Published in final edited form as:
Cell Mol Neurobiol. 2022 July ; 42(5): 1453–1463. doi:10.1007/s10571-020-01036-5.

Sidestream Smoke Affects Dendritic Complexity and Astrocytes After Model Mild Closed Head Traumatic Brain Injury

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Abstract

Mild traumatic brain injuries can have long-term consequences that interfere with the life of the patient and impose a burden on our health care system. Oxidative stress has been identified as a contributing factor for the progression of neurodegeneration following TBI. A major source of oxidative stress for many veterans is cigarette smoking and second-hand smoke, which has been shown to have an effect on TBI recovery. To examine the potential influences of second-hand smoke during recovery from TBI, we utilized a mouse model of closed head injury, followed by repeated exposure to cigarette smoke and treatment with a neuroprotective antioxidant. We found that neither the mild injuries nor the smoke exposure produced axonal damage detectable with amino cupric silver staining. However, complexity in the dendritic arbors was significantly

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Author contributions WAR performed animal experiments, analyzed and interpreted the data, created figures, and wrote the manuscript. JNS conceived and performed the experiments. KLK and DCD performed animal experiments and data analysis. KEM performed data analysis and interpretation. MO assisted with experiments related to the use of the smoking machine. RFM performed the Golgi staining and dendritic analysis. VD analyzed and interpreted data and contributed significantly to the writing to the manuscript. BAC conceived the studies, analyzed data, and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Conflict of interest The authors declare no competing interests. The contents do not represent the views of the Department of Veterans Affairs or the US Government and the opinions, interpretations, conclusions and recommendations are those of the authors and are not necessarily endorsed by the Department of Defense.

Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical Approval The animal protocol was approved by the Bay Pines VA Institutional Animal Care and Use Committee (IACUC) and performed in accordance with all institutional, agency, and governmental Animal Welfare Regulations.

reduced after mild TBI plus smoke exposure. In the hippocampus, there were astrocytic responses, including Cyp2e1 upregulation, after the injury and tobacco smoke insult. This study provides useful context for the importance of lifestyle changes, such as reducing or eliminating cigarette smoking, during recovery from TBI.

Keywords

Traumatic brain injury; Cigarette smoke; Oxidative stress; Mouse models; Astrocytes; Dendritic arbor

Introduction

Mild traumatic brain injuries (mTBIs) have been experienced by at least 15% of recently deployed military personnel, and the worldwide prevalence is approximately 0.5% per year (Hoge et al. 2008). Though the majority of TBIs are mild, even mild injuries can have long-term consequences that interfere with the life of the patient and impose a burden on our health care system. Neurodegeneration associated with mild TBI involves a variety of factors within the brain. We have previously identified inflammatory regulatory factors, including transcription factor Nrf2, as factors in the consequences of mild TBI (Hatic et al. 2012). Additionally, TBI results in increased reactive oxygen species, reactive gliosis, and activation of proinflammatory cytokines (Burda et al. 2016; Siedler et al. 2014). Oxidative stress has been identified as having a role in the progression of neurodegeneration in a mouse model of TBI (Tweedie et al. 2013).

Veterans are also disproportionately likely to be cigarette smokers when compared to the general population within the USA. Studies show that veterans of recent conflicts in Iraq and Afghanistan are twice as likely as other Americans to be cigarette smokers (Bastian and Sherman 2010; Brown 2010). This has led to significant interest from the US Department of Veterans Affairs in smoking cessation (Sherman 2008). Oxidative stress is known to be produced by cigarette smoking or exposure to second-hand cigarette smoke. Studies have shown neurogenerative effects of smoking in mouse models (Ho et al. 2012; Speck et al. 2011) and human studies (Cho et al. 2016; Yu et al. 2016). Cigarette smoking has also been shown to be correlated with reduced neurocognitive recovery following mTBI (Durazzo et al. 2013). There is, however, little known about how oxidative stress from cigarette smoking impacts TBI recovery on a cellular and molecular level and how we may be able to ameliorate these negative effects.

Our study utilized a non-invasive weight drop mouse model of closed head injury which accurately recapitulates the rotational forces, neuropathology, and cognitive deficits that are observed in TBI patients (Milman et al. 2005; Zohar et al. 2003). This model produces a mild injury that produces axonal, dendritic, and cell body changes as well as apoptotic and necrotic damage to neurons without non-specific brain edema (Saykally et al. 2012,2018; Tashlykov et al. 2007; Tweedie et al. 2007). Following this injury, we exposed mice to sidestream cigarette smoke using a custom-built apparatus (Huang et al. 2013,2009). With this model, we have been able to identify changes among the neurons and astrocytes of the hippocampus, associated with TBI and smoke exposure. Additionally, we have tested

the impact of treatment with an antioxidant, tert-butylhydroquinone (tBHQ). tBHQ is a common food additive which activates the Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) transcription factor and has been shown in previous studies to improve cognitive performance, as well as dendritic length and branching, in mouse models of TBI (Ratliff et al. 2020a,c; Saykally et al. 2012).

Inflammatory responses are certainly known with TBI and with smoke insults (Adhami et al. 2017) and subsets of astrocytes will exhibit the upregulated cytochrome P450 family member, Cyp2e1, involved in a variety of processes including oxidative stress. Cyp2e1 has been implicated as influencing neuroplasticity. Expression of cytochrome P450 (CYP) has been shown to correlate with rodent cognitive skills in hippocampal-dependent learning tasks (Gjota-Ergin et al. 2018). In soldiers exposed to blast during training, methylation of the CYP2E1 gene region and gene expression in serum were responsive to the blast exposure (Wang et al. 2020). Therefore, we also examined Cyp2e1 after the TBI and smoke exposure insults.

The goal of this study was to provide an improved understanding of the changes in the brain which occur as a result of TBI and how an additional insult (i.e. exposure to cigarette smoke) during the recovery period could impact the initial molecular response to injury and the eventual recovery. This study provides a context for the importance of lifestyle factors on traumatic brain injury recovery and whether increasing antioxidant signaling affects plasticity following injury. While traumatic brain injuries will not be fully preventable, understanding the molecular processes which occur following TBI and other insults can help us to develop treatments and identify lifestyle changes which may improve the overall prognosis.

Experimental Procedures

Animals

The animal protocol was approved by the Bay Pines VA Institutional Animal Care and Use Committee (IACUC) and performed in accordance with all institutional, agency, and governmental Animal Welfare Regulations, as previously described (Ratliff et al. 2019). Male CD-1 mice at 3 months of age and weighing between 31 and 34 g were obtained from Harlan Laboratories (Indianapolis, IN). They were housed 3–4 per cage in a 22 °C ± 0.5 °C temperature-controlled environment with a 12 h light/dark cycle. Food and water were available ad libitum and health checks were performed daily with weights taken weekly at a minimum. Power analysis (to 90%) was used to determine sample sizes for experimental and control groups. Animals were randomly assigned to numbered cages by VMU staff without input from the investigators. Each numbered cage was assigned to a group prior to investigators handling the mice.

Closed Head Injury

Mild Traumatic Brain Injury (mTBI) was induced using a concussive closed head injury weight drop model as described previously (Zohar et al. 2003). Briefly, mice were anesthetized with isoflurane and placed on a sponge, which allows for rotation of the head to

inflict a diffuse concussive injury (Milman et al. 2005). The injury apparatus was positioned directly over the head such that the inner diameter of the tube (13 mm) spanned the right hemisphere caudal to the eye and rostral to the ear. A 50 g cylindrical weight was dropped 80 cm through the length of the tube in injured mice. Sham mice did not receive weight drop portion of procedure. Mice were allowed to recover from anesthesia in a heated chamber.

Sidestream Smoke Exposure

Mice were exposed to sidestream smoke three times per day, 4 h apart, for 30 min beginning 4 h post-injury. Mice were exposed to smoke daily for 3 to 14 days on the day of and following injury (Fig. 1a). To expose mice to sidestream smoke, mice were placed into the mouse compartments within the exposure chamber of the smoking apparatus (Fig. 1b). The smoking apparatus was a custom-built unit (TE-2; Teague Enterprises, Davis, CA) which has been detailed previously (Huang et al. 2009). Next, two cigarettes were placed in the holders within the cigarette chamber. Cigarettes (3R4F) were obtained from the Reference Cigarette Program, Tobacco Health Research Institute, University of Kentucky (Lexington, KY). The machine was then turned on and the first cigarette was lit. The second cigarette was lit after 5 min. The machine alternates “puffs” from each cigarette every 60 s. Each cigarette lasted approximately 10–12 min and cigarettes were replaced as they were finished. Four cigarettes were used per session, for a total of approximately 30 min of smoke exposure. Total particulate matter produced in the smoke by each cigarette was 11 mg, and this was consistent with particulates collected on filters in tests that we performed with fixed volumes from the chamber. Sham mice were placed adjacent to the machine to receive exposure to machine noise without receiving smoke exposure.

Treatment

Mice were treated via intraperitoneal injection of tert-butylhydroquinone (tBHQ). To create the solution, 100 mg tBHQ powder was dissolved in 1 mL DMSO to create a 100 mg stock solution. This stock solution was then diluted in 0.9% saline to create a treatment solution of 5 mg/mL tBHQ. Volume of solution delivered varied based on mouse weight and was approximately 200–230 μL for an average mouse. Total dosage was 33.4 mg/kg/day for each mouse receiving treatment. Control mice received an equivalent volume of vehicle solution of 5% DMSO in 0.9% saline. Solutions were made fresh daily. Injections occurred on each smoking day, approximately 1 h after the first smoking session.

Golgi Stain

Golgi Stain was performed as described previously (Ratliff et al. 2020b). Mice were perfused with phosphate buffered saline followed by 4% neutral buffered formalin. Brains were removed and then placed in 10% formalin at 4 °C overnight. Brains were then cryoprotected in 15% sucrose and incubated at room temperature for an additional 24 h. The samples were then incorporated into formalin-fixed tissue blocks (2–3 mm thick in the coronal plane) and stained using the Rapid Golgi method. To do this, fixed tissue blocks were placed in potassium dichromate and osmium tetroxide for approximately 6 days, and then transferred to 0.75% silver nitrate for approximately 40 h. The blocks were then dehydrated using increasing concentrations of alcohol solutions and ethyl ether. Blocks were then infiltrated with increasing concentrations of nitrocellulose solutions (5%, 10%,

20%, 30%; 1–2 days each). Next, blocks were placed in plastic molds and hardened using chloroform vapors. Tissue sections were cut to a thickness of 120 microns in the coronal plane using an AO sliding microtome, cleared in alpha-terpineol, rinsed with xylene, and mounted on slides using Permount. Strict selection criteria were used to select neurons for analysis and all selection and analysis was performed by the same examiner. Neurons must have been well impregnated, with branches unobscured by other neurons, glia, blood vessels, or undefined precipitate (a staining by-product). Additionally, the soma must have been located within the middle third of the section. A Zeiss brightfield microscope with long-working distance oil-immersion objective lessee and drawing tubes was used to prepare camera lucida drawings for analysis.

Dendritic arbors were analyzed using two methods: the Sholl Analysis, which defines the amount and distribution of the dendritic arbor, as well as an estimate of the total dendritic length, and Branch Point Analysis (BPA) which analyzes the complexity of the arbor based on the number of dendritic bifurcations within the dendritic domain, as previously described (Diamond et al. 2006).

Histological Analysis

Mice underwent cardiac perfusion to preserve brain tissues. Mice were anesthetized using isoflurane and heart was surgically exposed. Approximately 50 mL of wash solution (0.8% NaCl, 0.4% dextrose, 0.8% sucrose, 0.023% $CaCl_2$, and 0.034% sodium cacodylate in water) was injected into the heart, followed by approximately 50 mL fix solution (4% formaldehyde, 4% sucrose, and 1.4% sodium cacodylate in water). Following perfusion, brains were carefully dissected out and placed in fix solution overnight. Brains were then transferred to PBS for transport to Neuroscience Associates (Knoxville, TN) for multibrain blocking, sectioning at 35 μm thick, and histological staining. The stains included amino cupric silver stain (Switzer 2000), Hoechst 33,342 and immunohistochemistry. Antibodies were used as follows: glial fibrillary acidic protein- rabbit anti-GFAP (Dako Z0334) visualized with donkey anti-rabbit Alexa 488 LT A21206 or chicken anti-GFAP (Encor CPCA-GFA) visualized with donkey anti-chicken Alexa 488 (Jackson Laboratories 703-545-155). The cytochrome P450 factor was stained with rabbit anti-Cyp2E1 (Abcam ab28146) detected with donkey anti-Rabbit Alexa Fluor Plus 555 at 1:1000 dilution (Thermofisher A32794).

Slides were examined by laser scanning confocal microscopy with Fluoview 1000 and 3000 systems (Olympus, Waltham, MA). Z stacks were collected and maximum Z projections were evaluated quantitatively. To assess astrocyte morphology, ipsilateral dentate gyrus locations were stored at a lower magnification without regard for staining and then z-series were captured using a 60 × oil objective at these positions for each brain at the same locations. Quantification was performed using the ImageJ plugin, Analyze Skeleton. The images were batch processed to yield maximum intensity z projections and the appropriate channels were made binary with an automated intermodes threshold, despeckled, and skeletonized for analysis of process lengths and endpoints (Morrison and Filosa 2013, 2016). Cell counting was performed with cellSens software (Olympus).

RT–PCR

Frozen tissue from the ipsilateral (right) cortex was homogenized with QIAzol Lysis Reagent (Qiagen, Valencia, CA) and phase separated with chloroform 3 days post-injury. RNA was precipitated with 70% ethanol. Purification of mRNA was performed using a RNeasy Mini Kit (74,104; Qiagen, Valencia, CA) per the manufacturer's instructions. RNA concentration was determined by NanoDrop (Thermo-Scientific, Waltham, MA). RT-PCR was performed using iTaq Universal SYBR Green One-Step Kit (Bio-Rad, Hercules, CA) and L346/L347 primers for BDNF (IDT, Coralville, IA).

Statistics

GraphPad Prism software was used for statistical analysis. Mean values were compared using the two tailed *t* test or 2-way ANOVA for multiple group experiments. For Golgi analyses, the values of individual neurons were averaged per brain and statistical analyses considered each biological sample (brain) per group. For Sholl and Branch Point Analyses, the Wilcoxon signed-rank test was used. Means are displayed ± SEM and in all tests $P < 0.05$ indicates significance.

Results

Axonal Stability

Mouse brains were perfused, dissected, and then embedded in a 5 × 5 array and sectioned with the multibrain blocking, sectioning, and staining by Neuroscience Associates using the amino cupric silver stain for axonal degeneration. Amino cupric silver staining did not reveal any significant axonal degeneration when compared to sham controls (Fig. 2a) in any of the eight groups, including mice exposed to both injury and smoke (Fig. 2b) at 3 days post-injury. Brains were also examined at 12 days post-injury and similarly showed no axonal degeneration (not shown).

Dendritic Analysis

To determine the extent of any changes in dendritic complexity, we performed Golgi staining of granule cells within the dentate gyrus of the hippocampus. The dendritic arbor and spines comprise over 95% of the volume of the neuron and assessment of these parameters is a highly sensitive index of neuronal damage. Formalin-fixed tissue blocks (2–3 mm thick) incorporating the cortical samples were stained by the Rapid Golgi method and evaluated for the amount and distribution of dendritic branching by Sholl and branch point analyses (Diamond et al. 2006). Camera lucida drawings of the Golgi stained neurons were utilized to determine dendritic measurements for analysis. Representative camera lucida drawings of neurons following TBI (Fig. 3a) and after TBI and smoke exposure (Fig. 3b) are shown. Sholl analysis indicated highly significant reductions in complexity within the inner two-thirds of the dendritic material with smoke exposure following TBI (Fig. 3c, d). Branch point analysis also revealed a significant reduction in dendritic complexity (Fig. 3e, f). Following treatment with tBHQ, there was no change in the dendrites by either Sholl analysis (Fig. 4a, b) or branch point analysis (Fig. 4c, d).

BDNF mRNA

mRNA of brain-derived neurotrophic factor (BNDF) were analyzed via qRT-PCR in ipsilateral cortex obtained 3 days post-injury. There were no statistically significant changes from sham controls in BDNF levels following TBI, smoke exposure, or tBHQ treatment (Fig. 5).

Astrocyte Responses

Multiblocked brains from 3 days post-injury underwent GFAP and Hoechst nuclear counterstaining. GFAP stained astrocytes within the dentate gyrus indicate elevated immunoreactivity compared to sham controls (Fig. 6a) following both mTBI alone (Fig. 6b) and mTBI plus smoke exposure (Fig. 6c). When we measured the density of astrocytes in the dentate gyrus granule layer, we found a significant increase only after the TBI (Fig. 6d). We did not observe any significant differences in Astrocyte morphology.

Mouse brain sections, stained with Hoechst 33,342 and antibodies to GFAP and to Cyp2e1 were examined in the dentate gyrus region by confocal microscopy and then maximum Z projections were generated. We found two subsets of Cyp2e1 positive astrocytes, those with moderated Cyp2e1 expression and a much smaller subset with strong immunoreactivity (Fig. 7). Although there seemed to be trends in the intense Cyp2e1 counts per area in response to the smoke insult, the only statistically significant finding involved lower intense Cyp2e1 densities in the tBHQ-treated groups (Fig. 8a). The moderate intensity Cyp2e1 cells were more common and the staining was confined to the soma. There was an elevation in the moderately stained cells after injury that was significantly reduced by the tBHQ treatment (injured-air-vehicle compared to injured-air-tBHQ, Fig. 8b).

Discussion

Traumatic brain injury has become increasingly common in recent years, especially among the armed forces. Among U.S. Forces, there have been approximately 384,000 brain injuries sustained since 2000 (Defense and Veterans Brain Injury Center 2018). Additionally, cigarette smoking among veterans of recent conflicts in Iraq and Afghanistan is significantly higher than that of the average US Citizen (Bastian and Sherman 2010; Brown 2010). In this study, we utilize a mouse model of closed head mild traumatic brain injury, combined with repeated sidestream smoke exposure to better understand how the oxidative stress induced by cigarette smoking may impact the brain’s ability to protect itself and recover from mTBI. There are several established models of direct and second-hand smoke exposure (Gairola 2006; Kaisar et al. 2017) and ours involved daily exposures three times per day (Huang et al. 2009). We also tested whether enhancing antioxidant response through our treatment could counteract any negative effects of TBI and/or smoke exposure.

We focused on the hippocampus because it seems particularly sensitive to insults. For example, combined smoke and alcohol exposures were found to decrease astrocyte marker GFAP in the CA1, CA3, and dentate gyrus regions of the hippocampus, while neither alcohol nor smoking exposure alone had an appreciable effect (Huf et al. 2019). In the same study, alcohol and smoking had an additive effect on the increased expression of cleaved

caspase-3, a marker of apoptosis, in CA3, CA2, and the dentate gyrus of the hippocampus but not in CA1 suggesting selective regional sus-ceptibility to combined insults (Huf et al. 2019).

While we did not note any axonal degradation in response to either TBI or smoke exposure, there were significant reductions in dendritic complexity within the dentate gyrus of the hippocampus with smoke exposure following TBI compared to TBI alone. We did not observe any further changes in dendritic complexity in response to treatment with tBHQ. The changes in dendritic architecture that we did observe indicated the possibility of dysregulation of trophic factors. It has been shown that prenatal cigarette smoke exposure decreases BDNF expression in brains of male pups in select nuclei (Machaalani et al. 2019). In adult mice exposed for 60 days to smoke, BDNF levels were found significantly lowered in the hippocampus (Tuon et al. 2010). Conversely, chronic smoke exposure elevated BDNF mRNA levels following severe TBI in rats (Lee et al. 2012). Although we did not find BDNF dysregulation in our samples, it is possible that changes do occur that are specific for the brain region, severity of the injury, and time relative to the insult.

GFAP staining indicated that astrocytes were responsive to the insult within the ipsilateral dentate gyrus when mice were exposed to either TBI or smoke when compared to sham controls. This phenotype was reversible with antioxidant treatment. In other reports, inflammatory cytokines were found increased in mice as early as 1 month after initiation of smoke exposure and this was followed by additional damage as the exposure continued (Adhami et al. 2017). Furthermore, studies with Nrf2 knockout mice and a Nrf2 activator showed that, activation of Nrf2 using a small molecule triterpenoid CDDO-imidazolide can attenuate smoke-induced cytotoxicity in the heart and lungs, however, this was not evaluated in the brain (Sussan et al. 2009). In other reports, mice chronically exposed to smoke and a subsequent TBI showed behavioral and motor deficits and diminished recovery at 1, 24, and 72 h post-injury (Sivandzade et al. 2020). Neuroprotective Nrf2 expression was upregulated in response to TBI and this upregulation was severely diminished by smoke exposure (Sivandzade et al. 2020).

Nrf2 signaling could certainly play a role because reactive oxygen species (ROS) are greatly enriched in tobacco smoke and chronic smoking has been shown to dysregulate normal blood brain barrier (BBB) function by activation of oxidative, inflammatory, and immune responses (Cataldo et al. 2010; Cojocaru et al. 2013; Kaisar et al. 2018; Paulson et al. 2010; Sivandzade and Cucullo 2019a, 2019b). Modulation of Nrf2 via treatment with tBHQ has been shown previously to reduce ROS following model brain injury (Zhang et al. 2020). These are likely contributors to diminished cognitive recovery of smokers than nonsmokers after a mild TBI as has been shown in human studies (Durazzo et al. 2013).

Finally, we did find evidence for upregulation of Cyp2e1 after insult. This is consistent with elevations of Cyp2e1 that have been found by other groups. For example, Cyp2e1 expression was increased in rat and gerbil astrocytes in vitro and in vivo along with inflammatory factors and induced by ischemic injury (Tindberg et al. 1996). The hippocampus was shown to be one of the brain regions that exhibit Cyp2e1 upregulation after insult (Zhong et al. 2012).

One of the most common long term-effects of mTBI involves memory problems (McDowell et al. 1997; Stuss et al. 1985). Our study indicates that there are several important changes to the astrocytes of the hippocampus in response to TBI and smoke exposure. These changes induced by cigarette smoke, suggest that smoke exposure during recovery may contribute to poorer cognitive out-comes following TBI, and memory deficits, in particular. Our study also provides some evidence that treatment with an antioxidant pathway activator, such as tBHQ, may be able to combat some of these negative effects. Further research is necessary, however, our study provides a unique cellular basis for the impact of lifestyle choices, such as cigarette smoking, on the impact and recovery from TBI.

Acknowledgements

We thank Andrea Smith and Tai Vo for expert animal assistance, Miseanne Tang for technical assistance, and Dr. John C. M. Tsibris for the loan of the smoking system.

Funding This study was supported by the Department of Veterans Affairs (Veterans Health Administration, Office of Research and Development, Rehabilitation Research and Development (I01RX001520)), the Assistant Secretary of Defense for Health Affairs through the Congressionally Directed Gulf War Illness Research Program (W81XWH-16-1-0626), the Florida Department of Health James and Esther King Biomedical Research Program (4KB14), and The Bay Pines Foundation. The funding sources had no involvement in study design; in the collection, analysis, or interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

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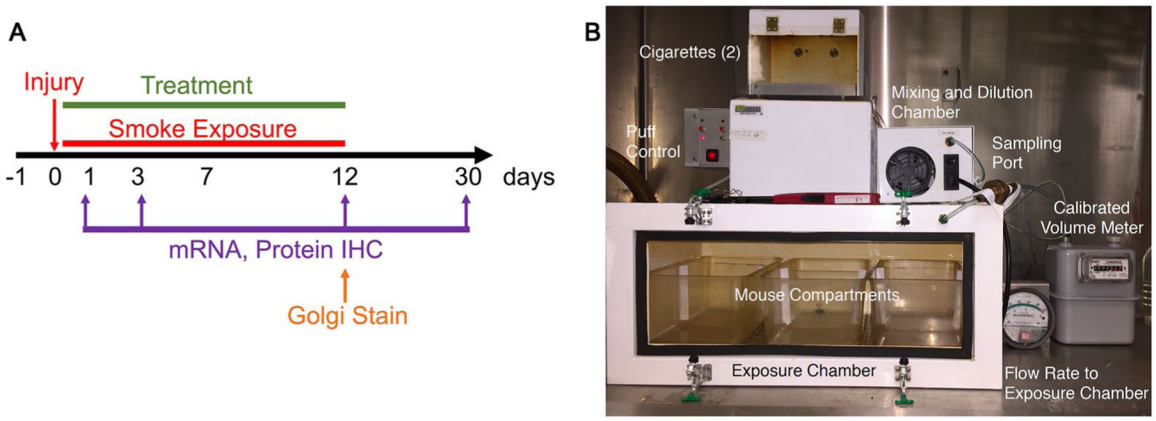

Fig. 1.

Mouse model of sidestream smoke exposure following mTBI. **a** Mice received a single closed head injury followed by up to 12 days of exposure to second-hand smoke and tBHQ treatment. Assays were performed at 1, 3, 12, and 30 days post-injury. **b** To expose the mice to second-hand smoke, they were placed inside the exposure chamber of the smoking apparatus. Cigarettes are loaded into the apparatus and "smoked" using the puff controller with smoke then being sent into the exposure chamber. Each smoking session lasted approximately 30 min and used 4 cigarettes. Sham mice were placed next to the apparatus for exposure to noise without being exposed to smoke

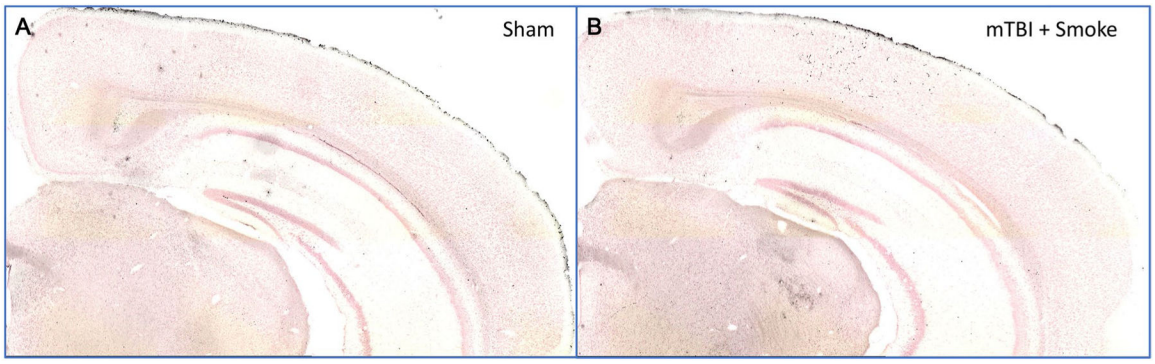

Fig. 2.

Analysis of axonal degradation. Perfused mouse brains were analyzed using the amino cupric silver stain for axonal degradation. When compared to sham controls (**a**), mTBI and smoke exposure did not induce any noticeable axonal degradation (**b**). Similarly, no axonal degradation was observed in any other groups

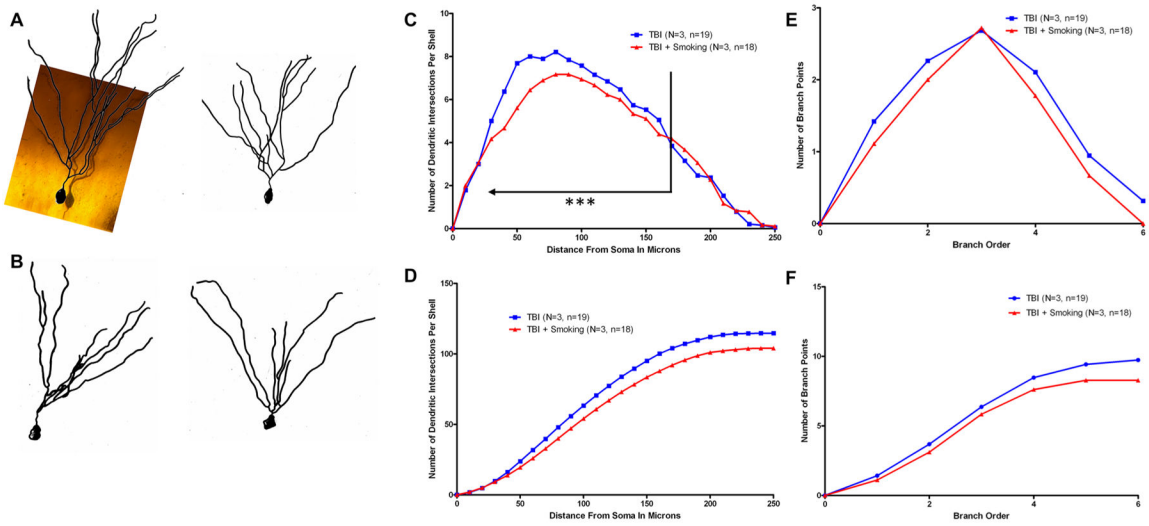

Fig. 3.

Dendritic analysis of injured and smoke exposed brains. Golgi staining was utilized to determine the extent of any changes in dendritic complexity following injury and smoke exposure. Camera lucida drawings of granule cells from the dentate gyrus were measured to determine the complexity of the dendritic arbor by Sholl and branch point analyses. Representative neurons from brains taken 12 days post-injury are shown from injured brains (**a**) or injured and smoke exposed brains (**b**). Sholl analysis indicates reduced complexity following mTBI and smoke exposure especially for the inner two-thirds, indicated on the graph, of the dendritic arbor ($P = 0.0009$) (**c**). Total differences in complexity can be seen more clearly in the cumulative graph (**d**). Branch point analysis indicated a similar trend ($P = 0.06$) (**e**) which can be seen clearly in the cumulative graph (**f**)

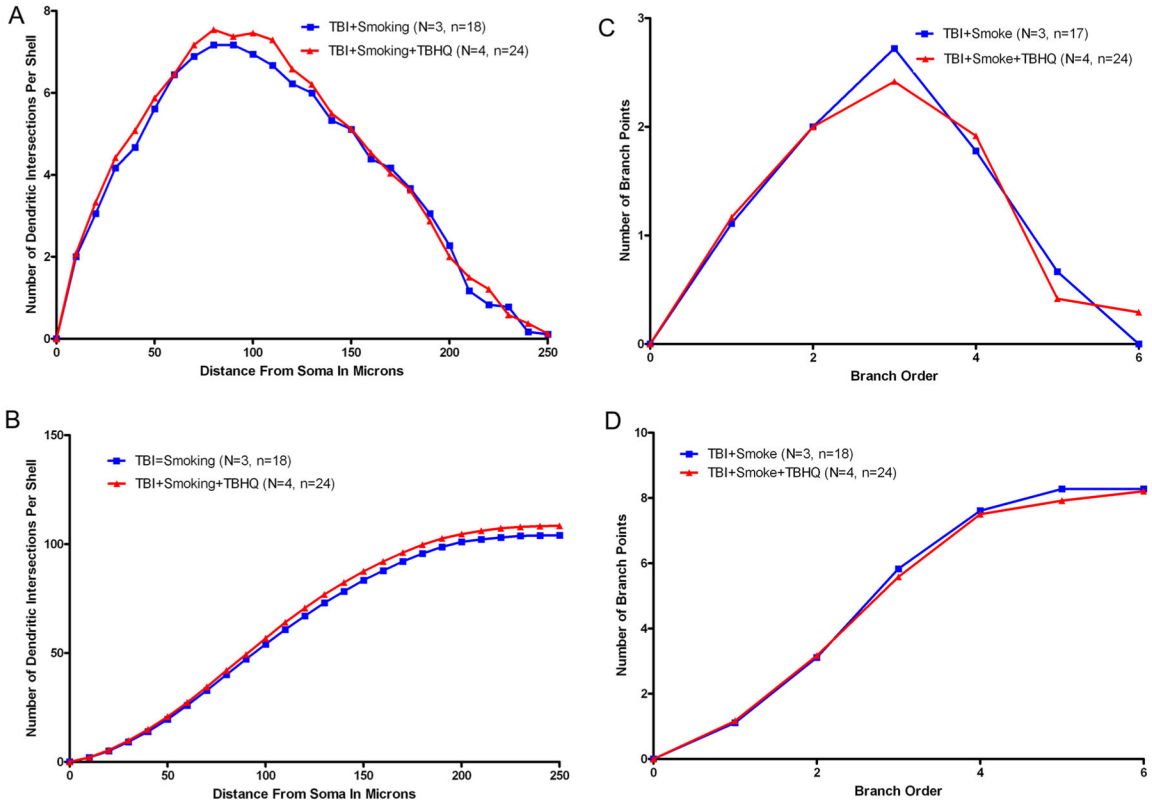

Fig. 4.

Dendritic analysis following treatment. Sholl and branch point analyses were performed on granule cells of the dentate gyrus 12 days post-injury. Sholl analysis did not indicate any significant change in dendritic complexity of mTBI and smoke exposed brains following treatment (**a**), this can also be seen in the cumulative graph (**b**). Similarly, branch point analysis also showed no statistically significant change with treatment (**c**), the cumulative graph also shows no change (**d**)

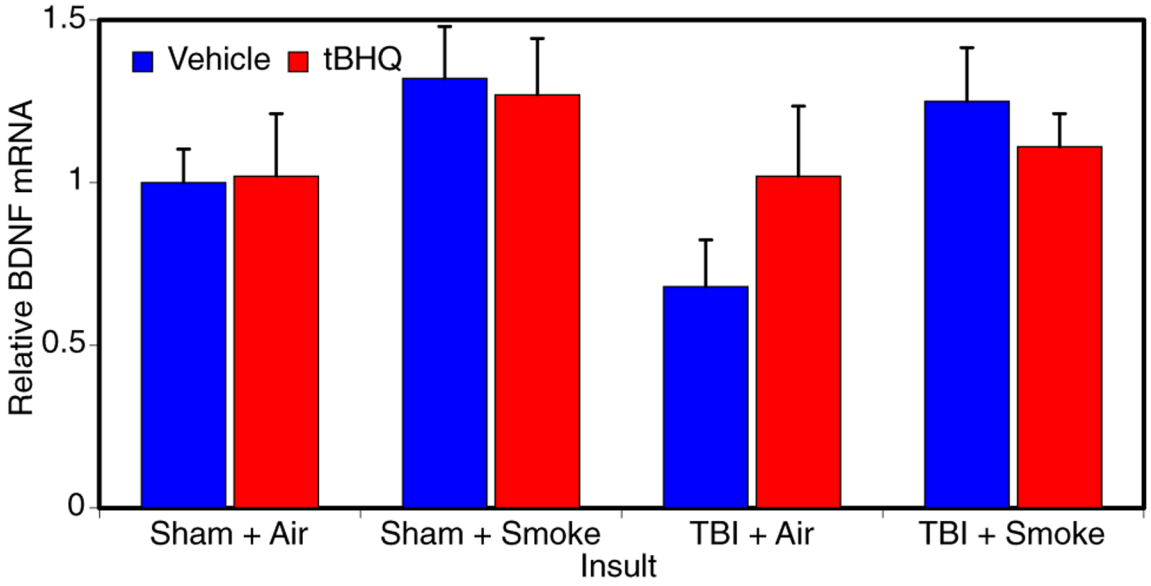

Fig. 5.

Analysis of BDNF mRNA. qRT-PCR analysis was utilized to determine if neuronal changes following injury, smoke, and treatment could be related to changes in BDNF expression in ipsilateral cortex at 3 days post-injury. mRNA samples from 4 mice per group were used for the analysis. These analyses indicated that there were no significant changes in BDNF mRNA levels

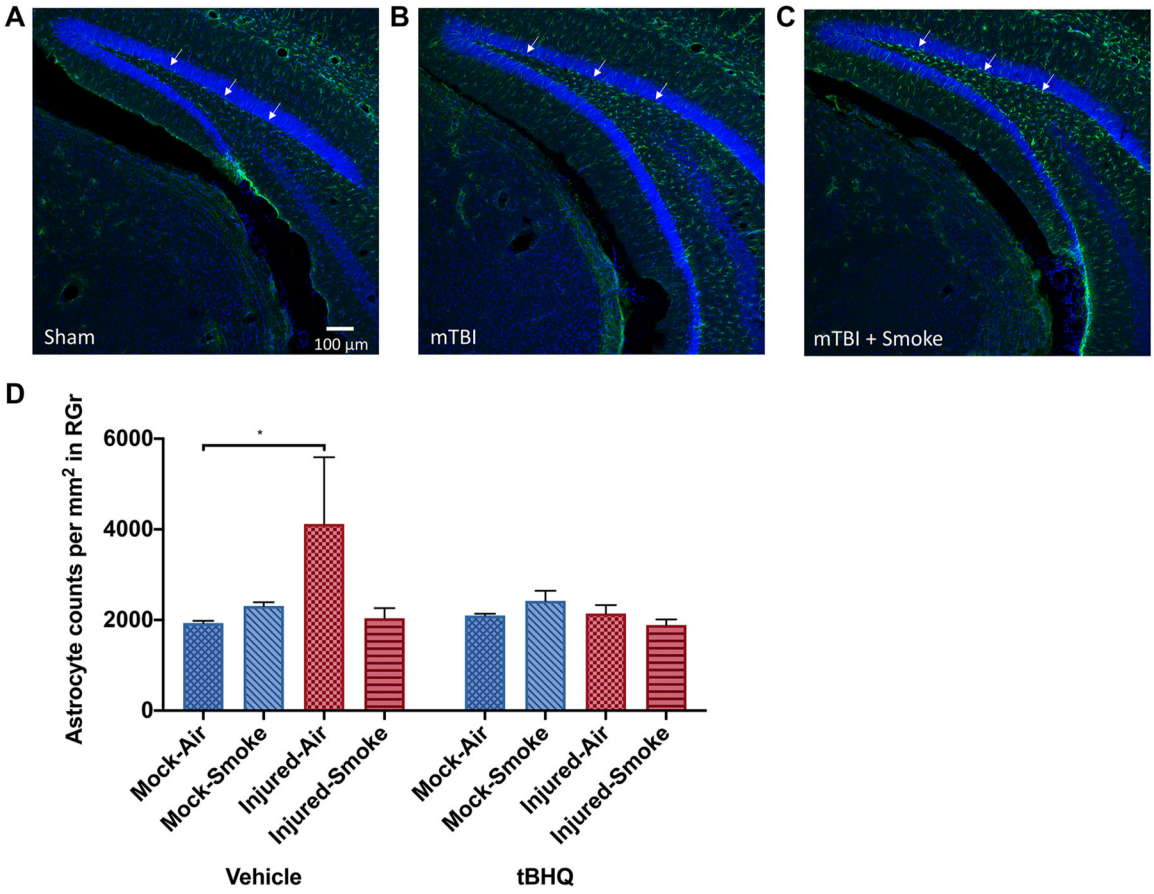

Fig. 6.

Reactive astrocytosis following injury and smoke exposure. GFAP staining (green channel) and Hoechst nuclear counterstaining (blue channel) were performed on multiblocked brains 3 days post-injury. When compared to sham controls (**a**) visual examination suggests elevated immunoreactivity following TBI (**b**) with even further elevation following TBI and smoke exposure (**c**). Quantitative analysis of the numbers of astrocytes within the granule layer of the dentate gyrus indicated that TBI resulted in additional astrocytes present (**P* = 0.035) (**d**)

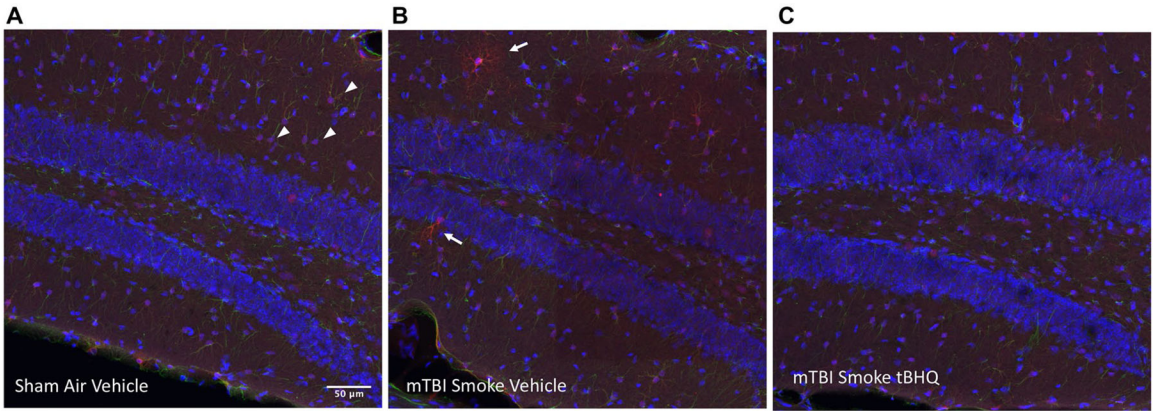

Fig. 7.

Cyp2e1 in the granular cell region of the ipsilateral hippocampus. Astrocytes in the dentate gyrus region of the hippocampus were frequently found to stain positively at a moderate intensity in the soma (arrowheads) and sometimes more intensely with immunoreactivity throughout the branched processes (arrows). Hoechst 33,342 staining of nuclei is shown in the blue channel and Cyp2e1 immunoreactivity in the red channel in these example sections from mice with no insult or treatment (**a**), mTBI + smoke exposure without treatment (**b**) or mTBI + smoke exposure with the tBHQ treatment (**c**). The region is from the right hippocampus closest to the closed head impact centered at − 2.9 mm from bregma

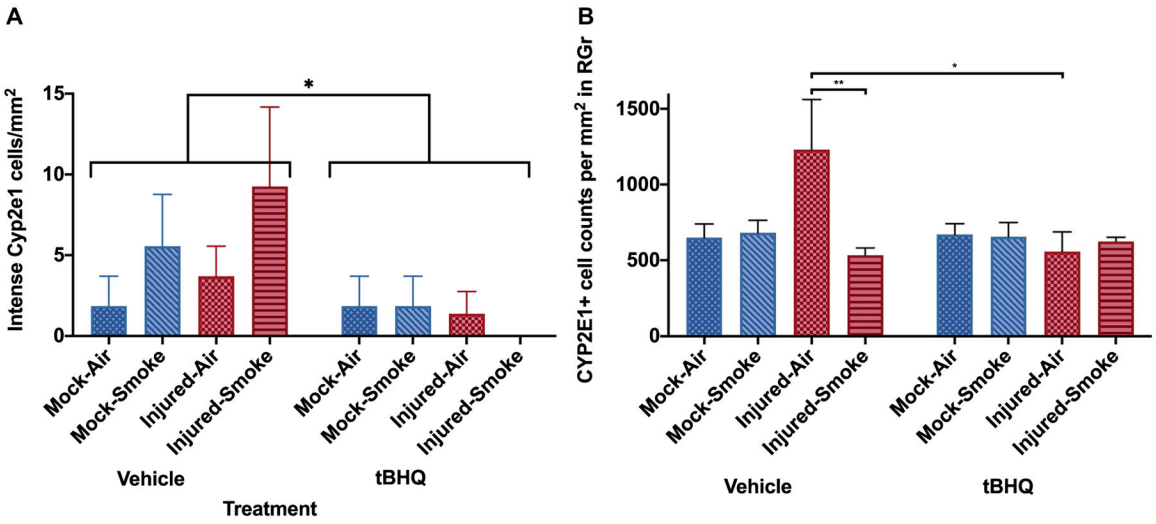

Fig. 8.

Cyp2e1 positive astrocyte densities. Analysis of the numbers of astrocytes displaying intense Cyp2e1 immunoreactivity in the dentate gyrus region (**a**) or moderate staining within the granule layer (**b**) indicated that there were varied responses to the insults and that in both cases, Cyp2e1 astrocyte densities were reduced by the tBHQ treatment (**a** **P* = 0.04 effect of tBHQ over all groups; **b** **P* = 0.045, ***P* = 0.0047, 2-way ANOVA was used for statistical analysis)