and Schnell, E. (2019) Neuronal network remodeling and Wnt pathway dysregulation in the intra-hippocampal kainate mouse model of temporal lobe epilepsy. *PLOS One*, 14(10): e0215789

In addition, during the performance of this award, our lab published additional manuscripts, that was based in part upon technical developments undertaken in preparation for this proposal. This has helped guide our work on this CDMRP proposal, and is evidence of the synergy that is possible when combining multiple translational models in a single lab. The citations for these works are noted here for your reference:

Hendricks, W. D., Westbrook, G. L., and Schnell, E. (2019) Early detonation by sprouted mossy fibers enables aberrant dentate network activity. *Proceedings of the National Academy of Sciences, U.S.A.*, 116(22):10994-10999.

Beeson, K. A., Beeson, R., Westbrook, G. L., and Schnell, E. (2020) $\alpha 2\delta$ -2 protein controls structure and function at the cerebellar climbing fiber synapse. *Journal of Neuroscience*, 40(12): 2403-2415 and Cover Illustration.

Chatzi, C., Zhang, Y., Hendricks, W. D., Chen, Y., Schnell, E., Goodman, R. H. and Westbrook, G. L. (2019) Exercise-induced enhancement of synaptic function triggered by the inverse BAR protein, Mtss1L. *eLife* 8:e45920.

During our lab shutdown, I led several hospital-based initiatives to conserve/decontaminate Personal Protective Equipment (PPE), and endeavored to provide guidance to the rest of the medical community. One manuscript is available as a preprint (citation below) and three additional manuscripts are in preparation. Even though the lab was closed, it was (and remains) a complex and busy time to be a clinician-scientist.

Schnell, E., Harrieff, M.J., Yates, J.E., Karamooz, E., Pfeiffer, C.D., McCarthy, J., Trapp, C.L., Frazier, S.K., Dodier, J.E. and Smith, S.M. (2020) Homegrown Ultraviolet Germicidal Irradiation for Hospital-Based N95 Decontamination during the COVID-19 Pandemic. *medRxiv*, 10.1101/2020.04.29.20085456v1 [Preprint]

7. PARTICIPANTS

Name:

Eric Schnell

Project Role:

Principal Investigator

Researcher Identifier (ORCID ID): 0000-0002-5623-5015
Nearest person month worked: 3
Contribution to Project: Dr. Schnell initiated the project, prepared preliminary data and proposal, hired staff, trained staff, analyzed preliminary data.
Funding Support: Dept of Veterans' Affairs

Name: Minghui Chen
Project Role: Post-doctoral Scholar
Researcher Identifier (ORCID ID): 0000-0002-0316-2289
Nearest person month worked: 8
Contribution to Project: Dr. Chen was specifically hired to work on this project, and has been breeding mice, performing TBI models in mice, and performing electrophysiologic recordings for this project (taking over for Ms. Wilson, below).
Funding Support: OHSU Department of Anesthesiology and Dept of Veterans' Affairs

Name: Laura Villasana
Project Role: Collaborator
Researcher Identifier (eRA Commons): VILLASAN
Nearest person month worked: 1
Contribution to Project: Dr. Villasana produced preliminary data for the project, is co-authoring a manuscript with the PI on work related to this proposal, and is (still) training new staff in procedures
Funding Support: OHSU Department of Anesthesiology

Name: Miranda Lim
Project Role: Collaborator
Researcher Identifier (eRA Commons): 100010401
Nearest person month worked: 2
Contribution to Project: Dr. Lim is a collaborator that will be assisting with EEG recordings from mice with post-traumatic epilepsy; she assembled a new 4-cage EEG recording apparatus (purchased by Dr. Schnell specifically for this CDMRP project) and has been testing it with validation cohorts so that it is fully operational for next years' recordings.
Funding Support: Dept of Veterans' Affairs

Name: Kathleen Beeson
Project Role: Graduate Student
Researcher Identifier (eRA Commons): KBEESON
Nearest person month worked: 6
Contribution to Project: Ms. Beeson continues to train and oversee staff in the procedures necessary for this project
Funding Support: OHSU Department of Anesthesiology

Name: Alexandria Wilson
Project Role: Research Assistant

Researcher Identifier: n/a
Nearest person month worked: 4
Contribution to Project: Ms. Wilson was specifically hired to work on this project, bred mice, performed TBI models in mice, stained/analyzed post-TBI tissue, and performed electrophysiologic recordings for this project; she had to end her employment due to personal/health reasons.
Funding Support: Dept of Veterans' Affairs

Changes in active other support of the PD/PI(s) or senior/key personnel since the last reporting period:

Nothing to report.

Changes in other organizations involved as partners:

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

Quad chart provided on next page.

Adult-Born Neurons and the Development of Posttraumatic Epilepsy

USAMRMC Proposal Number EP170012

W81XWH-18-1-0598

PI: Eric Schnell

Org: Oregon Health and Science University

Award Amount: \$500,000

Study/Product Aim(s)

- Assess adult-born hippocampal granule cell contributions to the formation of recurrent excitatory brain circuits after TBI
- Assess adult-born granule cell contribution to hippocampal hyperexcitability after TBI
- Assess whether modulation of post-traumatic neurogenesis by diazepam prevents hippocampal hyperexcitability and post-traumatic epilepsy

Approach

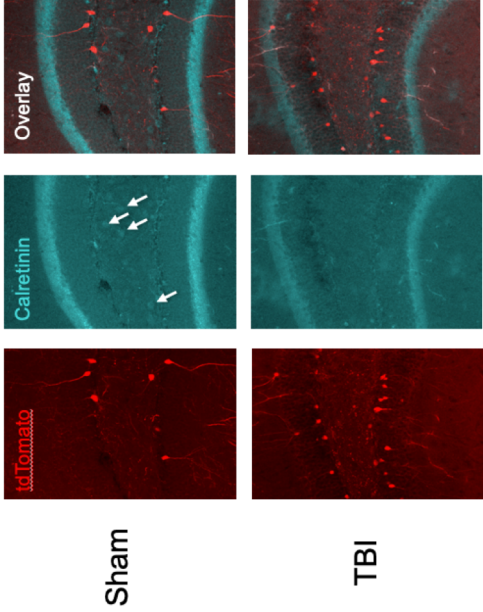
We are using a model of post-TBI epilepsy in a new line of genetically modified mice to investigate the neurobiology that causes epilepsy after head injury. We are imaging brain tissue, performing electrical recordings of brain circuit function, and performing mouse EEG recordings to characterize how seizures occur and to evaluate a treatment strategy to prevent epilepsy after TBI.

Timeline and Cost

Activities	CY	18	19	20	21
Identify circuit changes after TBI					
Identify how hyperexcitability occurs					
Strategy to prevent circuit changes					
Strategy to prevent post-TBI epilepsy					
Estimated Budget (\$K)		\$ 56	\$167	\$167	\$111

Updated: 9/29/2020

Mossy cell loss after TBI



Accomplishment:
Immunohistochemical validation of altered dentate granule cell inputs after TBI. Loss of hilar mossy cells (Calretinin-positive, teal) coincides with circuit rewiring noted in our recordings from new adult-born granule cells generated after TBI (tdTomato positive, red).

Goals/Milestones

CY18 Goal – Develop research model and hire staff

- ☒ Staff hired and trained, experiments in progress

CY19 Goals – Functional characterization of adult-born neurons

- ☒ Imaging of tissue (ongoing)
- ☒ Electrophysiologic recordings (ongoing)

CY20 Goals – Characterize hyperexcitability

- ☐ Recordings of output circuitry

- ☒ Establish EEG recordings (ongoing)

CY21 Goal – Test therapeutic strategy

- ☐ Test effects using EEG recordings from treated mice

Comments/Challenges/Issues/Concerns

- Pandemic-related lab closures and research limitations

Budget Expenditure to Date

Projected Expenditure: \$341K

Actual Expenditure: ~\$262K

9. APPENDICES

Please see the attached manuscripts:

Appendix 1 – Villasana et al. pg 14

Appendix 2 – Chen and Schnell pg 28

Appendix 3 – Gupta and Schnell pg 29

Diazepam Inhibits Post-Traumatic Neurogenesis and Blocks Aberrant Dendritic Development

Laura E. Villasana,¹ Austin Peters,¹ Raluca McCallum,² Chang Liu,¹ and Eric Schnell^{1,2}

Abstract

Traumatic brain injury (TBI) triggers a robust increase in neurogenesis within the dentate gyrus of the hippocampus, but these new neurons undergo aberrant maturation and dendritic outgrowth. Because gamma-aminobutyric acid (GABA)_A receptors (GABA_ARs) modulate dendritic outgrowth during constitutive neurogenesis and GABA_AR-modulating sedatives are often administered to human patients after TBI, we investigated whether the benzodiazepine, diazepam (DZP), alters post-injury hippocampal neurogenesis. We used a controlled cortical impact (CCI) model of TBI in adult mice, and administered DZP or vehicle continuously for 1 week after injury via osmotic pump. Although DZP did not affect the neurogenesis rate in control mice, it almost completely prevented the TBI-induced increase in hippocampal neurogenesis as well as the aberrant dendritic growth of neurons born after TBI. DZP did not reduce cortical injury, reactive gliosis, or cell proliferation early after injury, but decreased c-Fos activation in the dentate gyrus at both early and late time-points after TBI, suggesting an association between neuronal activity and post-injury neurogenesis. Because DZP blocks post-injury neurogenesis, further studies are warranted to assess whether benzodiazepines alter cognitive recovery or the development of complications after TBI.

Keywords: controlled cortical impact; diazepam; models of injury; neurogenesis; regeneration; traumatic brain injury

Introduction

IN 2010, APPROXIMATELY 2.5 MILLION PEOPLE in the United States sustained a traumatic brain injury (TBI), and over the past decade, the rate of TBI has increased in men and women by 63% and 49%, respectively.¹ As the rate of TBI-related death has declined, more people now live with TBI-related cognitive and emotional impairments.^{1–5} It was long believed that recovery from TBI was in part limited by the irreversible loss of neurons; however, we now know that the adult brain has a limited capacity for self-renewal through the process of adult neurogenesis.^{6,7} In the hippocampus, adult-born dentate granule cell neurons are important for learning and memory,^{8–10} and may also play important roles in emotional regulation.^{11,12}

TBI robustly increases hippocampal neurogenesis,^{13–18} and augmentation of this process after TBI could facilitate cognitive recovery.^{19–22} Alternatively, as neurons born after various neuronal injuries have functional and morphological abnormalities,^{23–27} they could drive negative outcomes such as the development of post-traumatic epilepsy.^{28–30} Either way, the net effect of post-TBI neurogenesis on recovery likely depends on the maturation and neuronal circuit integration of the neurons born after injury.

A variety of signaling mechanisms modulate adult hippocampal neurogenesis, including ionotropic neurotransmitter receptors.^{31–33}

Specifically, gamma-aminobutyric acid type A receptors (GABA_ARs) modulate the proliferation of neuronal stem cells and neuronal maturation.^{33–36} GABA_ARs are also the targets of sedative and anesthetic drugs³⁷ including benzodiazepines and propofol, which are often administered to hospitalized patients after severe head injury,³⁸ and thus could affect cell proliferation, maturation, and survival both during constitutive and post-injury adult neurogenesis.^{39–41}

Thus, we asked whether the prototypical benzodiazepine, diazepam (DZP), administered after TBI affects post-traumatic neurogenesis. We used a controlled cortical impact (CCI) model of TBI in wild-type and in transgenic *POMC-GFP* mice, in which green fluorescent protein (GFP) is selectively expressed by immature adult-born hippocampal neurons. DZP was administered continuously by osmotic pump for 7 days, beginning shortly after CCI or sham injury. We subsequently analyzed hippocampal neurogenesis and histopathological markers in injured and control brains to determine whether DZP modulates post-traumatic neurogenesis.

Methods

Animals

All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory

¹Department of Anesthesiology and Perioperative Medicine, Oregon Health & Science University, Portland, Oregon.

²Operative Care Division, VA Portland Health Care System, Portland, Oregon.

Animals and were approved by the Institutional Animal Care and Use Committee at Oregon Health & Science University (OHSU). Male and female C57Bl/6J wild-type mice were obtained from Jackson Labs, and heterozygotic *POMC-GFP* mice⁴² were obtained by crossing homozygotic mice (maintained on a C57 background) with wild-type mice.

Controlled cortical impact injury and diazepam administration

We used a CCI model of TBI in 2-month-old mice as previously described.^{23,43} Briefly, mice were placed under isoflurane anesthesia (2% spontaneously inhaled) and mounted on a stereotaxic frame. After sterile skin preparation and a midline scalp incision, a 4-mm craniotomy was made unilaterally bordering right of the midline between lambda and bregma, keeping dura intact. TBI was induced with a 0.9-mm deformation (4.4 m/sec; 800 msec dwell) delivered directly onto the exposed dura using an electromagnetic impactor with a 3-mm cylindrical tip (ImpactOne, Leica Microsystems). Sham mice underwent the same surgery and anesthesia minus the craniotomy and impact.

After CCI or sham treatment, the scalp was closed using sutures and Vetbond adhesive. While still anesthetized, each mouse was subcutaneously implanted with a mini-osmotic pump (AlzetAP2001; Cupertino, CA) containing either vehicle (1:1 dimethyl sulfoxide [DMSO]:propylene glycol) or DZP (added to vehicle for a dose of 15 mg/kg/day), and which had been pre-equilibrated in saline per the manufacturer's instructions. Mice were given ear punches for identification and allowed to recover in a warm padded chamber for 1 h. Osmotic pumps delivered solutions at a rate of 1 μ L/h for 1 week and were then surgically removed under isoflurane anesthesia. Residual volumes in the pump were all $\pm$ 10% of predicted volumes based on the implantation/equilibration time. *POMC-GFP* mice were sacrificed 2 weeks after sham/CCI for histological analysis. For any group, littermates were divided between sham and CCI treatment and between vehicle and DZP pump implantation.

The number of *POMC-GFP* mice for each group was: 10 sham-vehicle (7 females, 3 males), 9 CCI-vehicle (5 females, 4 males), 7 sham-DZP (3 females, 4 males), and 11 CCI-DZP (5 females, 6 males). The number of wild-type mice assessed 3 weeks after CCI (for BrdU labeling, DCX expression) was 6 sham-vehicle (3 females, 3 males), 7 CCI-vehicle (3 females, 4 males), 7 sham-DZP (4 females, 3 males), and 6 CCI-DZP (3 females, 3 males). The number of wild-type mice assessed 3 days after CCI included a total of 12 sham-vehicle (7 females, 5 males), 13 CCI-vehicle (8 females, 5 males), 14 sham-DZP (10 females; 4 males), and 13 CCI-vehicle (9 females, 4 males). A third cohort of 16 wild-type male mice was sacrificed 3 h after sham or CCI treatment with pump implantation, and used to determine the acute effects of DZP on neuronal activity and cell death. The number of mice per group is reported in the legend of the figure for each outcome.

BrdU protocol

Wild-type mice received two intraperitoneal injections (4 h apart) of the mitotic marker bromodeoxyuridine (BrdU, 300 mg/kg dissolved in saline) daily for 2 days, beginning 2 days after CCI or sham. This saturating BrdU dose provides long-term labeling of dividing cells and their progeny.⁴⁴ These mice were sacrificed at 3 weeks to assess the density of BrdU⁺ cells. Neuronal fate specification of BrdU⁺ cells was assessed using co-labeling with the neuronal marker doublecortin (DCX). To determine the effects of DZP on cell proliferation within the dentate gyrus, a separate cohort of wild-type mice was injected with BrdU (50 mg/kg) every 2 h for 6 h on the second day after surgery, and then sacrificed the following day to minimize the effects of differential early survival on the BrdU⁺ cell count.⁴⁵ This lower dose was chosen to provide a

continuous level of BrdU for an extended period during the labeling day, without the need to label multiple rounds of cell division given the shorter experimental time frame. We did not observe any sex differences in regard to post-traumatic neurogenesis or cell proliferation in any of our assays, and thus results for both sexes were combined.

Immunohistochemistry

Immunohistochemistry was performed as previously described²³ with some modifications. Following a terminal dose of avertin (1.2%, 1 mL, intraperitoneal [i.p.]), mice were transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Brains were harvested and post-fixed overnight. Free-floating coronal sections (150 μ m thick) were then prepared using a vibratome. Four sections containing the hippocampus (two dorsal: approximately -1.46 and -2.18 mm from bregma; and two ventral: approximately -2.54 and -2.80 mm from bregma) from each mouse were permeabilized in 0.4% Triton in PBS (PBST) for 45 min and then blocked for 30 min with 10% horse serum (HS) in PBST. The sections were then incubated overnight (4°C) with primary antibodies diluted in PBST with 1.5% HS. These included rabbit anti-GFP (Alexa Fluor 488 conjugated; 1:400, Invitrogen), goat anti-doublecortin (1:400, Santa Cruz), rat anti-BrdU (1:400, Abcam), rabbit anti-c-Fos (1:400, Santa Cruz), rat anti-Ki67 (1:400, eBioscience), and rabbit anti-GFAP (1:1000, DAKO). Sections stained with anti-BrdU were first denatured in 2N hydrochloric acid in potassium-PBS for 30 min (37°C) followed by a neutralization step (potassium-PBS pH 8.5) prior to staining.

After primary antibody incubation, sections were washed in PBST and exposed to fluorophore-conjugated secondary antibodies (Invitrogen) dissolved in PBST +1.5% HS for 4 h at room temperature. The sections were then washed in PBST containing DAPI (1:20,000) and mounted on slides with Fluoromount-G (Southern Biotech).

Cell death was assessed 3 days after surgery using Fluoro-Jade C (Histo-chem) staining following the manufacturer's protocol. Slices were mounted on gelatinized slides and allowed to air dry at room temperature before immersion in 100% ethanol (3 min), 70% ethanol (1 min), and deionized water (1 min). The slides were then immersed and gently shaken in 0.06% potassium permanganate for 15 min followed by 0.001% Fluoro-Jade C for 30 min. The slides were washed in water for 1 min 3 times before they were allowed to dry overnight. The next day, the slides were dipped in xylene 3 times (2 min each time) and cover-slipped with DPX (Electron Microscopy Sciences, Inc.). In a separate cohort of animals, cell death in the granule cell layer (GCL) and surrounding tissue was assessed in mice sacrificed 3 h after injury (using the same staining protocol), to determine whether DZP exerted an immediate effect on cell death after CCI.

Confocal microscopy and image analysis

Slides were coded, and the hippocampus of each mouse was imaged with a Zeiss LSM780 confocal microscope using a 10 $\times$ 0.45 NA or 20 $\times$ 0.8 NA lens, by an experimenter blinded to experimental condition. All subsequent analyses were performed on coded images with ImageJ software.

In BrdU-stained slices from wild-type mice taken either 3 days or 3 weeks after CCI, all DCX/BrdU double positive cells were counted throughout a 50- μ m image stack of the dentate GCL and the subgranular zone (SGZ), and normalized to the GCL volume. GCL volume was obtained by multiplying the cross-sectional area of the GCL in the first optical section by the depth of image stack. Quantification of Ki-67 staining from mice 3 days after CCI was performed similar to the BrdU quantification, but sections were imaged and counted using a 25- μ m image stack of dentate GCL and SGZ. To determine the effects of DZP and CCI on neuronal

activity, slices were stained for the activity-dependent gene c-Fos at early and late time-points during treatment. In the mice assessed 3 days after CCI, cells within the dentate gyrus positive for c-Fos were counted in a 30- μ m thick z-stack of the GCL. In a separate cohort of mice assessed 3 h after CCI, due to the high density of c-Fos⁺ cells within the dentate gyrus GCL, the mean intensity of all pixels corresponding to the GCL (determined via DAPI signal) within a single confocal section was used to compare group differences. Intensity values were normalized by dividing the mean pixel intensity for each image by the mean c-Fos pixel intensity from the sham/vehicle stained slices (which was set to 100%).

To compare glial fibrillary acidic protein (GFAP) staining between groups, the mean pixel intensity in images of the dentate hilus and the molecular layer was obtained with ImageJ, and all values were normalized to the mean GFAP pixel intensity from the sham/vehicle stained slices. To count Fluoro-Jade C⁺ cells, single confocal sections of the dentate gyrus from multiple slices (minimum of 3) bracketing the injury cavity were used for each mouse. The somatosensory cortex was also analyzed separately to assess Fluoro-Jade C⁺ cell counts and staining quality at the 3 h time-point. For all imaging experiments, samples were identically processed (side-by-side), imaging parameters were kept constant between slides, and blinded investigators performed imaging and image analysis.

To quantify post-traumatic neurogenesis in *POMC-GFP* mice, the ipsilateral hippocampus was imaged from tissue harvested 2 weeks after sham or CCI. This time-point was chosen to allow analysis of the same population of cells labeled by BrdU in wild-type mice, as GFP is expressed by immature, adult-born neurons between 10 and 14 days post-mitosis.⁴² GFP⁺ cells were counted on single confocal sections from 2 to 4 slices per mouse that included the molecular layer, the GCL, and the SGZ of the dentate gyrus. The cell count was normalized to the imaged GCL area of each slice to estimate GFP⁺ cell density.

The cortical cavity in mice that underwent CCI treatment was assessed 3 days after surgery by subtracting the area of the ipsilateral cortex immediately beneath the impact site (using a tiled image acquired with overlapping 4 $\times$ images) from the area of the contralateral cortex from the same slice. All anatomical measurements were collected and analyzed by an investigator blinded to the group assignment of each animal.

Cell migration and morphology

As adult-born neurons mature, they slowly migrate from the SGZ of the dentate gyrus outward into the GCL. To determine whether DZP influences cell migration, the linear distance from the center of each cell body to the SGZ/hilar border was measured in the confocal image plane traversing mid-nucleus, using ImageJ as previously described.²³ Cells that migrated backward into the hilus or out into the molecular layer (defined as migration >10 μ m away from the inner border of the SGZ or the outer border of the GCL, respectively) were counted separately to quantify ectopically migrated cells. Ectopically migrated cells were quantified using single confocal sections of the dentate gyrus.

To quantify dendritic morphology, we traced the morphology of GFP⁺ cells from confocal stack images using ImageJ as previously described.²³ GFP⁺ cell dendritic trees were imaged in their entirety (three dimensions) using a confocal stack, and traced in ImageJ. An average of 5 to 8 cells were traced for 3 to 4 mice/group, and these cell tracings were analyzed using the ImageJ Sholl analysis plug-in (Ghosh Lab, <http://labs.biology.ucsd.edu/ghosh/software/>). All analyses were performed by an experimenter blinded to experimental condition.

Open field analysis following CCI and during diazepam administration

The open field test was used to assess locomotor and exploratory behavior to determine whether the dose of DZP administered had

any gross effects on mouse activity levels. Three days following sham or CCI treatment (during DZP or vehicle delivery), mice were acclimated to single housing for 1 h in a holding room during the morning hours. After acclimatization, they were individually introduced to an open field (40-cm $\times$ 40-cm white chamber; 200 lux luminescence) for 10 min each. The distance traveled, rearing events (postured upright on rear legs), and velocity were assessed using video recording and tracking software (Noldus, Ethovision).

Experimental design and statistical analysis

Sample sizes were determined for each group through power analyses using estimates of effect magnitude and variability to detect significance at $p < 0.05$ with a power of 80%. For all statistical analyses, data normality was first assessed to determine use of parametric or non-parametric tests as indicated in the Results section. For parametric statistics, a two-way ANCOVA (analysis of co-variance) was used to compare group differences and potential interactions. The between-subjects variables consisted of CCI treatment, DZP exposure, and sex, and litter was used as a covariate to account for potential variability in cell labeling densities between litters. A repeated measures analysis of variance (ANOVA) was used to compare group differences in the Sholl analysis with the distance from the soma as the within-subject factor and CCI and DZP treatment as the between-subject factors. A Bonferroni post hoc test was used to compare specific distance points when there was a significant interaction between the variables. For non-parametric statistics, a Mann-Whitney test was used to compare CCI-treated groups with their respective vehicle or DZP controls. All figures and statistical analyses were conducted using Prism (GraphPad Software, La Jolla, CA) and SPSS (IBM SPSS, Armonk, NY) software. Data are expressed as mean $\pm$ standard error of the mean (SEM) and comparisons were considered significant at $p < 0.05$. The number of mice for each time-point and analysis are noted in the Methods section and figure legends.

Results

Diazepam inhibits post-traumatic neurogenesis

To determine the effect of DZP on post-traumatic neurogenesis, wild-type mice were implanted with osmotic pumps after CCI or sham procedures, to deliver DZP (or vehicle) continuously for 7 days at a sub-anesthetic dose of 15 mg/kg/day (Fig. 1A). This dose was chosen for its demonstrated pre-clinical efficacy,⁴⁶ and continuous delivery was used to model prolonged sedative exposure as well as to account for the short serum half-life of DZP in rodents (54–66 min).^{47,48} These mice were injected with the mitotic marker BrdU on post-procedure days 2 and 3, to coincide with the peak level of post-traumatic neurogenesis,^{13,18} and analyzed 3 weeks later using immunohistochemistry.

Consistent with prior reports,⁴⁹ CCI increased hippocampal neurogenesis (BrdU⁺/DCX⁺ cell density; see Methods section) in vehicle-treated mice (sham-vehicle vs. CCI-vehicle; $p = 0.001$, Mann-Whitney test; Fig. 1B,C). However, DZP treatment for 1 week after CCI completely prevented the CCI-induced enhancement in neurogenesis (sham-DZP vs. CCI-DZP; $p = 0.12$, Mann-Whitney test; Fig. 1B,C). Interestingly, DZP had no effect on neurogenesis rate in sham-treated mice (sham-vehicle vs. sham-DZP; $p = 0.81$, Mann-Whitney test; Fig. 1B,C). Thus, a sub-anesthetic dose of DZP prevented post-traumatic neurogenesis without affecting constitutive adult neurogenesis.

As these BrdU⁺ cells were generated during a 2-day time window early after injury, we broadened our analysis by quantifying overall DCX⁺ cell density, which would also include cells generated after DZP cessation, based on the known expression pattern of

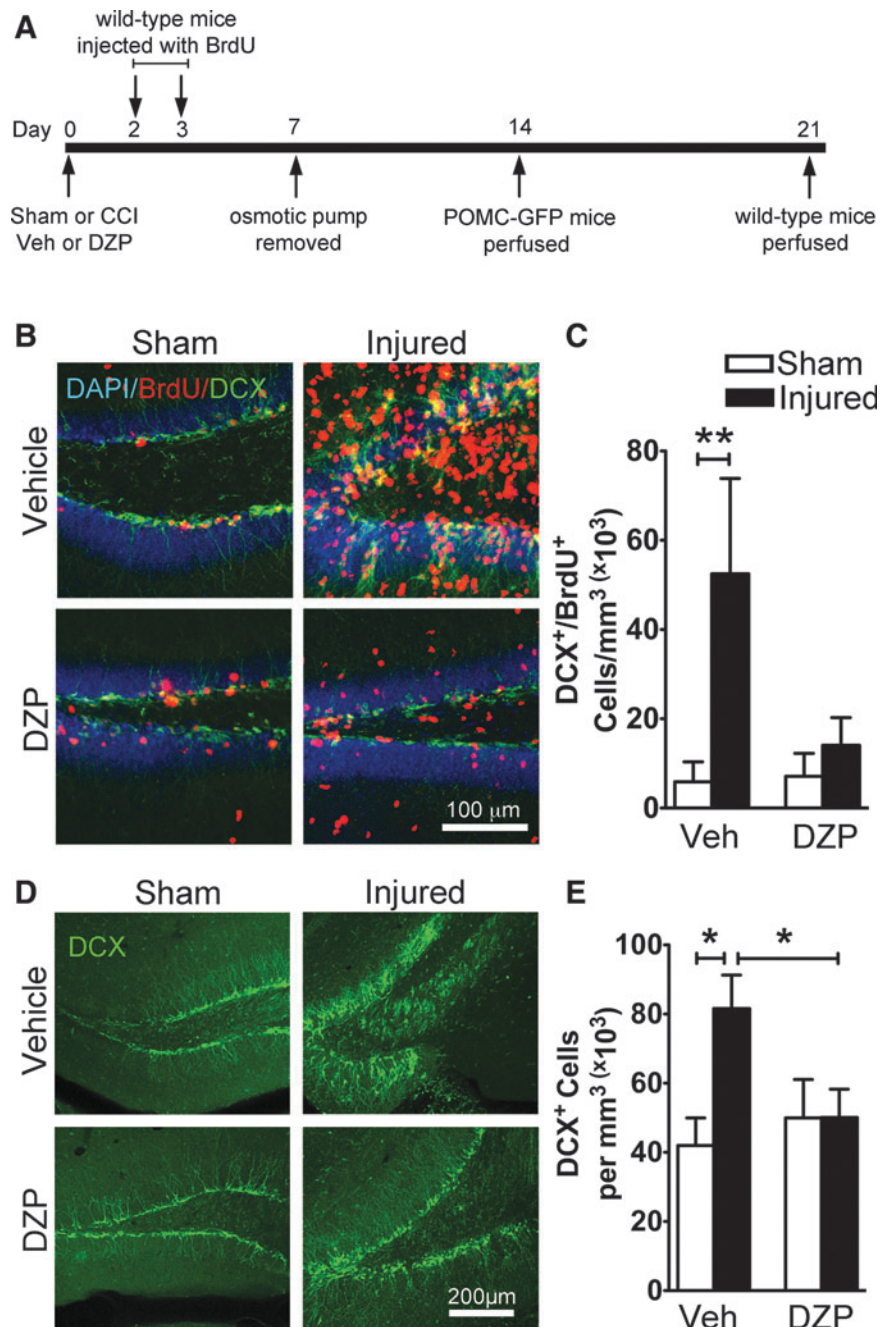


FIG. 1. Diazepam (DZP) inhibits post-traumatic hippocampal neurogenesis. **(A)** Experimental design. Two-month-old male and female *POMC-GFP* and C57Bl/6J wild-type mice underwent a controlled cortical impact (CCI) injury or sham procedure, and were subsequently implanted with osmotic pumps releasing vehicle (Veh) or DZP. On the second and third day after surgery, wild-type mice received two injections of BrdU per day. Osmotic pumps were left in place for 1 week and hippocampal neurogenesis was analyzed 2 or 3 weeks later (separate cohorts). **(B)** Confocal image projections of the dentate gyrus of wild-type mice perfused 3 weeks after injury, co-labeled with the neuronal marker doublecortin (DCX) and the mitotic marker BrdU. BrdU was overexposed (equally in all images) to clearly illustrate all BrdU⁺ cells, including dim cells, against the green DCX⁺ cells. **(C)** Quantification of BrdU⁺/DCX⁺ cell density within the granule cell layer of the dentate gyrus. BrdU⁺/DCX⁺ cell density was increased after CCI in vehicle-treated mice; however, this increase was prevented in injured mice that received DZP immediately after injury (** $p < 0.01$, sham-vehicle vs. CCI-vehicle treated mice; $n = 6-9$ mice/group). **(D)** Confocal images of the dentate gyrus of wild-type mice stained for DCX. **(E)** Quantification of DCX⁺ cell density within the granule cell layer of the dentate gyrus. CCI increased the density of DCX⁺ cells in vehicle-treated but not DZP-treated mice (* $p < 0.05$ CCI-vehicle vs. sham-vehicle and CCI-DZP treated mice, $n = 6-9$ mice/group).

DCX from 1 to 3 weeks post-mitosis.⁵⁰ Three weeks after CCI, DCX staining showed a similar pattern (Fig. 1D), as DZP prevented the CCI-induced increase in DCX⁺ cell density (drug $\times$ CCI interaction $F[1, 23] = 4.970$; $p = 0.036$; two-way ANCOVA; Fig. 1E). CCI increased the density of DCX⁺ cells in vehicle-treated mice (main effect of CCI, $F[1, 14] = 8.94$; $p = 0.011$; Fig. 1D,E), whereas CCI did not alter the density of DCX⁺ cells when followed by 1 week of DZP treatment (CCI-DZP vs. sham-DZP; $p = 0.941$; Fig. 1E). DZP treatment did not affect DCX expression in sham-treated mice (sham-vehicle vs. sham-DZP; $p = 0.430$; Fig. 1E), again indicating that a continuous sub-anesthetic dose of benzo-diazepine does not affect constitutive neurogenesis.

Diazepam does not inhibit cell proliferation after TBI

The DZP-mediated inhibition of post-traumatic neurogenesis could result from altered stem cell proliferation after CCI in the

presence of DZP, or alternatively via differential survival of neuronal precursors.⁵¹ To directly measure the effect of DZP on cell proliferation during the peak period of post-traumatic neurogenesis, we repeated our CCI protocol on a cohort of mice injected with BrdU on post-surgery day 2, and analyzed hippocampal tissue on post-surgery day 3.^{13,18} In vehicle-treated animals, CCI dramatically increased cell proliferation, as assessed by the density of newly post-mitotic cells in the GCL/SGZ ($p = 0.032$; sham-vehicle vs. CCI-vehicle; Mann-Whitney test; Fig. 2A,B). Surprisingly, DZP did not prevent the CCI-induced increase in cell proliferation ($p = 0.030$; sham-DZP vs. CCI-DZP; Mann-Whitney test; Fig. 2A,B), in sharp contrast with the DZP-mediated inhibition of neurogenesis noted at later time-points (Fig. 1). DZP also did not alter proliferation in sham-treated mice, as assessed by BrdU⁺ cell density ($p = 0.556$; sham-vehicle vs. sham-DZP; Mann-Whitney test; Fig. 2A,B).

To further investigate this observation with an independent assay, we stained hippocampal tissue for Ki67, a nuclear antigen that

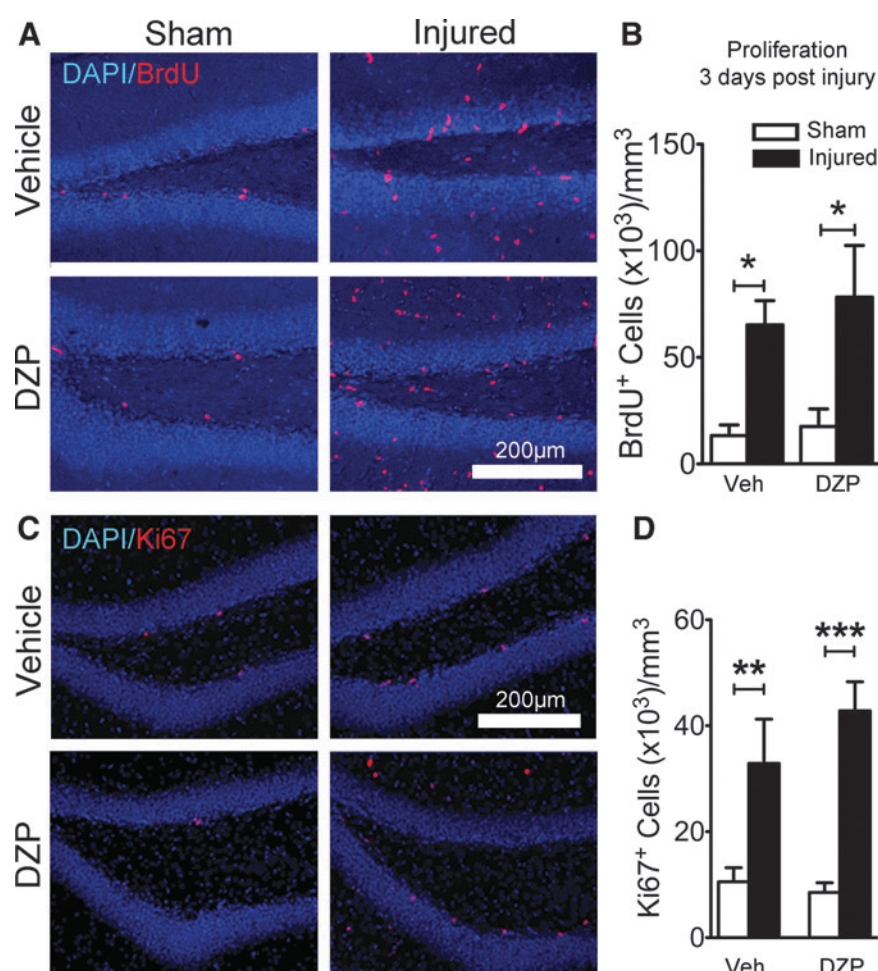
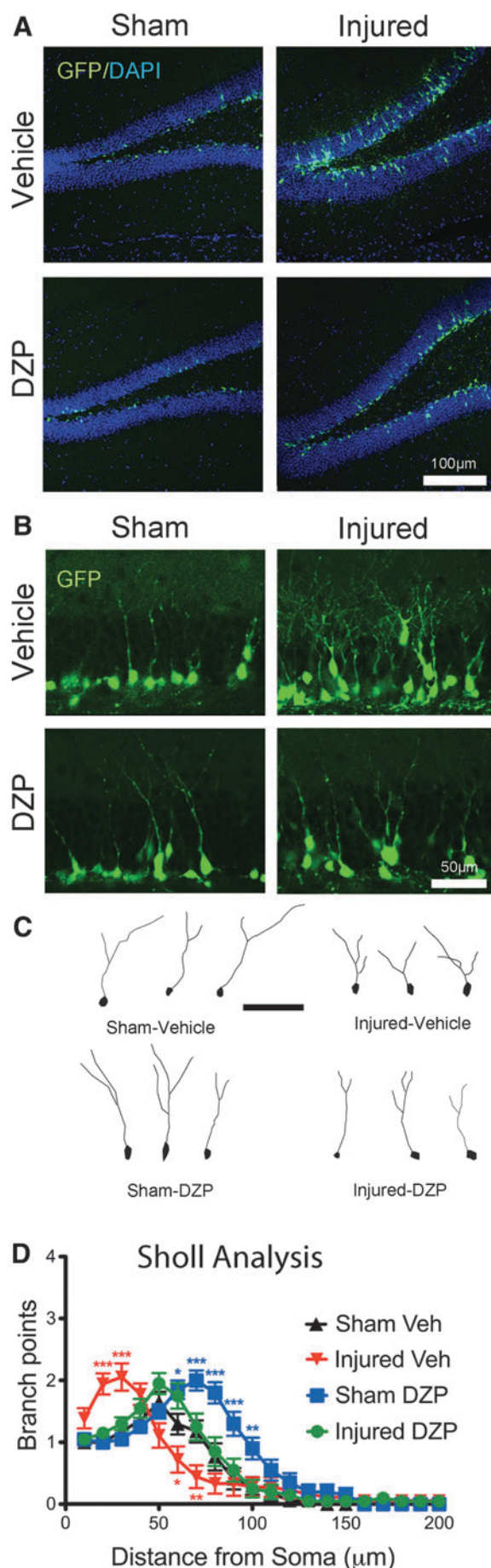


FIG. 2. Diazepam (DZP) does not affect cell proliferation early after controlled cortical impact (CCI). (A) BrdU-stained hippocampal tissue from sham and CCI-treated mice, exposed to either vehicle or DZP after sham/CCI. Mice were given BrdU 2 days after sham/CCI and fixed 1 day later to assess hippocampal cell proliferation. (B) Quantification of BrdU⁺ cell density in the granule cell layer and subgranular zone of the dentate gyrus. Cell proliferation increased similarly in both vehicle- and DZP-exposed mice after injury (* $p < 0.05$ sham-vehicle vs. CCI-vehicle; sham-DZP vs. CCI-DZP, $n = 4-6$ mice/group). (C) Hippocampal sections stained for the cell proliferation marker Ki67 (red) treated with either vehicle or DZP, 3 days after sham or CCI. (D) Quantification of Ki67⁺ cell density within the granule cell layer and subgranular zone of the dentate gyrus. Cell proliferation, as assessed by Ki67 expression, increased similarly in both vehicle- and DZP-exposed mice after injury (** $p < 0.01$ sham-vehicle vs. CCI-vehicle; *** $p < 0.001$ sham-DZP vs. CCI-DZP, $n = 8-10$ mice/group).



indicates cell proliferation.⁵² CCI dramatically increased Ki67 expression in the GCL of the dentate gyrus (Fig. 2C,D). Ki67 expression increased similarly in both CCI-vehicle ($p=0.011$; vs. sham-vehicle; Mann-Whitney test; Fig. 2D) and CCI-DZP groups ($p=0.001$; vs. sham-DZP; Mann-Whitney test; Fig. 2D), and importantly, did not differ between CCI groups ($p=0.130$; CCI-vehicle vs. CCI-DZP; Mann-Whitney test; Fig. 2D). This observation further supports the lack of an effect of DZP on post-injury hippocampal cell proliferation. Again, DZP did not affect cell proliferation in sham-treated mice, as assayed by Ki67 expression ($p=0.515$; sham-vehicle vs. sham-DZP; Mann-Whitney test; Fig. 2C,D). Together, these data indicate that although DZP inhibits post-traumatic neurogenesis, this is not mediated by a DZP-induced decrease in cell proliferation.

Diazepam prevents aberrant dendritic arborization of neurons born after TBI

POMC-GFP mice transiently express GFP in immature (10- to 14-day-old) hippocampal adult-born neurons, and allow for morphological assessment of immature neurons.⁴² Granule cells born after TBI exhibit dendritic branching abnormalities,^{23,24} characterized by the increased branching of dendrites close to the soma and within the inner molecular layer. As dendritic outgrowth is modulated by GABA_AR signaling during cell maturation,^{33,53} we analyzed whether DZP affected the dendritic morphology of neurons born after CCI using hippocampal tissue from *POMC-GFP* mice 14 days after CCI or sham treatment.

We first examined the magnitude of post-traumatic neurogenesis in *POMC-GFP* mice treated with DZP. Consistent with our data from wild-type mice (Fig. 1B–E), CCI injury increased the density of GFP⁺ immature neurons in vehicle-treated *POMC-GFP* mice (sham-vehicle = 987 ± 101 cells/mm²; CCI-vehicle = 1730 ± 290 cells/mm²; $p=0.04$, Mann-Whitney test, $n=9$ mice/group; Fig. 3A,B), and the CCI-induced increase in GFP⁺ neuron density was inhibited by DZP (sham-DZP = 956 ± 129 cells/mm²; CCI-

FIG. 3. Diazepam (DZP) inhibits post-traumatic neurogenesis and mitigates the effect of traumatic brain injury (TBI) on the dendritic morphology of adult-born neurons. **(A)** Representative images of the dentate gyrus of *POMC-GFP* mice 2 weeks after sham or controlled cortical impact (CCI) treatment, illustrating the increase in immature neuron (GFP⁺) density following CCI in vehicle-treated but not DZP-treated mice. **(B)** Higher-power images demonstrating GFP⁺ granule cell morphology after CCI or sham procedures in vehicle- and DZP-treated *POMC-GFP* mice. **(C)** Representative tracings of adult-born neurons from mice exposed to DZP or vehicle after CCI or sham treatment. **(D)** Sholl analysis comparing dendritic branching patterns of adult-born neurons generated in the different groups. Compared with sham-vehicle treatment (black line), adult-born neurons from injured mice treated with vehicle (red line) had more branches 20 μm and 30 μm from the soma, and had fewer branches 60 μm and 70 μm from the soma (red asterisks indicate comparison between CCI-vehicle and sham-vehicle, * $p<0.05$, ** $p<0.01$, *** $p<0.001$). This injury-induced branching pattern was prevented by DZP, as there were no significant differences in the branching patterns between sham-vehicle and CCI-DZP treated mice (black vs. green lines). DZP alone enhanced distal branch length of new neurons in sham mice with no effects on proximal branching (blue asterisk indicates comparison between sham-DZP and sham-vehicle, * $p<0.05$, ** $p<0.01$, *** $p<0.001$; compared with sham-vehicle) $n=17$ –20 cells from 3–4 mice/group).

DZP = 1447 ± 219 cells/mm²; $p = 0.72$, Mann-Whitney test, $n = 7$, 11 mice/group). Again, DZP had no effect on neurogenesis rate in non-injured *POMC-GFP* animals (sham-vehicle vs. sham-DZP; $p = 0.83$, Mann-Whitney test).

Morphological analysis of single GFP⁺ neurons from sham and CCI-treated mice demonstrated that CCI altered the dendritic branching of neurons born after injury in vehicle-treated mice ($F[5.189, 171.238] = 6.587$; $p < 0.001$, CCI by distance interaction, sham-vehicle vs. CCI-vehicle, repeated measures ANOVA; Fig. 3C,D). CCI-induced adult-born neurons had more branches at proximal regions of their dendritic trees and fewer branches at more distal regions from the soma ($20 \mu\text{m}$, $p < 0.001$; $30 \mu\text{m}$, $p < 0.001$; $60 \mu\text{m}$, $p < 0.05$; $70 \mu\text{m}$, $p < 0.01$; Bonferroni post hoc test; Fig. 3D), consistent with prior reports.^{23,24} In contrast, aberrant proximal dendritic branching of neurons born after CCI was completely prevented by DZP treatment, which restored the normal (more distal) branching pattern of these cells and showed no difference from sham-vehicle controls in the branching patterns at any single point (Fig. 3B–D).

Consistent with the previously described facilitatory role of GABA_AR activation on dendritic outgrowth,²⁹ DZP itself caused a mild but significant increase in dendritic length of immature neurons in sham-treated animals, with no effect on proximal branching patterns ($p > 0.05$ at $10\text{--}60 \mu\text{m}$, $p < 0.001$ at $70 \mu\text{m}$, $80 \mu\text{m}$, and $90 \mu\text{m}$, $p < 0.01$ at $100 \mu\text{m}$, sham-vehicle vs. sham-DZP, Bonferroni post hoc test; $F[4.007, 140.245] = 5.656$; $p < 0.001$, drug by distance interaction, sham-vehicle vs. sham-DZP, repeated measures ANOVA, Fig. 3D). Also, as expected for neurons 10 to 14 days post-mitosis,⁴² we did not observe dendritic spines on neurons in any treatment group. As dendritic spines begin to appear on immature neurons at approximately 16 days post-mitosis,⁵⁴ this suggests that neither CCI nor DZP treatments accelerated neuronal maturation in

any group, and that no GFP⁺ cells were generated prior to CCI/sham procedures.

Diazepam does not attenuate the enhanced migration of adult-born neurons after TBI

Aberrant migration of adult-born neurons occurs after various forms of brain injury, including seizures, strokes, and TBI.^{18,23–26,55} In our experiments here, neurons born after TBI also migrated greater distances from the SGZ (sham-vehicle vs. CCI-vehicle; $p = 0.0005$, Mann-Whitney test; Fig. 4A,B). However, in contrast to its other effects on post-injury neurogenesis, DZP did not prevent the aberrant migration of neurons born after CCI (sham-DZP vs. CCI-DZP treated mice; $p = 0.0007$, Mann-Whitney test; Fig. 4A,B). DZP also did not prevent the CCI-induced increase in ectopic cell migration into the dentate molecular layer (sham-vehicle vs. CCI-vehicle, $p = 0.003$; sham-DZP vs. CCI-DZP, $p = 0.002$; Mann-Whitney test; Fig. 4C, left) and actually enhanced the migration of a subset of adult-born neurons into the hilus (sham-DZP vs. CCI DZP; $p = 0.017$, Mann-Whitney test; Fig. 4C, right).

Diazepam does not alter neuronal injury, astroglial activation, or locomotor activity after CCI

The magnitude of post-traumatic neurogenesis is related to injury severity.^{15,56} Thus, if DZP had neuroprotective effects, it could reduce post-traumatic neurogenesis indirectly through mitigation of the CCI injury. To assess for a potential effect of DZP on brain injury, we first assessed the degree of tissue damage 3 days after CCI. In brain sections at the epicenter of the CCI injury, there were no differences in the CCI-induced cortical cavity between vehicle- and DZP-treated mice ($p = 0.56$, Mann-Whitney test; Fig. 5A,B), indicating that DZP did not decrease the amount of cortex lost to injury.

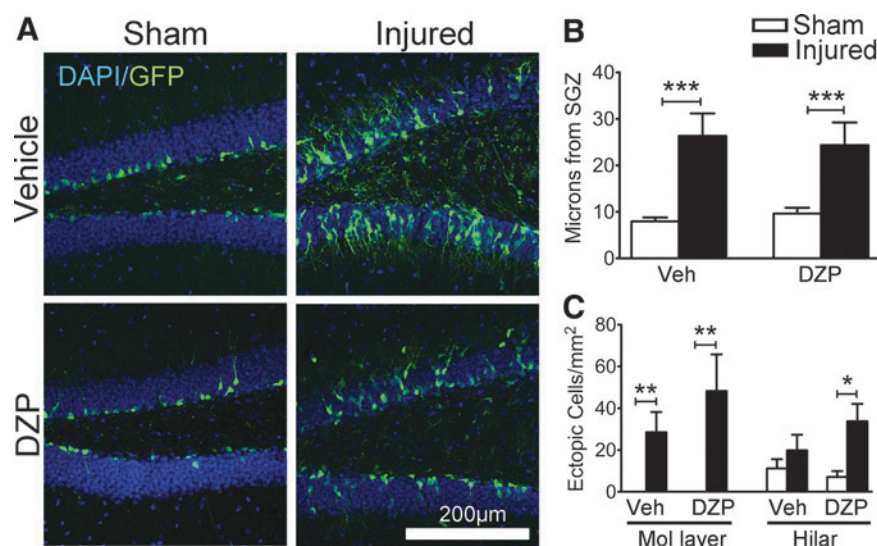


FIG. 4. Outward migration of adult-born neurons after controlled cortical impact (CCI) was not affected by diazepam (DZP). (A) Images of the dentate gyrus from *POMC-GFP* mice illustrate the migration of adult-born neurons away from the subgranular zone (SGZ) in sham and injured mice treated with DZP or vehicle. (B) Quantification of migration distance of adult-born neurons from the SGZ. GFP⁺ neurons generated after injury migrated greater distances from the SGZ compared with those born under sham conditions, and this was not prevented by DZP ($***p < 0.001$; $n = 7\text{--}11$ mice/group). (C) Quantification of ectopic adult-born neuron migration into the hilus or molecular layer of the dentate gyrus. CCI increased the number of ectopically migrated adult-born neurons in the molecular layer similarly in both vehicle- or DZP-exposed mice ($**p < 0.01$; $n = 7\text{--}11$ mice/group; left). Injured mice treated with DZP actually had a greater density of adult-born neurons in the hilus compared with their sham-DZP treated control group ($*p < 0.05$), whereas injured mice not treated with DZP were no different from their sham-vehicle treated control group ($p = 0.61$; sham-vehicle vs. CCI-vehicle treated mice; $n = 7\text{--}11$ mice/group; right).

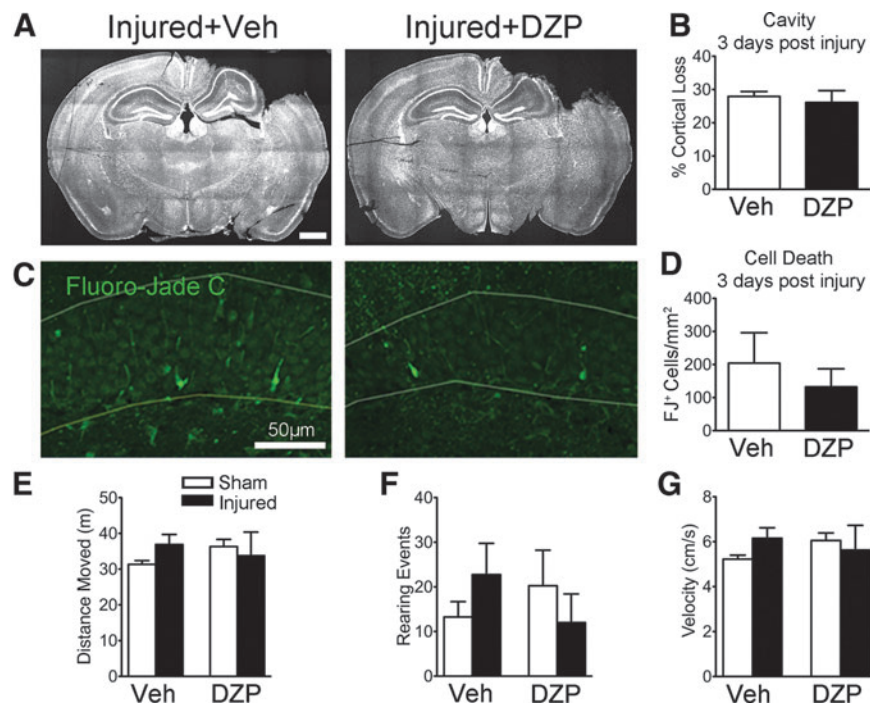


FIG. 5. Diazepam (DZP) does not decrease neuronal injury or alter locomotor activity after controlled cortical impact (CCI). (A) Images depicting typical cortical injuries in mice exposed to vehicle or DZP. Scale bar: 1 mm. (B) Quantification of cortical loss after injury. There were no differences between vehicle- and DZP-treated mice ($p=0.56$, $n=6-10$ mice/group). (C) Fluoro-Jade C staining of degenerating cells in the dentate granule cell layer from injured mice exposed to vehicle or DZP. (D) Quantification of Fluoro-Jade-C⁺ cell density in the granule cell layer of the dentate gyrus, 3 days after injury. There were no differences in the density of Fluoro-Jade C⁺ cells between injured mice exposed to vehicle and DZP treatment ($n=9-11$ mice/group). (E) Open field analysis performed 3 days after sham or CCI demonstrates that mice moved similar distances in the 10-min session in all groups, independent of vehicle versus DZP treatment ($p=0.809$, effect of drug, $n=4$ mice/group). (F) Rearing events during the open field testing did not differ in frequency between groups ($p=0.916$). (G) Open field mean velocities from distance traveled also did not differ between groups ($p=0.809$).

As this gross anatomical measurement might not be sensitive to cell death within the parenchyma, we examined cell death with Fluoro-Jade C after CCI in both vehicle- and DZP-treated mice 3 days after injury. There was no difference in cell death between CCI-injured mice given vehicle versus DZP (Fig. 5C–D). We also examined a separate cohort of injured brains 3 h after injury, and again found no differences in the density of Fluoro-Jade C⁺ cells between DZP- and vehicle-treated mice in either the GCL (vehicle, 54.5 ± 13.9 cells/ μm^2 ; DZP, 19.7 ± 6.3 cells/ μm^2 ; $p=0.11$, Mann-Whitney test, $n=4$ mice/group) or peri-lesional cortex (vehicle, 1075 ± 334 cells/ μm^2 ; DZP, 1248 ± 140 cells/ μm^2 ; $p=0.49$, Mann-Whitney test, $n=4$ mice/group).

As neurogenesis is enhanced by locomotor activity,⁶² we assayed whether DZP may have altered post-injury neurogenesis by reducing mouse locomotor activity levels. First, gross daily observation of mice did not reveal any sedating effects of our DZP dose in either CCI- or sham-treated mice (data not shown). However, as gross observations rely on qualitative assessments, we quantified open field activity in mice 3 days after injury, to coincide with the time-point used for our analyses of neurogenesis/mitosis. DZP did not alter locomotor activity of mice after CCI, based on the lack of group differences in the total distance moved ($p=0.809$, effect of drug; two-way ANOVA, $n=4$ mice/group; Fig. 5E); rearing events ($p=0.916$, vehicle vs. drug; Mann-Whitney test; Fig. 5F); or in mean velocity ($p=0.809$, effect of drug; two-way ANOVA; Fig. 5G). These data indicate that differences in loco-

motor activity do not account for DZP's specific effects on post-injury neurogenesis.

Astroglia are activated by neuronal injury and can release factors that modulate the survival and maturation of adult-born neurons.^{57–59} Astroglia also express benzodiazepine-sensitive GABA_ARs, which can modulate glial activation.⁶⁰ Thus, if DZP altered glial function after injury, either by directly modulating glial activation or glial proliferation, or by reducing post-injury signaling to astroglia, it could affect post-injury neurogenesis through DZP-induced changes in astroglial function.

To assess astroglial activation, we stained hippocampal sections for the acute phase marker glial fibrillary acidic protein (GFAP), which increases in expression during glial activation. As has been commonly observed,^{15,61} CCI increased the density of GFAP immunofluorescence when compared with sham-treated mice receiving vehicle ($p=0.029$; sham-vehicle vs. CCI vehicle; Mann-Whitney test; Fig. 6A,B). This increase in GFAP staining after CCI was similar in DZP-treated animals ($p=0.0087$; sham-DZP vs. CCI-DZP; Mann-Whitney test; Fig. 6A,B), and was not different between CCI-vehicle and CCI-DZP treated mice ($p=0.413$, Mann-Whitney test). The data not only indicate that astroglial activation was not reduced by DZP, but also provide further evidence that injury magnitude was not affected by DZP.

Additionally, mice were weighed just before and 1 week after DZP exposure to globally assess the potential of DZP-induced weight changes, which could indicate other effects on mouse health

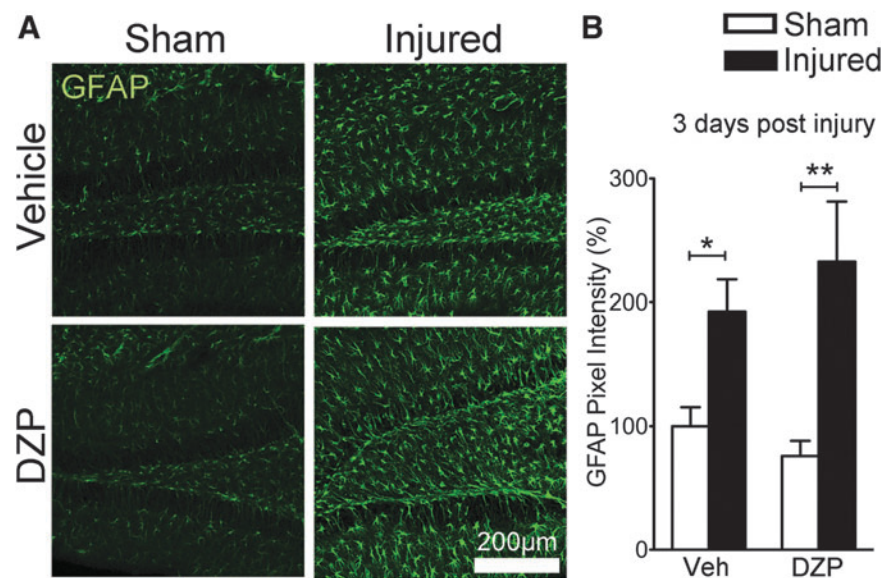


FIG. 6. Diazepam (DZP) does not attenuate glial activation after controlled cortical impact (CCI). **(A)** Images of glial fibrillary acidic protein (GFAP)-stained hippocampal tissue from sham- or CCI-treated mice exposed to vehicle or DZP. **(B)** Quantification of GFAP immunoreactivity within the dentate gyrus (molecular layer and hilar pixel intensities were combined, as there were no regional differences), normalized to mean pixel intensity of sham/vehicle-treated sections. CCI injury increased GFAP intensity irrespective of drug treatment (* $p < 0.05$, sham-vehicle vs. CCI-vehicle; ** $p < 0.01$, sham-DZP vs. CCI-DZP, $n = 4$ –6 mice/group).

or nutritional status. Although post-injury weight gain was slightly lower in both groups of CCI-treated mice, mouse weight was unaffected by DZP (change in weight during the week of pump exposure: sham-vehicle = 0.68 ± 0.14 g; CCI-vehicle = 0.44 ± 0.29 g; sham-DZP = 1.09 ± 0.26 g; CCI-DZP = 0.26 ± 0.16 g; two-way ANOVA, $F[1,25] = 4.242$, $p = 0.0499$ main effect of CCI; $p = 0.666$ for effect of DZP, $n = 7$ mice/group).

Diazepam inhibits the injury-induced burst of neuronal activity in the dentate gyrus

TBI induces dramatic changes in neuronal activity, both directly at the time of injury, and in subsequent weeks due to neuronal death and dysfunction.^{23,63} In humans, TBI can cause clinical or sub-clinical seizures at or near the time of injury.⁶⁴ As neuronal activity and seizures can positively modulate neurogenesis,^{32,65,66} we postulated that DZP might reduce post-traumatic neurogenesis by reducing neuronal activity. To assay neuronal activity during the early post-injury period, we stained hippocampi from CCI- and sham-treated mice for the activity-dependent gene c-Fos.

As previously described,^{15,67,68} injury transiently increased neuronal activity in the hippocampal dentate gyrus in vehicle-exposed mice within hours after injury (Fig. 7A,B; $p = 0.029$, Mann-Whitney test, $n = 4$ /group). At this same time-point, DZP-treated mice did not demonstrate a statistically significant increase in c-Fos⁺ expression after CCI (Fig. 7A,B; $p = 0.40$, Mann-Whitney test, $n = 3$, 3–4 mice) indicating that DZP had attenuated the early injury-induced burst of hippocampal neural activity, shortly after osmotic pump implantation.

Three days after injury, DZP caused an overall reduction of neuronal activity within the GCL of mice in both sham and CCI groups, as quantified using c-Fos⁺ cell density ($F[1,33] = 25.63$, $p = 0.00002$; main effect of drug, two-way ANOVA; Fig. 7C,D,F). Independently, CCI was associated with reduced c-Fos⁺ activation in the dentate gyrus in both drug treatment groups ($F[1,33] = 13.2$,

$p = 0.0009$; main effect of CCI, two-way ANOVA; Fig. 7E), which may be the immunohistochemical manifestation of post-traumatic neural depression that has been described in both humans and animals after TBI.⁶⁹ Thus, at both early and late phases after injury, DZP inhibited neuronal activity throughout the dentate gyrus, which was associated with both quantitative and qualitative normalization of post-traumatic neurogenesis.

Discussion

DZP prevented post-traumatic neurogenesis and inhibited the aberrant dendritic development of neurons born after TBI. This not only has implications for our mechanistic understanding of injury-induced neurogenesis, but will facilitate future investigation into the importance of post-traumatic neurogenesis to recovery after brain injury.

Activity-dependent mechanisms in neurogenesis

Constitutive neurogenesis is strongly regulated by network activity, as environmental/behavioral contingencies and the direct activation of neurotransmitter-binding receptors can increase the proliferation of stem cells and survival of adult born neurons.^{32,66,70–73} Thus, it is plausible that these mechanisms could sculpt the neurogenic response after injury as well. TBIs are known to induce seizure-like activity early after injury,⁷⁴ and seizures increase neurogenesis and alter the dendritic structure of adult-born neurons.^{26,27} Thus, the inhibition of post-traumatic neurogenesis by DZP could be due to inhibition of circuit hyperactivity after injury, and in particular via suppression of sub-clinical seizure activity after TBI. Although we did not directly measure seizure activity after CCI in our mice, our c-fos staining indicated that DZP reduced activity during both early and late phases after injury, consistent with the known seizure-suppressing effects of benzodiazepines.⁷⁵

Our finding that DZP normalized the dendritic development of cells generated after injury is surprising, due to the time course of

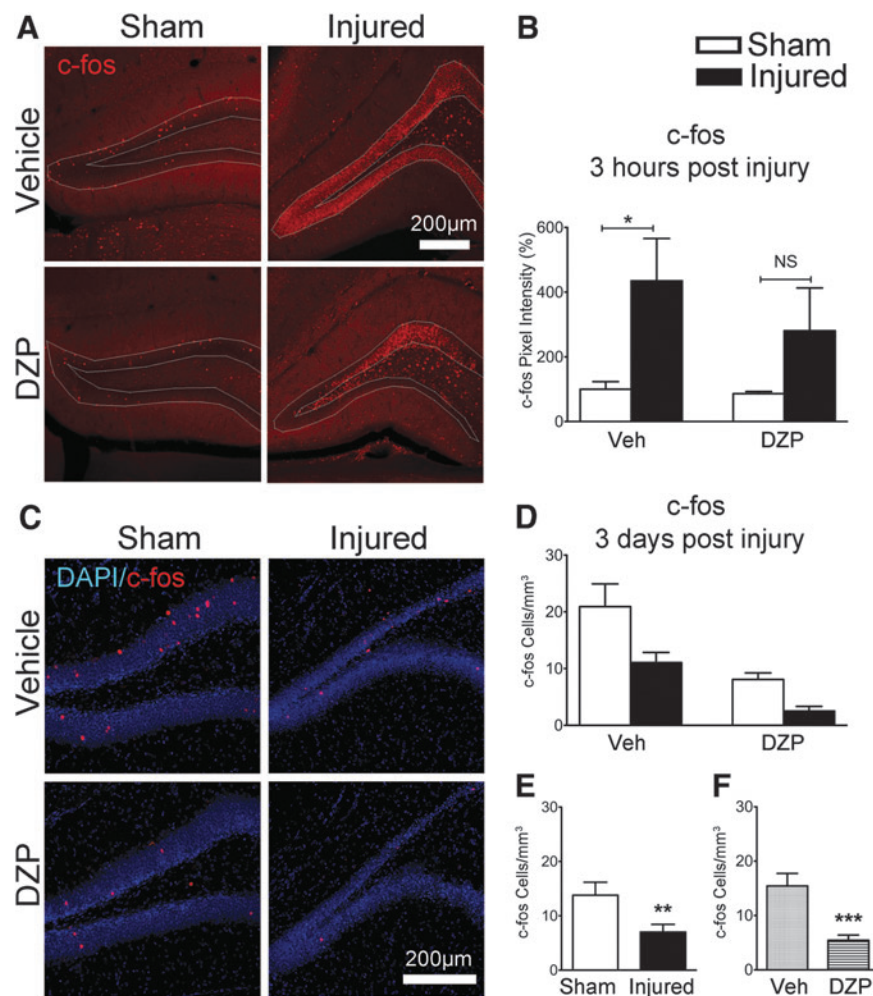


FIG. 7. Diazepam (DZP) reduces neuronal activity after CCI. **(A)** Images of the dentate gyrus of sham or injured mice treated with DZP or vehicle, 3 h after treatment. White lines delineate the traced granule cell layer (GCL) used for quantification. **(B)** Mean pixel intensity of c-fos immunoreactivity within the GCL, normalized to mean pixel intensity of sham/vehicle-treated sections. Injury significantly increased c-fos expression in vehicle-treated, but not DZP-treated, mice (* $p < 0.05$, sham-vehicle vs. CCI-vehicle; $p = 0.40$; sham-DZP vs. CCI-DZP, $n = 3-4$ mice per group). **(C)** c-Fos staining (red) in the dentate gyrus of sham and CCI-injured mice treated with DZP or vehicle, 3 days after sham or CCI injury. DAPI (blue) shows the outline of the GCL of the dentate gyrus. **(D)** Quantification of c-fos⁺ cell density. **(E)** c-fos⁺ cell density was reduced by CCI injury independent of drug treatment (** $p < 0.001$) and by **(F)** DZP independent of CCI treatment (** $p < 0.0001$; $n = 8-10$ mice/group).

drug administration relative to dendritic outgrowth. DZP was removed after the first post-injury week, before the majority of the dendritic outgrowth by this population of cells would have occurred,⁵⁴ yet the cells still exhibited dendritic branching patterns that were similar to cells born in uninjured brains. This suggests that DZP induced longer-lasting changes in the hippocampal signaling environment, or caused other changes in gene expression in these neurons⁷⁶ that persisted after DZP exposure ended.

DZP blocked post-traumatic neurogenesis without affecting the early burst of cell proliferation after injury, and inhibited the aberrant dendritic development of these cells without preventing their enhanced outward migration. Thus, our data suggest that post-traumatic neurogenesis is influenced by multiple different signals, which drive both an early hyperproliferative response as well as subsequent alterations in neuronal survival, cell migration, and dendritic arborization. As glial activation and neuronal cell death were unaffected by DZP, we propose that glial-derived or other

injury-related signals drive the increase in hippocampal stem cell proliferation,^{23,77-81} after which neuronal activity or GABA_AR-mediated signaling is required for the successful maturation of these cells into neurons. This is consistent with the notion that survival signaling can involve both an activity-independent early phase and an activity-dependent late phase.^{45,50,82,83} Similarly, our observation that DZP does not block the TBI-induced migration of adult-born cells suggests that migration of these cells after injury is controlled by other injury-induced alterations in signaling gradients, such as reelin, which were not affected by DZP.

Finally, it must be noted that although we found an association between the DZP-induced reduction in neuronal activity and the normalization of neurogenesis, our work does not establish a causal relationship between these two observations. Although this would be difficult to prove *in vivo*, we suspect that further studies of the specific signaling mechanisms involved will shed light on this question.

GABA_AR-mediated modulation of neurogenesis

DZP could also have directly affected the survival of adult-born neurons by modulating GABA_ARs on individual adult-born neurons in a cell-autonomous manner. Functional GABA_ARs are expressed on the membranes of neuronal precursor cells and radial glial cells,^{39,84} and DZP, a GABA_AR allosteric modulator, could increase signaling via those receptors. Although GABA_ARs control the rate of adult-born neuron maturation and dendritic outgrowth,³³ data suggesting that GABA_AR activity directly modulates cell proliferation and survival are less clear.

Prior studies have obtained conflicting results regarding the effects of DZP on neurogenesis rates in healthy animals, with either no or minimal effect,^{85–87} an increase in cell proliferation,⁴⁰ or a net decrease in neurogenesis.^{39,72} In apparent contrast to results obtained by Song and colleagues,³⁹ we did not observe an effect of DZP in sham-treated animals, although we employed a different dosing strategy (continuous administration vs. once-daily i.p. injection). We chose to administer DZP via pump given the short (~ 1 h) half-life of DZP in rodents,^{47,88} and to model continuous drug administration, as might be expected for humans receiving prolonged sedative infusions during post-injury intensive care. These different dosing strategies drive differential gene expression patterns *in vivo*,⁸⁹ and thus could explain this discrepancy.

Our results are reminiscent of a recent study in which DZP completely prevented the early phases of post-ischemic neurogenesis when administered for just 3 days after stroke.⁸⁷ This study, however, did not examine cells after neuronal differentiation and early maturation had commenced, so it is unclear whether other aspects of post-ischemic neurogenesis, such as the aberrant maturation of adult-born granule cells,²⁵ were also affected.

Future work will hopefully determine whether the effect of DZP on adult-born granule cells is cell autonomous or mediated by changes in network activity. In this regard, it would be of interest to determine whether other GABA_AR agonists used in the management of acute head injury, such as propofol or etomidate, have similar effects on post-injury neurogenesis during prolonged administration, as they modulate GABA_ARs at different binding sites and have different off-target effects.^{90,91}

Implications for functional recovery

Although neurons born after TBI likely contribute to hippocampal information processing after injury,²³ it is unknown whether increased neurogenesis is beneficial or maladaptive, particularly given the abnormal morphology and aberrant migration of cells born after injury.^{23,24} Because neurons born after other brain injuries aberrantly integrate into the hippocampal network^{25,26} and might play a role in driving neuropathology,²⁹ it is possible that aberrantly developed neurons could drive long-term circuit dysfunction after TBI, for example, by driving hippocampal hyperexcitability and post-traumatic epilepsy, or via depletion of the stem cell pool.^{30,92}

Previous attempts to assess the functional significance of post-traumatic neurogenesis have done so via genetic or pharmacological inhibition of neurogenesis, followed by behavioral assessment of cognitive performance.^{93,94} However, these studies inhibited neurogenesis below baseline levels, which alone causes cognitive impairments.^{8,95–97} An alternative approach has involved the administration of growth factors to increase neurogenesis after TBI^{20,21,98–100}; however, growth factors can modulate other neuronal functions, such as synaptic plasticity.¹⁰¹ As DZP appeared to normalize post-traumatic neurogenesis both quantitatively and

qualitatively, it will facilitate analysis of whether the alterations in neurogenesis after TBI positively or negatively affect long-term outcomes.

The contribution of hippocampal neurogenesis to recovery after human TBI is unknown. Although hippocampal neurogenesis has been demonstrated in adult humans using a variety of different techniques,^{7,102–104} there is a lack of data on human neurogenesis after TBI, which likely results from difficulty in obtaining samples at the appropriate post-injury interval. One study examined pediatric brains after TBI with a very short, mean post-injury survival of just 33 h, and did not see evidence of increased neurogenesis.¹⁰⁵ However, this is before increased neurogenesis is observed in rodent models¹⁰⁶ and thus, these samples may have been obtained before the initiation of post-injury neurogenesis. Interestingly, the one sample from this study obtained 7 days after injury demonstrated a notable increase in hippocampal neurogenesis. As histochemical studies have inherent limitations due to tissue availability and processing, perhaps non-invasive quantification of neurogenesis¹⁰⁷ could one day conclusively characterize the course of post-injury neurogenesis in humans.

Acknowledgments

This material was supported in part by the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development, Biomedical Laboratory Research and Development CDA-2 Award 005-10S (ES), a Department of Veterans Affairs Merit Review Award I01-BX002949 (ES), a Department of Defense CDMRP Award W81XWH-18-1-0598 (ES), the Oregon Clinical and Translational Research Institute (OCTRI) NIH TL1TR000129 from the National Center for Advancing Translational Sciences at the National Institutes of Health (RM), the NIH F32NS083109 (LEV), a BIRCIW K12HD043488 Award made possible through the Eunice Kennedy Shriver National Institute of Child Health and Human Development and the Office of Research on Women's Health (LEV), and P30NS061800 (OHSU) Awards. We thank Dr. Gary Westbrook of the Vollum Institute for comments on our manuscript, Dr. Stefanie Kaech-Petrie of the OHSU Advanced Light Microscopy Core for assistance with imaging, and our lab volunteer Khanh Doan and research assistants Sarah Mader and Alexandria Wilson for outstanding technical support. The contents of this manuscript do not represent the views of the U.S. Department of Veterans Affairs or the United States Government.

Author Disclosure Statement

No competing financial interests exist.

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conduct statistical testing. The data are reported as percent change and mean $\pm$ standard deviation.

Results: TTX-mediated inactivation of PFC increased the time to emergence from sevoflurane anesthesia by 95.9% [CTRL = 516.2 ± 98.6 s vs. TTX = 1011 ± 394.5 s] while there was a negligible effect of PC inactivation on the increase (3.98%) in emergence time [CTRL = 452.3 ± 167.2 s vs. TTX = 470.3 ± 48.1 s]. Inactivation of PFC and PC produced similar decreases in the time to loss of consciousness [PFC: 45.6% decrease, CTRL = 232.8 ± 21.4 s vs. TTX = 126.7 ± 11.02 s; PC: 40.8% decrease, CTRL = 377.7 ± 152.6 s vs. TTX = 223.7 ± 101.02 s]. Inactivation of PFC or PC had a comparable effect on BSR [PFC: 10.6% increase, CTRL = 19.8 ± 10.7 vs. TTX = 21.9 ± 16.8 ; PC: 27.08% increase, CTRL = 14.4 ± 8.4 vs. TTX = 18.3 ± 2.3].

Conclusions: While the increase in emergence time and decrease in induction time after PFC inactivation supports a causal contribution of PFC in level of arousal, these findings warrant further investigation with a greater sample size.

Funding: NIH R01 GM111293 to G.A.M. and D.P., and Department of Anesthesiology.

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[SNACC-370] Imbalanced Excitatory and Inhibitory Inputs Onto Hippocampal Dentate Granule Cels Born After Traumatic Brain Injury

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Background: Post-traumatic epilepsy can be treatment-resistant, so understanding the underlying pathophysiology will allow for the development of novel therapeutic approaches. Hippocampal granule cels born after traumatic brain injury (TBI) develop more dendritic branches in the inner molecular layer (IML) of the dentate gyrus, which is known to contain recurrent inputs. This ongoing study is evaluating whether granule cels born after TBI receive altered input from fibers within IML, which could contribute to their participation in recurrent positive feedback loops.

Methods: Heterozygotic Doublecortin-CreERT2:Rosa26-tdTomato (DcxCre/tdT) mice were used to visualize adult born granule cels. We labeled adult-born granule cels in two-month old DcxCre/tdT mice by administering tamoxifen (TAM) a week after controlled cortical impact (CCI) or sham surgery. The doublecortin (Dcx) promoter is selectively active in immature hippocampal granule cels, and immature neurons at the time of TAM injection become permanently labeled with the tdT fluorescent marker. Eight weeks after CCI, we obtained whole-cell voltage clamp recordings from tdT+ cels in acutely prepared hippocampal slices, used a bipolar electrode to evoke IML synaptic responses, and used SR95531 (10 μ M) in the external solution to block GABA_A receptors (GABAARs).

Results: The serial resistance (sham: 18 ± 1 M Ω , n = 11, CCI: 19 ± 3 , n = 9, $P = 0.62$) and membrane input resistance (sham: 436 ± 38 M Ω , CCI: 356 ± 44 M Ω , $P = 0.18$) of tdT+ cels exhibited no statistical difference between two groups, indicating that these two groups of cels had similar electrophysiological properties. After blocking GABAARs with SR95531, the evoked responses represented AMPA receptor (AMPA)-mediated currents. Evoked GABAAR-mediated currents were derived by subtracting AMPA-mediated currents from the combined evoked post-synaptic current before SR95531 perfusion. The ratio of AMPA-to GABAAR-mediated currents at a given stimulus intensity was significantly increased in the CCI group

compared to shamgroup (ratio of charge transfers: sham = 0.19 ± 0.03 , n = 11, CCI = 0.73 ± 0.26 , n = 9, $P = 0.034$; ratio of amplitudes: sham = 0.60 ± 0.14 , CCI = 1.69 ± 0.39 , $P = 0.01$). AMPA-to-NMDA ratio did not change significantly (sham: 1.28 ± 0.13 M Ω , n = 11; CCI: 1.64 ± 0.20 , n = 9; $P = 0.14$).

To compare the overall strength of excitatory inputs between conditions, we increased the stimulus strength from 100 μ A to 900 μ A in 100 μ A increments, while recording evoked currents in the presence of SR95531. Preliminary results suggest that the slope of the I/O relationship is similar in the two groups (sham = -0.81 ± 0.22 , n = 6; CCI = -1.13 ± 0.33 , n = 4; $P = 0.44$), but more recordings are needed to confirm this observation.

Conclusion: The results suggest that granule cels born after TBI receive reduced feed-forward inhibition in the IML, which might cause a net imbalance between excitation and inhibition onto cels born in the post-injury brain. Ongoing studies are examining whether this phenomenon present in al granule cels in the dentate after TBI, and to determine whether therapeutic attempts to alter synaptic connectivity in the IML could lead to improved outcomes after head injury.

[SNACC-371] Visual Evoked Potentials Breakdown Under Anesthesia

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How general anesthetics block sensory perception remains a mystery. Interestingly, even under general anesthesia, evoked sensory responses are readily recorded from primary sensory cortices.^{1,2} This suggests that perception relies on the interaction among primary and higher order cortical networks. The long range integration of cortical signaling can often be seen as a complex evoked response that long outlasts stimulus presentation and propagates across the cortical surface.^{3,4} Yet, how such spatiotemporal responses are generated, maintained, and modulated by brain state remain a mystery. To quantify the effect that anesthetic induced brain state has on the spatiotemporal dynamics of visual-evoked activity, we performed high density electrocorticography recordings in head-fixed mice that were given isoflurane, propofol, ketamine and awake. We recorded spontaneous and evoked local field potentials as mice were presented with simple visual stimuli. We find that there is much trial-to-trial and animal-to-animal variability in visual evoked potentials (VEPs). However, one consistent feature of VEPs across trials, animals and anesthetics is an early (< 250 msec) visual-evoked gamma band oscillation (20-60 Hz). This coherent gamma oscillation organizes into standing, traveling and rotational waves that cover large swaths of cortical surface. In mice under isoflurane and ketamine, gamma waves begin in the primary visual cortex (V1) and extend into the posterior parietal association and the somatosensory cortices. These gamma waves extend even farther over the cortical surface when the same animals are awake. In contrast, under propofol, the gamma waves decay quickly and fail to reliably extend outside of V1. Our results illuminate the presence of long range coordinated visual evoked gamma waves under waking and anesthetic states. This suggests that even under some anesthesia agents, there is organized intercortical processing of visual stimuli outside of V1. Moreover, anesthetic and behavioral state can modulate parameters of visual evoked gamma waves.

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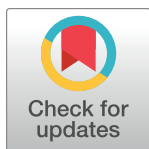
RESEARCH ARTICLE

Neuronal network remodeling and Wnt pathway dysregulation in the intra-hippocampal kainate mouse model of temporal lobe epilepsy

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OPEN ACCESS

Citation: Gupta K, Schnell E (2019) Neuronal network remodeling and Wnt pathway dysregulation in the intra-hippocampal kainate mouse model of temporal lobe epilepsy. PLoS ONE 14(10): e0215789. <https://doi.org/10.1371/journal.pone.0215789>

Editor: Stephen D. Ginsberg, Nathan S Kline Institute, UNITED STATES

Received: April 4, 2019

Accepted: September 20, 2019

Published: October 9, 2019

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This material was supported in part by the Department of Veterans Affairs Merit Review Award I01-BX002949 (ES), a Department of Defense CDMRP Award W81XWH-18-1-0598 (ES), a Neurosurgery Research and Education Foundation Fellowship (<https://www.nref.org>) ANEUS0125 (KG) and P30NS061800 (OHSU) awards.

Abstract

Mouse models of mesial temporal lobe epilepsy recapitulate aspects of human epilepsy, which is characterized by neuronal network remodeling in the hippocampal dentate gyrus. Observational studies suggest that this remodeling is associated with altered Wnt pathway signaling, although this has not been experimentally examined. We used the well-characterized mouse intrahippocampal kainate model of temporal lobe epilepsy to examine associations between hippocampal neurogenesis and altered Wnt signaling after seizure induction. Tissue was analyzed using immunohistochemistry and confocal microscopy, and gene expression analysis was performed by RT-qPCR on RNA extracted from anatomically micro-dissected dentate gyri. Seizures increased neurogenesis and dendritic arborization of newborn hippocampal dentate granule cells in peri-ictal regions, and decreased neurogenesis in the ictal zone, 2-weeks after kainate injection. Interestingly, administration of the novel canonical Wnt pathway inhibitor XAV939 daily for 2-weeks after kainate injection further increased dendritic arborization in peri-ictal regions after seizure, without an effect on baseline neurogenesis in control animals. Transcriptome analysis of dentate gyri demonstrated significant canonical Wnt gene dysregulation in kainate-injected mice across all regions for Wnt3, 5a and 9a. Intriguingly, certain Wnt genes demonstrated differential patterns of dysregulation between the ictal and peri-ictal zones, most notably Wnt5B, 7B and DKK-1. Together, these results demonstrate regional variation in Wnt pathway dysregulation early after seizure induction, and surprisingly, suggest that some Wnt-mediated effects might actually temper aberrant neurogenesis after seizures. The Wnt pathway may therefore provide suitable targets for novel therapies that prevent network remodeling and the development of epileptic foci in high-risk patients.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Mesial temporal lobe epilepsy can develop in humans after a number of neurologic insults, including trauma [1], infection [2], stroke [3] and febrile seizures [4]. Anti-epileptic drugs (AEDs) in these conditions can reduce seizure occurrence, however up to 40% of patients with epilepsy are considered medically refractory [5]. AEDs have been tested in a preventative fashion in post-traumatic epilepsy and effectively reduce early seizures after injury [6]; however, no treatment exists to prevent the development of delayed epilepsy after neurologic insults. Thus, an understanding of neuronal circuit remodeling after neurologic insults is necessary to optimally design preventative treatments.

Rodent models recapitulate key hallmarks of human temporal lobe epilepsy, including mesial temporal sclerosis, gliosis, mossy fiber sprouting and loss of hippocampal pyramidal and hilar neurons, and have contributed greatly to our understanding of mechanisms underlying epileptogenesis [7, 8]. In many of these models, an initial seizure is triggered using either focal (intraparenchymal) or global (systemic) manipulations, and is followed by a latent period during which rodents develop spontaneous recurrent seizures, similar to clinical observations in a subset of human patients [9].

It is increasingly recognized that circuit changes in epilepsy involve more than just the ictal zone where seizure activity initiates, and that peri-ictal regions are also remodeled to alter seizure threshold, creating secondary foci and wider epileptic networks [10, 11]. These peri-ictal regions and subsequent epileptic networks may provide additional therapeutic targets in the treatment of clinical epilepsy [12]. Consistent with a potentially wider epileptogenic zone, unilateral intrahippocampal kainate injection causes epilepsy characterized by bilateral hippocampal seizures in mouse models [11, 13], and is associated with increased neurogenesis in both the contralateral hippocampus and distant ipsilateral hippocampus [13, 14]. The contribution of these distant changes to epileptogenesis remains unknown, and the underlying transcriptional and molecular mechanisms that initiate these changes both in the ictal onset zone and the wider peri-ictal epileptogenic zone are poorly defined.

Recent work has begun to describe early transcriptional changes in both rodent models of epilepsy and human clinical samples, in hopes of identifying potential effectors of circuit remodeling during epileptogenesis. One potential effector system involves the Wnt (wingless/integrated) signaling pathway, which encompasses a large family of 19 secreted Wnt protein ligands, which bind a family of 10 membrane frizzled receptors and co-receptors. These regulate downstream canonical and non-canonical pathways, including the planar cell polarity and calcium pathways [15]. These signals have been implicated in neurogenesis and dendrite formation in the adult rodent hippocampus [16, 17], and dysfunction of these processes are associated with epileptogenesis [18–20]. Dysregulation of hippocampal Wnt and mTor (mechanistic target of rapamycin) pathways was observed using microarray analysis in a hypoxic neonatal mouse seizure model; in this study, beta-catenin was dysregulated in an activity-dependent manner independent of the hypoxic insult, suggesting that canonical Wnt signaling may play an early role in epileptogenesis [21].

The association between Wnts and epileptogenesis is only beginning to be explored and may offer novel therapeutic targets that prevent the development of epilepsy after neurologic insults [22, 23]. Here, we investigate hippocampal Wnt signaling changes during epileptogenesis, using the mouse intra-hippocampal kainate model of epilepsy. First, we characterize seizure-induced alterations in hippocampal neurogenesis after kainate using the *POMC-EGFP* transgenic mouse, in which newly born dentate granule cells transiently express eGFP (enhanced green fluorescent protein) [24]. We subsequently evaluate the role of the Wnt pathway in these early changes by administration of XAV939, a canonical Wnt antagonist [25],

and transcriptionally profile the whole dentate gyrus to characterize changes in Wnt gene transcription during early epileptogenesis.

Materials and methods

Animal husbandry

Experiments were performed utilizing the -13/+8POMC-EGFP transgenic mouse line (MGI: 3776091), in which newborn dentate granule cells are labeled with eGFP for up to 2 weeks after birth [24]. Homozygous and heterozygous male mice were studied between 3–4 months of age, as female mice have been shown to have neither a latent period nor hippocampal discharges with intrahippocampal kainate [26]. Mice were housed according to local IACUC guidelines, with food and water *ad libitum*. All procedures and animal handling were performed in accordance with the *Guide for the care and use of laboratory animals* and were approved by the Oregon Health & Science University Institutional Animal Care and Use Committee (IACUC).

Stereotactic intrahippocampal kainate injection

After induction with isoflurane by spontaneous respiration, mice were anesthetized with isoflurane by nose cone, head-shaved and secured in the stereotactic apparatus with ear bars. The scalp was sterilized with betadine and topical lidocaine gel was applied for local anesthesia. A single midline sagittal incision was performed with a #10 scalpel blade and the bregma was visualized under stereoscopic magnification. Drill coordinates were acquired relative to bregma. A single burr hole was placed at X +1.8, Y -2.1 mm and debris was cleared with sterile saline irrigation. The injection needle was slowly inserted to target, Z -1.7 mm from the dura. Sterile normal saline vehicle or kainate (Cayman Chemicals, 0.5mg/ml in normal saline) were delivered by Hamilton syringe connected to a Quintessential Stereotaxic Injector (Stoelting); 100nl were injected over 1 minute. The needle was left in place for 2 minutes to prevent reflux of the injection, and then slowly withdrawn. The skin was closed with dermal glue (Vetbond, 3M) and the mouse allowed to recover in a warmed chamber. Seizures were scored by a modified Racine scale for 2 hours after injection; stages 1 and 2 demonstrated freezing, mastication and head nodding, stage 3 demonstrated forelimb clonus, stage 4 demonstrated rearing, stage 5 demonstrated rearing and falling, stage 6 demonstrated “popcorn” type seizures [27, 28]. Convulsive status epilepticus is well described after intrahippocampal kainate injection [29–31]. Therefore, early, kainate-induced seizures were quantified by Racine’s behavioral criteria without the use of EEG, as described in other studies [14, 23, 32–36]. Only mice that were observed to undergo at least one behavioral seizure measuring 3–6 on the Racine scale were included for study in the kainate group [36], and were grouped together for analysis. After recovery, mice were provided food saturated with diluted pediatric acetaminophen (3.2mg/ml) for 24 hours and assisted feeding with soft food daily until sacrifice.

Drug preparation and intra-peritoneal injection

XAV939 (Millipore Sigma) was dissolved in filter-sterilized 10% DMSO / 45% saline / 45% poly-ethylene glycol-400 (Affymetrix) at a final concentration of 1mg/ml. Animals received intraperitoneal injections daily for 14 days with vehicle or XAV939 (5 mg/kg) after recovery from surgery, as this dose effectively inhibits beta-catenin signaling in translational models [37–39].

Immunohistochemistry

Mouse brains were retrieved after terminal anesthesia and trans-cardiac perfusion with 4% paraformaldehyde, post-fixed overnight, embedded in 2% agarose and sectioned at 100 μ m thickness on a vibratome. Slices were blocked and permeabilized in PBS containing 5% goat serum and 0.4% Triton-X for 1 hour. Primary antibodies included anti-GFP (A-21311 polyclonal rabbit antibody, Alexafluor 488 pre-conjugated, Thermo Fisher Scientific), anti-c-Fos (2250, monoclonal rabbit antibody, Cell Signaling Technology) and anti-ZnT3 (197 004, polyclonal guinea pig antibody, Synaptic Systems Germany), which were applied at 1:500 dilution overnight at 4°C; Alexafluor-conjugated secondary antibodies (donkey isotype, Thermo Fisher Scientific) were applied at 1:1000 dilution overnight at 4°C. DAPI was applied at 1:10,000 dilution for 15 minutes and sections were mounted on slides with Fluoromount-G (Thermo Fisher Scientific).

Immunohistochemical analysis

Immunohistochemically stained sections were imaged with confocal microscopy (Carl Zeiss LSM 780, Jena, Germany). Four to five animals were used in each experimental group. For dendritic arbor and migration analysis, images were acquired using a 20x objective. Two sections per region per mouse were analyzed; image stacks were 22.0 μ m thick with 1.1 μ m steps. eGFP+ cell counts were performed manually in ImageJ using image stacks of 23.4 μ m thickness using a 10x objective, from two sections for each location in each animal. Dorsal and ventral regions were identified anatomically. For contralateral dentate gyrus, all parameters measured were similar between both contralateral dorsal and ventral hippocampus, therefore data and figures are displayed for dorsal contralateral hippocampal sections. An eGFP+ cell was considered within the granule cell layer if the center of its soma was between 10 μ m below the inferior border of the granule cell layer (to include the subgranular zone) to the outer granule cell layer border. Granule cell layer width and volume were measured using ImageJ (NIH). Dendritic arbors of eGFP+ newborn dentate granule cells were measured using FilamentTracer (Imaris BitPlane, Zurich, Switzerland). Mean arbor length per cell was calculated by dividing the total arbor length for a given section by the number of eGFP+ cells within that section. Migration analysis was performed manually in ImageJ (NIH), by measuring the distance of the eGFP+ cell body from the hilus-subgranular zone border.

Gene expression analysis

Bilateral dentate gyri were anatomically microdissected in an RNase-free environment. The ipsilateral dentate was hemisected into dorsal and ventral parts, the contralateral dentate was processed whole. Tissue was then placed in Qiazol (Qiagen), macerated with an RNase-free pestle (Kimble Chase) and stored at -80°C until processing. RNA was isolated using the Universal Plus mini Kit (Qiagen) with QIAcube automation. RNA quality assessment was performed using the Agilent 2100 Bioanalyzer with a Eukaryote total RNA Nano chip. All samples received an RNA integrity score of >9. Reverse transcription (RT) was performed using the SuperScript VILO cDNA synthesis kit (Life Technologies) with 650ng of input RNA per 80 μ l reaction. Following reverse transcription, 2 μ l of cDNA was used in the PCR reaction with 10 μ l TaqMan universal master mix and 1 μ l of 20x gene specific TaqMan assay in a total volume of 20 μ l and loaded onto the QuantStudio instrument. The qPCR assays were performed on the QuantStudio Real-time PCR System (Life Technologies) using a single master-mix per TaqMan probe set, for Wnt3, Wnt5A, Wnt5B, Wnt7A, Wnt7B, Wnt8A, Wnt8B, Wnt9A, WLS (wnt ligand secretor) and DKK-1 using TUBA1A [40] as the endogenous control. Four 384-well plates were used; each plate was setup to contain a complete biologic group

in order to minimize variation. Additionally, a calibrator sample (pooled RNA) was used in each plate assayed, to ensure concordance between plates. Data were collected using Applied Biosystems QuantStudio™ 12K Flex Software v1.2.2.

Statistics

All statistical analyses were performed using Prism 7 (GraphPad, La Jolla, CA, USA). Histologic data were analyzed by 2-way ANOVA (analysis of variance) parametric testing with repeated measures. RT-PCR were analyzed by the $\Delta\Delta\text{CT}$ method [41]. In brief, data were normalized to a reference house-keeping gene (TUBA1A) as follows: $\Delta\text{Ct} = \text{Ct}_{\text{target}} - \text{Ct}_{\text{reference}}$. $\Delta\Delta\text{Ct}$ was calculated by the difference between mean $\Delta\text{Ct}_{\text{seizure}}$ and mean $\Delta\text{Ct}_{\text{control}}$. Fold change in gene expression was calculated by the formula $2^{(-\Delta\Delta\text{Ct})}$. Baseline data within each region were analyzed by 2-tailed t-test. Analysis of individual gene expression between the different regions were calculated by 2-way ANOVA. Tukey's multiple comparisons test was used for multiple comparisons. All tests were used two-sided; $p \leq 0.05$ were considered significant. Data are presented as mean $\pm$ standard error.

Results

Unilateral intra-hippocampal kainate injection causes early bilateral hippocampal activation and delayed regional differences in dentate histology

We used the intrahippocampal kainate injection model of temporal lobe epilepsy to study post-kainate neurogenesis in the hippocampus of adult male *POMC-EGFP* mice. As previously described [29], unilateral injection of kainate into the hippocampal CA1 region elicited modified Racine stage 3–6 seizures in mice [27], including behavioral freezing, prolonged mastication, clonic movements of the forelimbs and hindlimbs, rearing, and “popcorn” type seizures. Kainate-injected animals incur robust status epilepticus throughout the bilateral hippocampus [13, 42], as well as a series of morphological changes in the dentate gyrus of the hippocampus including granule cell dispersion [33, 43], mossy fiber sprouting [44, 45], region specific neuronal loss [30, 46] and neurogenesis [19, 47]. In comparison, unilateral hippocampal injection of saline (control) had no effects on dentate morphology or caused behavioral seizures. After kainate or saline injection, mice were allowed to recover and maintained for up to 2 weeks prior to analysis (Fig 1A).

We first examined expression of c-Fos, a well-established marker of neuronal activation after seizure induction [48–51], 3 hours after kainate injection. When compared with saline-injected controls, unilateral kainate injection caused diffuse and widespread c-Fos expression throughout both the ipsilateral and contralateral dentate gyrus, in both the dorsal and ventral hippocampus (Fig 1B). Thus, although kainate was injected unilaterally, there was diffuse bilateral activation of neurons in the hippocampal dentate gyrus, indicating generalized seizure activity that propagated to both hemispheres.

The hippocampus was examined 2 weeks after intrahippocampal injection to determine the delayed effects of focal kainate injection on the histology of the dentate gyrus (Fig 1C). We defined the ictal zone as dentate gyrus near the site of kainate injection (ipsilateral dorsal hippocampus), which is known to be the predominant site of kainate-induced neuronal activity [29], and compared it with neighboring peri-ictal regions in both the ipsilateral (ipsilateral ventral) and contralateral dentate gyrus. At this 2-week timepoint, we observed marked differences in histology between the dentate gyrus adjacent to the site of injection, the ictal zone, and the neighboring peri-ictal zones.

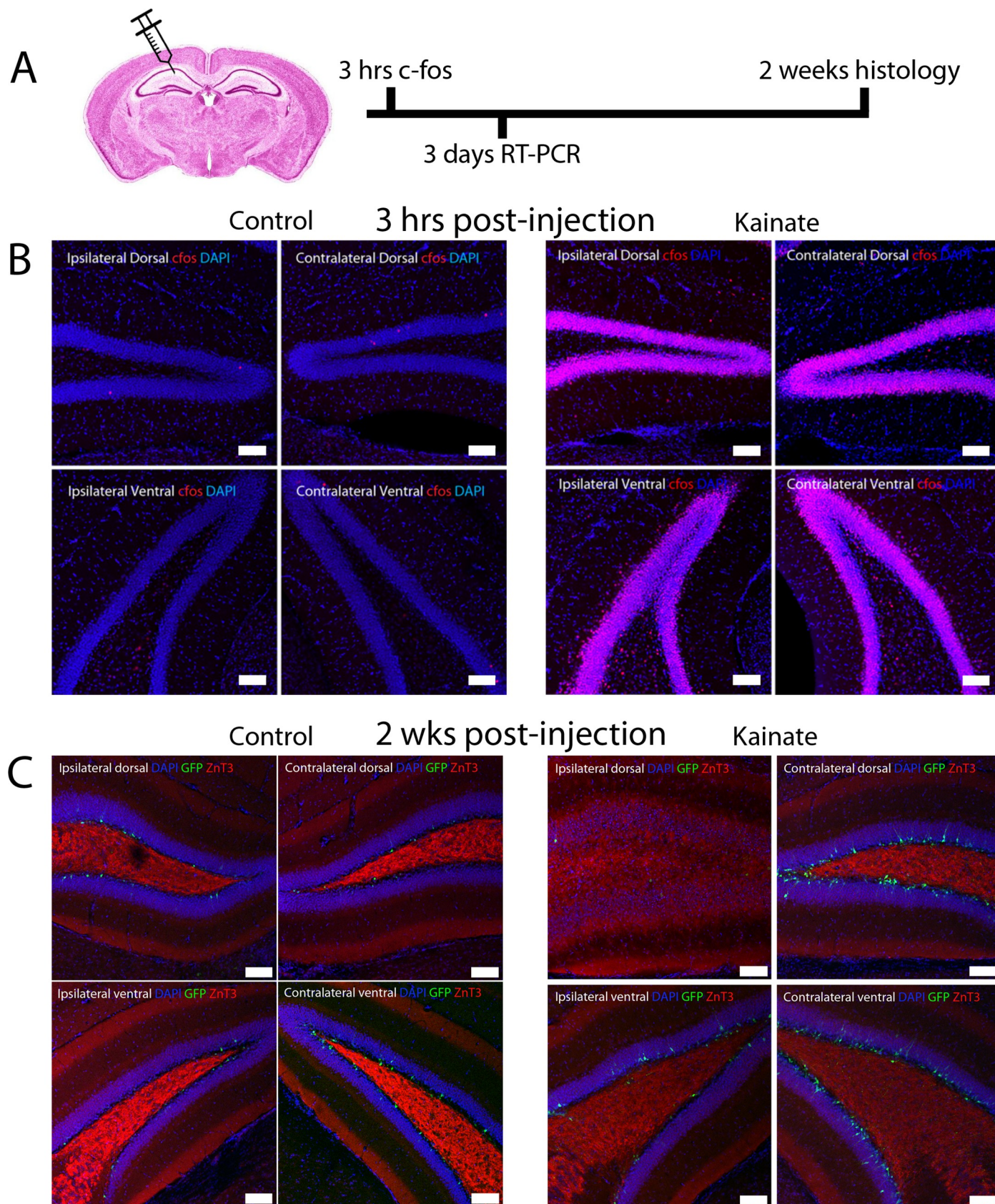


Fig 1. Intrahippocampal kainate injection causes widespread bilateral dentate granule cell activation, followed by delayed focal mossy fiber sprouting. (A) Experimental timeline after intrahippocampal injection. (B) c-Fos expression (red) in the dentate gyri from hippocampal quadrants 3hrs after saline (control; left) or kainate (seizure; right) injection into the ipsilateral/dorsal region CA1. Images show extensive bilateral c-Fos expression 3hrs after kainate in the dentate gyrus. (C) The mossy fiber terminal marker ZnT3 is localized to the dentate hilus as expected 2wks after saline injection (control; left), and localizes to the granule cell and molecular layers adjacent to the site of kainate injection in post-kainate *POMC-EGFP* animals. Scale bar 100µm.

<https://doi.org/10.1371/journal.pone.0215789.g001>

We observed granule cell dispersion, a hallmark of temporal lobe epilepsy [14, 29, 33, 43], only in the ictal zone but not in peri-ictal regions (Fig 1C). Similarly, mossy fiber sprouting into the granule cell and molecular layers of the dentate gyrus, another finding strongly associated with epilepsy [44, 45, 52], was also only observed in the ictal region (Fig 1C). Thus, despite robust bilateral neuronal activation induced by kainate activation, subsequent histologic changes induced by seizure activity differed between the ictal and peri-ictal zones. This suggested differences in the molecular and cellular signaling events between these regions, which could relate to the differential development of histological changes post-seizure induction as well as peri-ictal epileptogenic foci.

The novel Wnt antagonist XAV939 does not quantitatively alter neurogenesis in control animals

Wnt signaling controls neuronal migration during development as well as dendritic outgrowth from immature neurons [15]. Global Wnt pathway dysregulation has been demonstrated after seizures [21, 53], and there is growing interest in the mechanisms by which specific Wnt pathway mediators may contribute to epileptogenesis [22]. As post-kainate neuronal network remodeling includes a host of cellular changes potentially mediated by the Wnt pathway, including neurogenesis [19, 47], dendrite outgrowth [36, 54] and granule cell migration/dispersion [14, 30, 43], we hypothesized that changes in Wnt signaling after seizures might alter neuronal circuit remodeling in the dentate gyrus. We administered XAV939, a novel small molecule canonical Wnt antagonist [25], daily by intra-peritoneal injection for 2 weeks to determine whether this affects neuronal remodeling after seizures. XAV939 inhibits tankyrase 1 and 2, which then leads to actin stabilization and beta-catenin degradation, and blockade of downstream canonical Wnt pathway signaling [25].

First, we examined the effects of canonical Wnt inhibition on the early development of adult-born dentate granule cells in saline injected (control) *POMC-EGFP* animals treated with vehicle or XAV939 for 2 weeks (Fig 2A–2D), to determine whether canonical Wnt signaling plays a role in the early phase of constitutive neurogenesis. *POMC-EGFP* mice express eGFP in immature adult-born hippocampal granule cells, and can be used to both quantitatively and qualitatively assess adult neurogenesis [24]. Based on our analysis of eGFP-expressing immature adult born granule cells, XAV939 treatment in control animals did not significantly change adult-born dentate granule cell dendrite arbor length (Fig 2B), cell count (Fig 2C) or cell migration (Fig 2D). This suggests that these constitutive processes are not dependent upon intact canonical Wnt signaling pathways under baseline conditions.

We subsequently examined whether Wnt inhibition by XAV939 affected gross structural changes in the dentate gyrus after kainate-induced seizures. As previously observed [29, 30, 43], seizure induction by kainate markedly increased dentate granule cell layer dispersion in the ictal zone (Fig 3A). Ictal zone granule cell layer dispersion was unaffected by XAV939 treatment (Fig 3B), indicating that it occurs independent of canonical Wnt signaling. Granule cell dispersion was not observed in peri-ictal regions after seizures; and in these regions, granule cell layer thickness was also not affected by XAV939 treatment (Fig 3B). Importantly, XAV939 did not drive granule cell layer dispersion in any region in control/saline treated

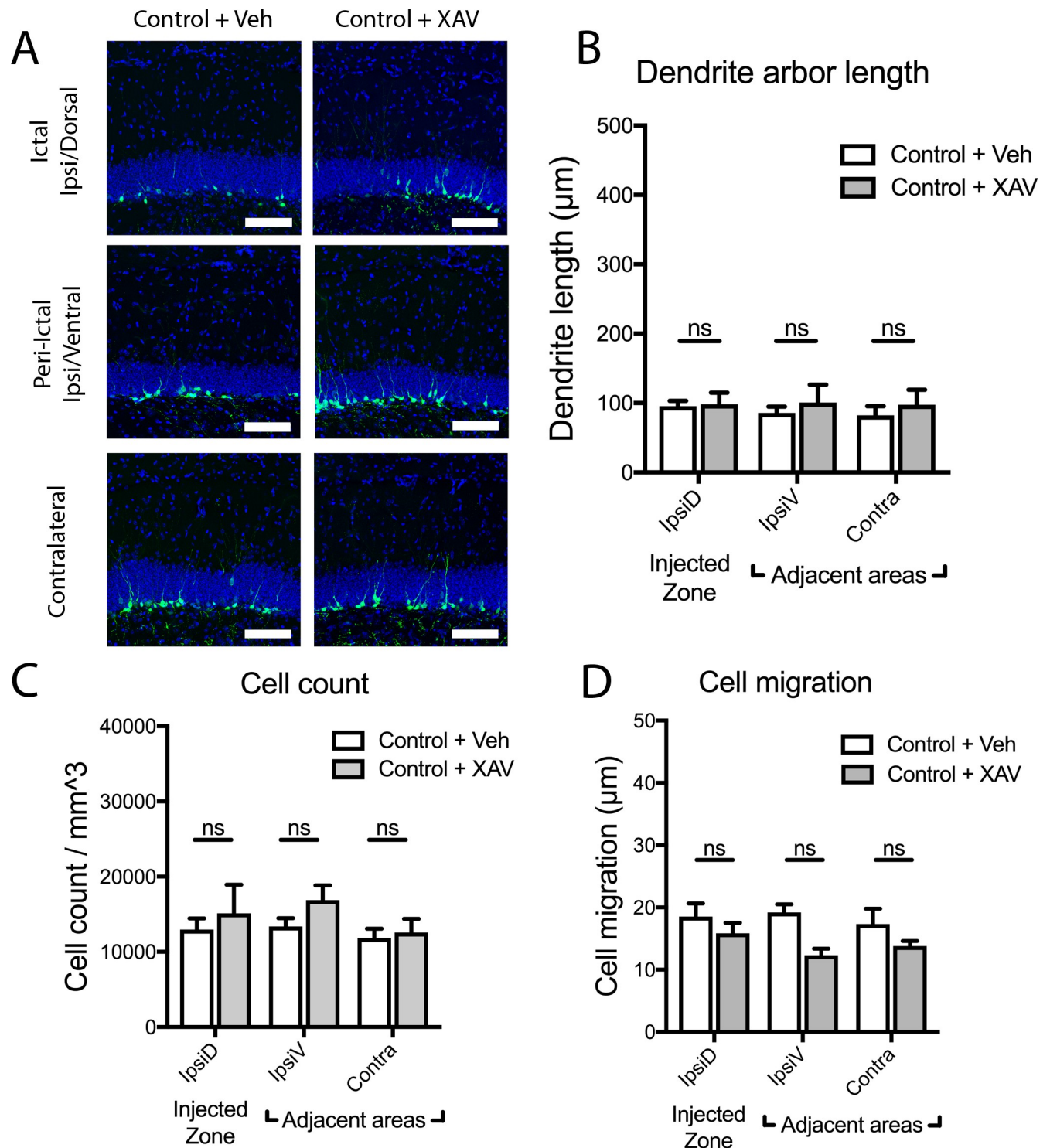


Fig 2. Treatment with novel Wnt antagonist XAV939 does not alter newborn dentate granule cell maturation in control animals. (A) Images demonstrate eGFP+ newborn dentate granule cells two weeks after intrahippocampal saline injection (control) in the ipsilateral dorsal, ipsilateral ventral, and contralateral dentate gyrus, of mice treated with vehicle vs. XAV939 daily for two weeks after injection. Scale bars 100 μm . (B-D) In saline-injected control animals, continuous Wnt inhibition by XAV939 treatment does not alter newborn dentate granule cell arbor length (B), cell density (C), or cell migration (D), when compared with vehicle-treated control mice (ns = not significant).

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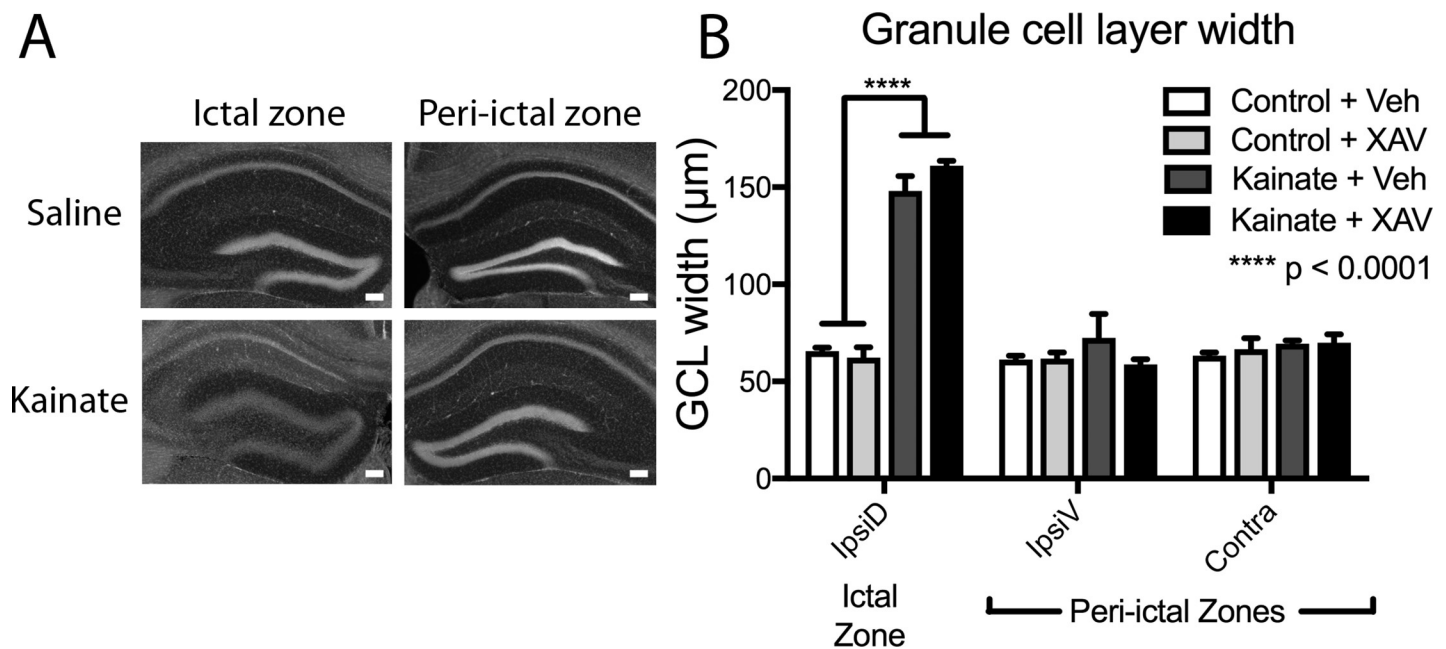


Fig 3. Granule cell dispersion in the ictal zone is not affected by XAV939 treatment. (A). DAPI-stained images of coronal whole dentate gyrus after saline (control) and kainate injection in the ictal (injected) and peri-ictal (non-injected) regions of the dentate gyrus. Scale bar 100µm. (B). Dentate granule cell layer dispersion is only seen in the ictal zone after seizure induction by kainate, and this was not affected by daily administration of Wnt antagonist XAV939 for two weeks after kainate.

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animals (Fig 3B), suggesting that constitutive canonical Wnt signaling is not needed to maintain granule cell layer organization in healthy mice.

XAV939 increases dendrite growth by newborn neurons in peri-ictal regions after seizure

Seizures drive several forms of hippocampal neuronal remodeling in a variety of rodent epilepsy models, including neurogenesis [47, 55], mossy fiber sprouting [44, 45, 52], CA3 and CA1 region neuronal loss [14, 29, 46], granule cell dispersion [14, 30, 43], and apical/basal dendrite formation [36, 54]. These changes may relate to the subsequent development of epilepsy. We therefore investigated whether seizure induction was associated with region-specific changes in the morphologic maturation of dentate granule cells born after seizure. We used *POMC-EGFP* mice to allow for both quantitative and morphologic assessment of newborn dentate granule cells, as they express eGFP for up to 2-weeks post-mitosis in both control and epileptic mice [24, 36]. Although neither seizures [36] nor XAV939 (Fig 2) appear to alter the selectivity of eGFP expression in *POMC-EGFP* mice, we further confirmed that EGFP+ neurons still predominantly represent the population of immature neurons in each of these conditions by co-staining tissue for DCX, an immature neuronal marker. In all conditions, EGFP expression was still almost entirely restricted to immature hippocampal granule cells (*POMC-EGFP*+/*DCX*+ co-positive neurons as a percentage of total *POMC-EGFP*+ neurons: saline/vehicle 89.9±1.9%, kainate /vehicle 91.2±0.9, saline/XAV939 96.2±2.7%, kainate/XAV939 87.4±5.2%; vehicle vs XAV939, saline p = 0.22, kainate p = 0.63), indicating that XAV939 treatment does not fundamentally alter the maturation of *POMC-EGFP*+ neurons in either control or post-kainate mice.

Prior work using the systemic pilocarpine model of epilepsy in mice demonstrated that seizures dramatically increased dendrite arbor length of newborn granule cells in the molecular

layer of the dentate gyrus [36]. However, in the intrahippocampal kainate model, we observed increased dendritic arbor length of newborn dentate granule cells only in the peri-ictal regions, and not in the ictal region (Fig 4A and 4B). Interestingly, the seizure-induced dendrite growth was very similar in both the ventral ipsilateral hippocampus and the contralateral hippocampus. As this peri-ictal dendrite growth closely resembled the phenotype observed in the pilocarpine model, we believe that close proximity to the ictal (kainate injection) zone produced a region-specific effect on the dentate in this model that was qualitatively different than that which occurred in the remainder of the hippocampus.

Wnt antagonism had an unanticipated effect on the growth of dentate granule cell dendrites after seizures, as XAV939 treatment significantly increased growth of dendrites by newly born cells in the peri-ictal regions after seizures (Fig 4A and 4B). This was not a general effect of XAV939 on dendritic arborization, as it had no effect on dendritic branching in control/saline treated animals (Fig 2A and 2B). This suggests that the effects of canonical Wnt signaling on neuronal remodeling primarily manifested after seizures, and that Wnt signaling in the peri-ictal dentate gyrus actually restricts aberrant growth of dendrites from granule cells after seizures.

XAV939 modulates post-kainate dentate neurogenesis and neuronal migration

Consistent with previous reports [13, 56, 57], intrahippocampal kainate injection significantly decreased the density of newborn dentate granule cells in the ipsilateral dorsal dentate gyrus / ictal zone, adjacent to the site of kainate injection (Fig 5A and 5B). This decreased neurogenesis in the ictal zone was not altered by XAV939 treatment (Fig 5A, B). However, kainate-induced seizures did not increase neurogenesis in the contralateral or ipsilateral ventral regions (Fig 5A and 5B), unlike previously reported [13]. XAV939 treatment increased the cell count in the ipsilateral peri-ictal region after kainate-induced seizure, which was not observed in the contralateral dentate gyrus (Fig 5B).

Kainate-induced seizures significantly increased the migration of surviving immature neurons from the subgranular zone in the ictal, but not peri-ictal, regions (Fig 5A and 5C). Again, XAV939 treatment after kainate had region-specific effects, such that XAV939 increased neuronal migration in the ictal region, whereas neuronal migration was unaffected by XAV939 in the peri-ictal regions (Fig 5A and 5C). This suggests that canonical Wnt signaling after kainate-induced seizures again appeared to primarily normalize aberrant phenotypes after seizures, as inhibition of this pathway led to a more dramatic phenotype.

Transcriptional profiling of ictal and peri-ictal dentate gyri

To characterize the changes in Wnt signals that might be involved in post-kainate dentate gyrus remodeling, we performed a gene expression analysis of candidate Wnt molecules from the dentate gyri of intrahippocampal saline and kainate injected mice, based on prior reports of their involvement in neurogenesis, neuronal morphogenesis or dentate specific expression in the Allen Brain Atlas (Allen Institute for Brain Science) [53, 58–60]. Dentate gyri were anatomically micro-dissected from mice that experienced intrahippocampal kainate-induced seizures, and from control mice that had received intrahippocampal saline injection (Fig 6A and 6B). To help determine how Wnt signals might differentially be associated with the profoundly different structural phenotypes between ictal and peri-ictal regions, we subdivided dentate gyri anatomically, corresponding to the ipsilateral dorsal dentate gyrus at the injection site (ictal zone), and 2 peri-ictal regions, the ipsilateral ventral dentate and contralateral dentate. Profiling was performed via quantitative RT-PCR of tissue 3-days after kainate vs. saline

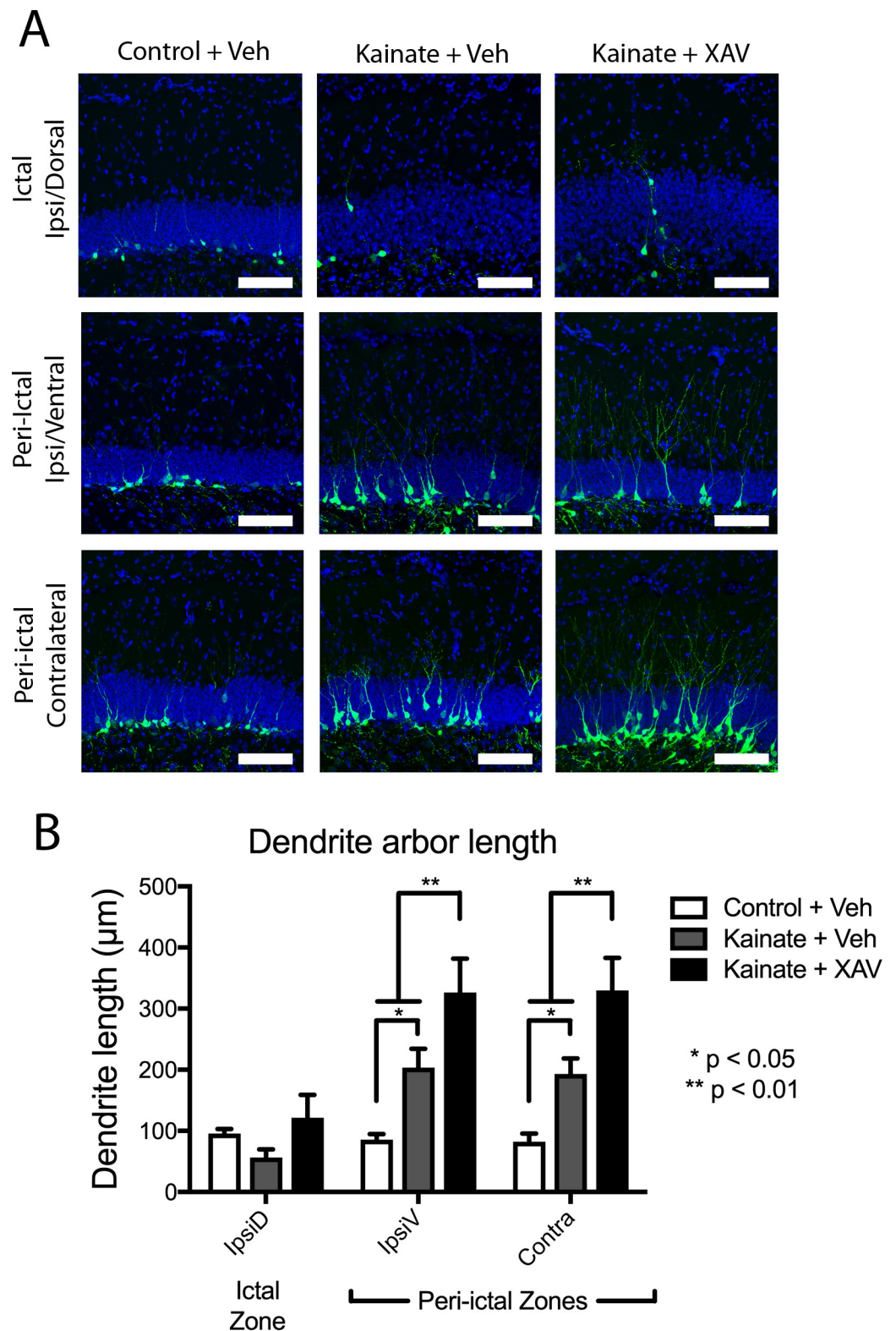


Fig 4. Post-kainate increase in newborn granule cell dendrite length is augmented by Wnt inhibition. (A) Images of *POMC-EGFP+* adult-born dentate granule cell neurons from indicated hippocampal regions two weeks after intrahippocampal kainate or saline injection, followed by daily systemic treatment with XAV939 vs. vehicle control. Scale bars 100µm. (B) In peri-ictal regions, mean dendritic arbor length per eGFP+ cell increased significantly after kainate-induced seizure in peri-ictal zones, and was further increased by XAV939 treatment.

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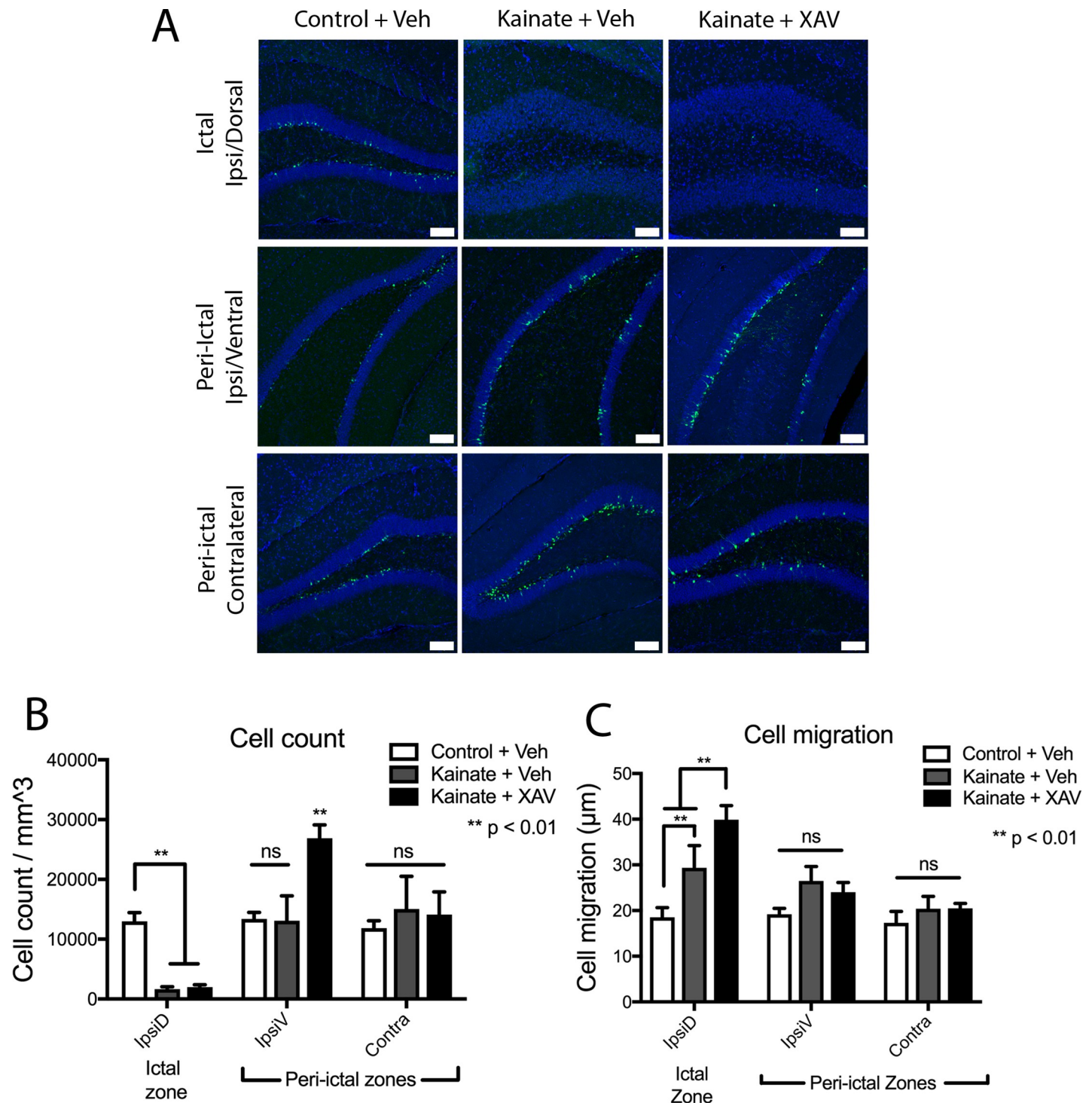


Fig 5. Modulation of neurogenesis and cell migration by the Wnt antagonist. (A) *POMC-EGFP*⁺ newborn dentate granule cells 2wks after kainate-induced seizure, followed by vehicle or XAV939 treatment, in ictal and peri-ictal regions. Scale bars 100µm. (B) Newly born (*POMC-EGFP*⁺) granule cell density decreased in the ictal zone, and was not rescued by XAV939 treatment. In the ipsilateral peri-ictal region, cell density increased after XAV939 treatment only in kainate-treated mice. No XAV939-induced change in cell density was observed in the contralateral dentate. (C) Newborn dentate granule cell migration increased in the ictal zone 2wks after seizure induction, which was further increased by XAV939 treatment. Cell migration was unchanged after kainate in the peri-ictal zones.

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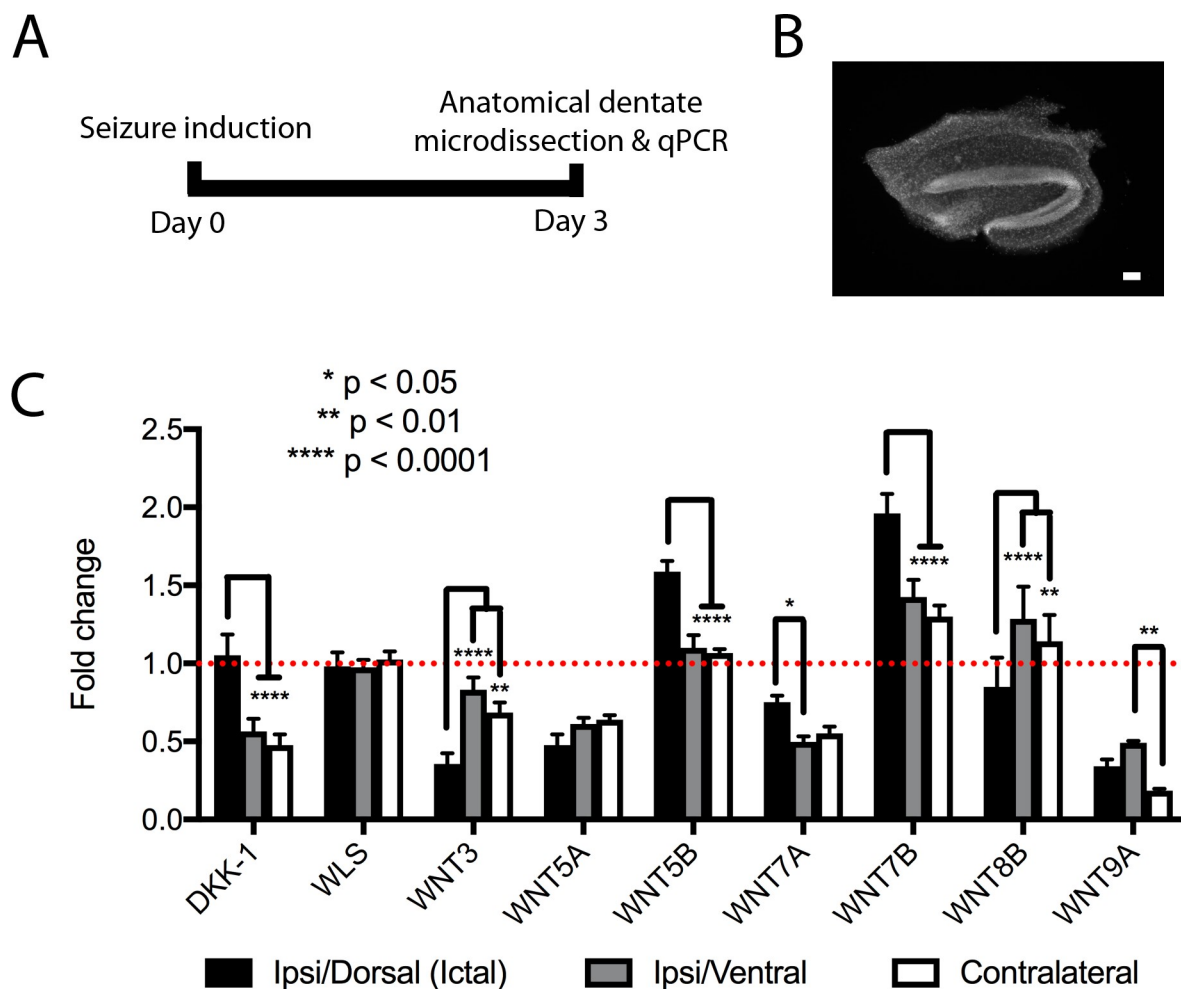


Fig 6. Transcriptional profiling of the whole dentate gyrus 3 days after seizure induction. (A) Timeline of transcriptional analysis. (B) Image of a representative DAPI-labeled cross-section of micro-dissected dentate gyrus used for transcriptional analysis. Scale bar 100 μ m. (C) Relative Wnt pathway gene transcription in the ictal and peri-ictal regions 3d after seizure induction. Various patterns are observed, whereby certain genes are selectively upregulated in the ictal zone (Wnt5b and Wnt7b), selectively downregulated in ictal (Wnt3) or peri-ictal regions (Dkk-1), or relatively unchanged from baseline (WLS, Wnt8b).

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injection, to determine whether kainate-induced seizure altered expression of any of these genes at a timepoint that would be expected to affect the development of dentate granule cells born after seizure induction [36, 47, 61]. Transcriptional data for each individual gene and region are reported in [S1 Table](#); comparative data for each gene are demonstrated in [Fig 6C](#).

In the ictal zone, the largest changes in transcriptional regulation were seen in Wnt3 and Wnt7b, whereas in the peri-ictal zones, the largest changes in transcriptional regulation were seen in DKK-1 and Wnt7a ([Fig 6C](#)). Interestingly, changes in individual Wnt gene expression in both peri-ictal regions showed striking similarities in their patterns of dysregulation, such that these genes were either upregulated or downregulated in both peri-ictal regions, and often in direct contrast to the transcriptional changes (or lack thereof) in the ictal region (e.g., DKK-1, Wnt5b, [Fig 6C](#)). This pattern matches the distinct phenotypes noted between ictal and peri-ictal regions ([Figs 3–5](#)), as well as the striking similarity between the ipsilateral/ventral and contralateral dentate gyri. Certain genes were similar in all regions after seizure, being either unchanged from control (WLS and Wnt8b), or downregulated (Wnt5a and Wnt9a, [S1 Table](#)),

but the majority of Wnt genes analyzed showed similar differential expression patterns between ictal and peri-ictal (ipsilateral/ventral and contralateral) regions (Fig 6C).

Discussion

It is increasingly recognized that not only are remote regions of the dentate gyrus and hippocampus affected by focal seizures, but that seizure-related alterations in the neuronal circuit connectivity of these regions might also contribute to brain hyperexcitability [11, 62]. For instance, in epileptic humans, epileptic foci are associated with structural changes and reduced seizure thresholds in neighboring brain, and these neighboring regions potentially act as independent epileptogenic regions [63–65]. Using the *POMC-EGFP* mouse to visualize granule cells born after an acute seizure event, we demonstrate that structural abnormalities occur in peri-ictal hippocampus after seizures, which include changes in newborn neuron migration and dendrite arbor formation, and that these post-kainate structural changes are affected by Wnt modulation.

Modulation of post-seizure hippocampal remodeling by Wnt signaling

Aberrant circuit rewiring in the hippocampal dentate gyrus is a hallmark of temporal lobe epilepsy in humans, and may contribute to the formation of epileptic foci [10, 66]. This rewiring includes altered granule cell migration, axonal arborization, and dendritic morphology, much of which involves qualitative and quantitative changes in post-seizure neurogenesis [18, 36, 47, 55, 67]. Two weeks after focal kainate-induced seizures, we observed marked heterogeneity in cellular responses between different regions of the hippocampus. These included marked granule cell dispersion and decreased neurogenesis in the dentate gyrus adjacent to the site of kainate injection (ictal zone), and upregulation of dendritic growth in the peri-ictal regions distant from the injection site. These findings are consistent with prior observations, which demonstrated similar differential effects on neurogenesis between ictal and peri-ictal zones using BrdU-based mitotic cell labeling and cell filling [13]. However, despite the marked reduction of neurogenesis in the ictal region, we still observed a remaining small number of *POMC-EGFP*⁺ in the dentate gyrus, consistent with other reports demonstrating a marked, but not complete, region-specific loss of neurogenesis early after kainate [14, 33].

In addition to increased overall granule cell dispersion in the ictal zone [29, 68], migration of the few remaining newly born dentate *eGFP*⁺ granule cells also increased in the ictal zone 2 weeks after kainate-induced seizures (Fig 5). As Wnts control the migration of neuroblasts during development [17, 69, 70], we had hypothesized altered Wnt signaling may contribute to this accelerated migration. Surprisingly, however, we observed that migration of newly born dentate granule cells in the ictal zone increased with pharmacologic antagonism of canonical Wnt signaling using the novel Wnt antagonist XAV939 [25]. Thus, our data are more consistent with a model in which canonical Wnt signaling actually inhibits the aberrant migration of newly generated granule cells, and that some other signal (or loss thereof, for instance, reelin [71]) causes aberrant newborn granule cell migration during epileptogenesis.

Interestingly, canonical Wnt antagonism by XAV939 also increased the length of newborn dentate granule cell dendrites after seizure, specifically in the peri-ictal regions. Again, in this region, Wnt signaling after seizures appeared to counteract some signal which was driving increased dendritic outgrowth. We base this interpretation on the observation that XAV939 did not accelerate dendritic outgrowth in control conditions (intra-hippocampal control saline injections), indicating that canonical Wnt signaling is not required for dendritic outgrowth during constitutive neurogenesis. Given prior evidence that specific Wnt signals control dendritic morphogenesis during neuronal development [59, 72, 73], we were surprised that

canonical Wnt antagonism did not noticeably affect constitutively-generated adult-born granule cells under control conditions. This suggests that Wnts may play differential roles for granule cells generated during early neonatal development from those generated in adults.

Under control conditions, Wnt antagonism with XAV939 did not affect neurogenesis. Constitutive beta-catenin activation has previously been shown to mediate expansion of Tbr2 + intermediate neurons [69], and lentivirus-mediated Wnt3 transduction has been shown to mediate expansion of DCX+ neurons in the dentate gyrus in 15wk old rats [17]. Our findings that small chemical Wnt antagonism did not affect baseline neurogenesis appear inconsistent with these over-expression studies, however other compensatory mechanisms may be present.

In contrast to prior BrdU-labeling studies after intrahippocampal kainate, we did not observe an increased number of eGFP+ cells in the peri-ictal regions [55]. It is not clear what caused this discrepancy, but perhaps since eGFP+ cells represent the aggregate population of newly born granule cells generated during the preceding 2 weeks [24], the lack of change may reflect the overall integrated sum of post-kainate neurogenesis over time, encompassing both the initial increase and subsequent decrease in neurogenesis rates that both occur within this two week window [14, 55]. Jessberger et al [47] utilized systemic kainate in rats and determined that when assayed 8 days after seizure induction, BrdU labeling was increased in the hippocampus. This was followed by increased BrdU/NeuN co-labeling 4w and 3mo after seizure induction, providing evidence that at least part of this proliferative response was neuronal. Somewhat surprisingly, however, GFAP+/nestin+ cell count was not increased, implying no change in baseline precursor proliferation. Choe et al [69] reported that Wnt signaling increased proliferation of intermediate precursors via CXCR4; the hypothesis that seizure-induced Wnt signaling specifically increases proliferation of transient amplifying cells, rather than neural stem cells per se, may therefore be consistent with our findings in the POM-C-EGFP mouse. Therefore, although we did not observe effects of canonical Wnt inhibition on constitutive neurogenesis, our analysis was limited to morphological features of adult-born granule cells during their early maturation, and does not preclude Wnt modulation of later stages of neuronal maturation.

In another study, Qu et al [23] suggest that seizure induction by kainate injection in rat hippocampus increases neurogenesis by Wnt3a mediated beta-catenin/cyclin D1 signaling. In their study, the ipsilateral hippocampus was studied in isolation, however, the increased BrdU labeling they observed may reflect glial proliferation (also observed previously [14, 33]), as BrdU+ cells were not phenotyped with cell type-specific markers, and the granule cell layer was not apparent in the images shown. Additionally, their finding that beta-catenin inhibition reduced the number of BrdU-labeled cells was not selective to Wnt3a, as their inhibition of beta-catenin would inhibit multiple other Wnt mediators. However, despite the different time-points and focus, this study provides further evidence that Wnt signaling via beta-catenin is implicated in hippocampal remodeling after seizure.

Granule cell dispersion after intrahippocampal kainate primarily affects differentiated dentate granule cell layer neurons, and occurs independently of neurogenesis [14, 33, 43, 74]. This is consistent with our observations, as marked dentate granule cell dispersion occurred in the injected region, which saw a dramatic decrease in neurogenesis and newborn dentate granule cells. However, although granule cell dispersion after kainate was unaffected by XAV939 treatment, it remains possible that Wnt signaling modulates mature neurons as well, which could perhaps be elucidated with more detailed structural and functional analysis of mature granule cells in future studies.

Similarly, our data also do not rule out Wnt-mediated control of neurogenesis via alternate downstream Wnt signaling pathways, such as the calcium or planar cell polarity pathways, or Wnt-mediated effects on circuit function that do not involve neurogenesis. Finally, although

XAV939 has been shown to be selective for the Wnt pathway and to not affect the CRE (cAMP response element), NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells), or TGF β (transforming growth factor beta) pathways in reporter lines [25], and was effective in both *in vivo* and *in vitro* assays involving mice and neural cell types at the same dose used in this study [37–39], unexpected off-target drug effects must still be considered.

Transcriptional changes in Wnt mediators after seizure

We examined transcriptional changes in the dentate gyrus in the early post-kainate period and found dysregulated expression of key Wnt genes known to modulate neuronal growth and architecture [17, 58, 59, 75–77]. Perhaps the most striking finding in the analysis of these transcriptional changes involves how the parallel histologic changes in peri-ictal and contralateral remodeling were accompanied by similar transcriptional patterns within Wnt pathway genes, both of which were very distinct from transcription in the ictal zone.

In terms of specific genes, Wnt3 was downregulated in the ictal zone and unchanged in peri-ictal regions. As Wnt3 positively regulates hippocampal neurogenesis, as well as spinal cord neurogenesis and neurite outgrowth [17, 77], its downregulation in the ictal zone is consistent with our observation of reduced neurogenesis in this region, and suggests that post-kainate downregulation of Wnt3 might contribute to the reduction of neurogenesis in the zone. Wnt7b is expressed primarily in the dentate gyrus of the hippocampus during the course of early post-natal development, and appears to drive dendritic growth and branching of hippocampal neurons via Dvl (disheveled segment polarity protein homolog) and Rac1 (Ras-related C3 botulinum toxin substrate 1) [59]. We observed an overall upregulation of Wnt7b in the ictal and peri-ictal zones, which is consistent with a possible contribution of Wnt7b to enhanced post-kainate dendrite growth. In this case, however, as the canonical Wnt antagonist XAV939 did not prevent, but actually enhanced, dendritic outgrowth, any potential roles of Wnt7b in driving dendritic outgrowth after seizures would likely be mediated by a non-canonical downstream Wnt pathway.

Our transcriptional characterization of Wnt signaling changes during early epileptogenesis, however, does not clearly implicate a specific gene, and it does not exclude multiple Wnt pathways acting in concert. Additionally, we performed our profiling at 3 days after kainate to analyze changes in Wnt genes during early epileptogenesis, but Wnt pathways will need to be examined further in a detailed fashion using specific Wnt gene modulation in specific hippocampal cell types at serial time-points to obtain a more complete picture of dynamic changes in Wnt signals that might occur over longer timeframes. We hope to address this in future studies in which we can perform longitudinal transcriptional profiling (as previously reported [21, 53]) in conjunction with morphologic analyses at expanded numbers of timepoints. Furthermore, although the pattern of associations between structural phenotypes and Wnt signaling suggest that these changes may be related, we are unable to make causative association between individual Wnt genes and any specific structural circuit changes. Future studies involving manipulations of specific Wnt signals and mediators will hopefully allow us to ascribe specific functional roles of individual candidate molecules to specific post-kainate changes in dentate structure, and eventually, function.

Conclusions

The intrahippocampal kainate model is a well-established model of temporal lobe epilepsy, in which kainate-injected mice manifest spontaneous seizures after several weeks [9]. Wnt antagonism with XAV939 altered the course of post-kainate dentate remodeling, however it remains to be seen how this drug, or Wnt signal modulation in general, would affect the eventual

development and severity of subsequent spontaneous seizures. Future studies with XAV939, and eventually with more targeted approaches specific to individual Wnts, together with EEG-based quantification of seizures after the development of epilepsy, will allow us to extend our data to determine how Wnts might be involved in epileptogenesis. Better understanding of Wnt pathway dysregulation in epilepsy may identify therapeutic targets that in high-risk human patients prevent the development of focal epilepsy in response to inciting conditions such as trauma, tumor, infection and others.

Supporting information

S1 Table. Wnt pathway transcriptional data. Transcriptional data of Wnt genes 3 days after seizure induction, demonstrating fold-change in gene expression in seizure group compared to control.
(DOCX)

Acknowledgments

This material was supported in part by the Department of Veterans Affairs Merit Review Award I01-BX002949 (ES), a Department of Defense CDMRP Award W81XWH-18-1-0598 (ES), a Neurosurgery Research and Education Foundation Fellowship (KG) and P30NS061800 (OHSU) awards. We thank Dr. Gary Westbrook of the Vollum Institute for comments on our manuscript, Dr. Stefanie Kaech-Petrie of the OHSU Advanced Light Microscopy Core for assistance with imaging, and research assistants Sarah Mader and Alexandria Wilson for technical support. The contents of this manuscript do not represent the views of the U.S. Department of Veterans Affairs or the United States Government.

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