Title: Nicotine Inhalant via E-Cigarette Facilitates Sensorimotor Function Recovery by Upregulating Neuronal BDNF-TrkB Signaling in Traumatic Brain Injury

Running Title: Nicotine improves TBI recovery via BDNF pathway.

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Abstract

Background and Purpose: Traumatic brain injury (TBI) imposes life-long physical, psychological, and financial burdens on affected individuals. The current study investigated the effects of chronic nicotine exposure via E-cigarette (E-cig) on TBI-associated behavioral and biochemical changes.

Experimental Approach: Adult C57/BL6J male mice were subjected to controlled cortical impact (CCI) followed by daily exposure to E-cig vapor for six weeks. The effects of chronic nicotine exposure on sensorimotor functions, locomotion, and sociability were evaluated by nesting, open field, and social approach tests, respectively. Immunoblots were performed to assess changes in the expression of mature brain-derived neurotrophic factor (mBDNF) and associated downstream signaling proteins. Histological analyses were performed to evaluate the effects of chronic nicotine exposure on microglia-mediated neuroinflammation.

Key Results: Post-injury chronic nicotine exposure significantly improved nesting performance in CCI mice. Histology analysis revealed that chronic nicotine exposure increased the survival of cortical neurons in the perilesion cortex. Immunoblots of cortical tissue revealed that chronic nicotine exposure significantly upregulated mBDNF, p-Erk, and p-Akt expression in the perilesion cortex of CCI mice. Additional IF microscopy revealed that elevated mBDNF and p-Akt expression was predominantly localized within cortical neurons of CCI mice. Furthermore, immunolabeling of Iba1 showed that chronic nicotine exposure attenuates microglia-mediated chronic neuroinflammation in the perilesional cortex of CCI mice.

Conclusions and Implications: Post-injury chronic nicotine exposure via vaping facilitates sensorimotor function recovery by upregulating neuroprotective mBDNF/TrkB/Akt/Erk signaling. Results from this study support the neuroprotective properties of nicotine, but further investigation is needed due to its highly addictive nature.

Key Words: Nicotine, E-cigarette, Traumatic Brain Injury, Sensorimotor Function, BDNF, Neuroprotection.

Bullet point summary：

What is already known

- Premorbid use of tobacco/E-cig adversely influences functional outcomes in TBI.
- Nicotine modulates multiple biological activities and has been shown to afford neuroprotective effects in neurodegenerative diseases, including Alzheimer’s Disease and Parkinson's Disease.

What this study adds

- Chronic nicotine exposure after TBI promotes sensorimotor function recovery by upregulating neuronal BDNF/TrkB signaling pathway in the cortex.
- Nicotine Withdrawal and CCI induce a compounding effect on social approach behavior.

What is the clinical significance

- nAchR-selective agonists could serve as a therapeutic target for neuroprotection against traumatic brain injury and other forms neurodegenerative diseases without inducing addictive side effects associated with chronic nicotine use.

1. INTRODUCTION

Traumatic brain injury (TBI) is one of the leading causes of disability and death in the United States. According to the Centers for Disease Control and Prevention (CDC), more than 220,000 TBI-related hospitalizations were documented in 2019. In 2020 alone, 64,362 deaths were attributed to TBI. The primary injury from TBI is caused by a strong and abrupt mechanical force that deforms and damages the brain parenchyma. Hemorrhage, neuronal damage, and disruption of the blood-brain barrier (BBB) are common conditions observed immediately after the primary injury, followed by secondary injuries due to impaired BBB, chronic inflammation, and excitotoxicity, which further exacerbate neuronal cell death and trigger neurodegeneration, leading to increased risks of neurodegenerative disorders (Brett et al., 2022). While it is clear that premorbid use of tobacco adversely influences neurocognitive and functional outcomes in TBI, primarily due to systemic effects that can compromise brain functions and weaken the integrity of the BBB (Alasmari et al., 2022; Durazzo et al., 2013), the effects of smoking post-TBI remain unknown. A recent study that recruited 336 veterans with TBI reported that 28% of participants were actively smoking after TBI, and notably, 12% of preinjury non-smokers became smokers at follow-up (Brown, 2010; Silva et al., 2018). In consideration of a significantly higher smoking rate among veterans and active military members, this unique demographic group may be particularly vulnerable to the comorbid effects of TBI and nicotine dependence.

Nicotine, the active ingredient in E-cig, exerts its psychoactive effects via binding to nicotinic acetylcholine receptors (nAchRs) in the brain. In the CNS, nAchRs plays crucial roles in mediating cholinergic transmission, neurotransmitter and growth factor release (Castillo-Rolon et al., 2020; Reid et al., 1999), and have been found to be involved in a wider range of CNS disorders including TBI, AD, PD, etc. Therefore, the approach to restore lost cholinergic signaling in various neurological conditions via selective activation of α7 nAchRs has gained increasing attention in the recent years (Alhowail, 2021; Dineley et al., 2015; Gatson et al., 2015; Nicholatos et al., 2018; Posadas et al., 2013; Wang et al., 2020). While most of the studies suggested that nicotine exposure could provide neuroprotective effect in PD and stroke, whether nicotine containing product use after TBI the recovery of TBI is not clear, due to the strong effect of prior tobacco use as a risk factor prior TBI in many of clinical studies. Thus, a more comprehensive understanding of post-injury nicotine exposure is urgently needed.

The aim of the current study is to: 1) provide a more comprehensive neurobehavioral analysis on the effects of chronic nicotine exposure following TBI; 2) elucidate the molecular mechanisms underlying nicotine-induced behavioral changes in TBI mice; 3) examine the effects of post-injury chronic nicotine exposure on TBI-induced pathophysiology. Considering the current epidemic of nicotine abuse through electronic cigarettes (E-cig) in our society (Jones & Salzman, 2020), the current study utilized an E-cig vapor exposure model to mimic human vaping sessions to examine the impact of chronic nicotine exposure after TBI.

2. METHODS and MATERIALS

2.1 Animals

Adult 3-month-old male C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and treated in compliance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, Bethesda, MD, USA). Animal use protocols (#20-021) was approved by the Veterans Administration San Diego Healthcare System Institutional Animal Care and Use Committee (IACUC). Mice were housed on a 12h:12h light-dark cycle (lights on at 6 a.m) in a temperature and humidity-controlled room with a sufficient supply of water and food. Mice were housed in standard cages (28.4 x 18.4 x 12.5 cm) in an individually ventilated caging system.

2.2 Experimental Design

Mice were randomly divided into six groups: Sham-Vehicle (Veh), Sham-Nicotine (Nic), Sham-Nicotine Withdrawal (Nic Withdrawal), CCI-Vehicle, CCI-Nicotine, and CCI-Nicotine Withdrawal. Mice underwent either Sham surgery or CCI surgery first, followed by baseline assessment for social preference using the three-chambered social approach (SA) test one-week after surgery. Next, mice received daily vehicle or nicotine-containing vapor exposure for 6 weeks. Considering the short half-life of nicotine, post-exposure behavioral

tests, including nesting, SA test, and open field, were performed during the last week of exposure for nicotine-exposed groups. Behavioral tests for the Withdrawal groups were conducted one week after the completion of the 6-week exposure period (**Figure 1A**).

2.3 Controlled Cortical Impacts (CCI) Model

CCI procedure was performed as described previously (Egawa et al., 2017). Mice were anesthetized with isoflurane (1.5% with oxygen at 1 L/min) and kept on a 37 ºC heating pad during the surgery. Mice were stabilized in a stereotaxic frame, and a 4 mm x 4 mm cranial window (AP: +1 mm to -3 mm, ML: +0.5 mm to +4.5 mm) was created above the right hemisphere. A 3-mm-diameter stereotaxic flat-tip impactor (Impact one; myNeuroLab.com, Richmond, IL, USA) was centered on the dura and accelerated to 3 m/s to impact the brain at a depth of 1 mm below the cortical surface. Those coordinates were chosen to target the sensory and motor cortices specifically to induce major deficits in sensorimotor function. Sterilized cover glass was placed over the cranial window, and surgical clips were used to close the incision. The animals were allowed to recover for one week before baseline behavior testing and vapor exposure.

2.4 Vapor Exposure Chamber Model

Stick V9 Max vape pen kit was used to aerosolize nicotine-containing E-liquid. The stick battery provides an output voltage of 2.1V to 4.1V and an output power of 60W. An E-liquid tank of 8.5 ml volume capacity is attached to the battery. The E-liquid vehicle contains 70% Vegetable Glycerin and 30% Propylene Glycol. Nicotine is added to the vehicle solution to achieve a concentration of 24 mg/ml. A paired Baby V2 S2 0.15 Ohm coil is used to create clouds of vape smoke. The mouthpiece of the vape pen is connected to the exposure chamber via PVC and metal tubing for effective delivery of vape smoke. A vacuum pump set at 50 psi is used to provide ventilation and draw in vapor into the chamber. Heating tapes are secured around the chamber for temperature control. A temperature probe is inserted into the chamber providing constant readouts. Each exposure session lasts 40 minutes, and each single puff cycle consists of 5s of heated E-cig vapor exposure followed by 25s of air.

2.5 Determination of optimal temperature and exposure duration

Previous studies have shown that chamber temperature affects the size of aerosolized particles and smoke delivery efficiency (Lechasseur et al., 2019); we thus first tested the efficiency of vapor delivery at varying chamber temperatures. Mice were exposed to vehicle vapor containing Evans Blue (EB) fluorescent dye at four different temperatures (21, 28, 32, 36 ℃) within the animal's thermal neutral zone. Animals were euthanized immediately after exposure, and the whole lungs, along with the trachea, were dissected, inflated, and air-dried for imaging by IVIS Spectrum In Vivo Imaging machine (level = high, Em = 700, Ex = 640, Epi-illumination, Bin:(M)8, FOC:13.2, f2, 15s). To determine the optimal exposure duration, mice were exposed to nicotine-containing vapor for 5, 20, 40, and 60 minutes respectively. Plasma and lungs were collected to assess cotinine concentration using the Cotinine Direct ELISA kit (Calbiotech, CO096D).

2.6 Behavioral tests and analyses

Open Field (OF)

The Open Field (OF) test was performed to assess general locomotion and anxiety-like behavior. Mice were placed in a square arena (41 x 41 x 34 cm enclosures) illuminated by a bright light. A computerized video tracking system (Noldus Ethovision XT 7.1, Leesburg, VA, USA) was used for recording during a 10-minute test session. Distance moved (cm), velocity (cm/s), and time spent in the center of the arena (s) were recorded as previously described (Wang et al., 2021).

Nesting

Nest building is an innate and highly motivated behavior performed by rodents to provide adequate thermo-regulation and to support pup survival (Gaskill et al., 2012). Since this behavior requires intricate coordination between sensory feedback and motor outputs, which includes pulling, carrying, and fluffing of the nesting material, it has been used to assess sensorimotor function in rodents (Deacon, 2006; Fleming et al., 2004; Paumier et al., 2013; Yuan et al., 2018). To measure nest building, mice were housed in single cages

approximately 1 hour before the start of the dark cycle. Three grams of a square-shaped nestlet (Ancare, NES3600) was placed in each cage with no other cage enrichment. Nests were scored the next day at 8 a.m. A nesting score is given based on the weight of the unused nestlet, and the overall shape of the nest (1: >90% of nestlet intact; 2: 50–90% of nestlet intact; 3: 10–50% of nestlet intact; 4: <10% of nestlet intact, the nest is flat; 5: <10% of nestlet intact, the nest is identifiable with high walls).

Social Approach (SA)

Neuropsychiatric disorders such as mood dysregulation and elevated social anxiety are commonly seen following TBI and nicotine exposure (Audrain-McGovern et al., 2014; Jobson et al., 2019). Thus, we assessed social preference before and after chronic nicotine exposure to evaluate the effects of nicotine exposure on sociability in CCI mice. The social approach (SA) test consists of a habituation stage followed by a test stage. In the habituation phase, the test mouse was given 10 minutes to explore the three-chambered arena with empty, inverted stainless steel wire cups (Galaxy Cup Inc. Streetsboro, OH) placed in the two outer chambers. In the test stage, a stranger mouse (pre-pubescent male) was placed under one cup located in either left or right chamber, and an object was placed under the other cup located in the opposite outer chamber (**Figure 4A**). The test mouse was then placed in the center chamber and allowed to explore the entire arena for 10 minutes. Time spent with the stranger mouse and the non-social stimulus (object) was recorded. The sociability of the animals was evaluated by the social preference index defined by the difference between time with stranger and time with object divided by the total time exploring both (Rein et al., 2020).

2.7 Immunoblotting (IB)

Freshly dissected cortical tissue was homogenized in cold 500 mM sodium carbonate buffer (pH 11.0; Protease and phosphatase inhibitor cocktail included) and sonicated three times for 10s (Wang et al., 2021). Bradford assay was performed to standardize sample concentration to ~1.0 ug/ul. Tissue homogenates were immunoblotted using primary antibodies for BDNF (Protein Tech #28205, 1:500), GAPDH (Cell Signaling Technology #2118S; 1:1000), p-TrkB (Cell Signaling Technology #9141S, 1:500); TrkB (BD Biosciences 610102; 1:1000), p-Akt (Cell signaling Technology #9271L, 1:1000), Akt (Cell signaling Technology #9272S, 1:1500), p-ERK (Cell Signaling Technology #4370, 1:1500), and ERK (Cell Signaling #9102, 1:1500) overnight at 4 degree. Subsequently, the membrane was incubated with HRP-linked anti-rabbit IgG (Cell Signaling Technology #7074S, 1:1000) for one hour at room temperature (RT). Lumigen ECL Ultra (Lumigen TMA-6) was used to visualize the signal, and densitometry analysis was performed using Photoshop. All bands were normalized to GAPDH, and all phosphorylated proteins were normalized to the respective total proteins.

2.8 Lesion volume and neuronal survival assessment

Mice were perfused transcardially with cold PBS followed by 4% PFA. The brains were then carefully removed for post-fixation in 4% PFA at 4°C overnight, followed by dehydration in 30% sucrose on the following day. Serial 40-μm coronal sections were collected and stained with cresyl violet stain solution (0.1%). Lesion area and neuronal count were analyzed using Photoshop. Lesion size was quantified by normalizing the size of the lesion area in the ipsilateral hemisphere to the intact contralateral hemisphere to control for unwanted sources of variations from dehydration and rehydration processes during tissue processing. Lesion size measurements were calculated by using a total of 5 sections with 1mm intervals starting from AP +1.4 mm to AP -2.6 mm as illustrated in **Supplemental Figure 5A**. Lesion volume was determined by averaging lesion size from all five sections. Neuronal count analysis was performed using 40x image captured by Keyence All in One (BZ-X700, Keyence Corporation of America, IL, USA). Nissl-stained neurons were counted manually in the motor cortex at the perilesion area. Mean values were calculated from two approximately equidistant slices.

2.9 Immunofluorescence microscopy (IF)

Floating sections (40um thickness) were blocked in 10% goat serum in TBS-Triton 0.25% solution for 1 hour at RT. Subsequently, sections were incubated with primary antibodies: anti-p-Akt (Cell signaling #9271L, 1:100), anti-Iba 1 (Wako #019-19741, 1:500), and anti-NeuN (Millipore Sigma #ABN91, 1:1000) diluted in the blocking solution at 4°C for 48 hours followed by incubation with species-specific fluorescent secondary antibodies in the dark for 2 hours at RT. For p-akt and BDNF staining, sections were heated in 10 mM sodium

citrate buffer (pH 6.0 and 0.05% Tween 20) for 20 minutes at 95-100°C for antigen retrieval. Next, slices were pretreated with hydrogen peroxide for 30 minutes prior to overnight anti-BDNF (Protein Tech #28205, 1:2000) incubation. On the following day, sections were incubated with anti-rabbit biotinylated secondary antibody (Jackson Immunoresearch. Laboratories) for 2 hours followed by 30 minutes of incubation in an Avidin-Biotin Complex solution (Vector Laboratories, PK6100). Next, sections were treated with a biotinyl tyramide solution for 20 minutes followed by incubation with streptavidin 647 (Invitrogen, S21374, 1:500) for 2 hours in the dark.

3.0 Data Analysis

Data were analyzed by Student *t*-tests, one-way analysis of variance (ANOVA), or two-way ANOVA followed by Fisher's LSD or Bonferroni's multiple comparisons tests as appropriate using GraphPad Prism 10 (La Jolla, California). Data were presented as mean ± SEM, and significance was assumed when * $p < 0.05$. Experimental groups were blinded to the observer, and the code was broken for analysis.

3. RESULT

3.1 Confirmation of nicotine delivery efficacy via a new semi-automated vapor exposure chamber

The current study utilized a semiauto vapor exposure chamber to mimic human vaping (**Figure 1B**). As shown in **Supplemental Figure 1,** fluorescent imaging of inflated lungs displayed no significant difference in EB intensity at all four temperatures after 30 minutes of vapor exposure. Next, the optimal exposure duration was determined as shown in **Figure 1C**. Plasma cotinine level significantly increased after 5 minutes, reaching a peak level of approximately 58 ng/ml at 40 minutes, comparable to other rodent studies that employed similar settings (Alasmari et al., 2022). Cotinine concentrations in lung homogenates exhibited a similar trend (**Figure 1D**). Thus, 40 min E-cig exposure at 28 ºC was chosen for all following experiments.

3.2 Chronic nicotine exposure induces transient hyperlocomotion and decreases thigmotactic behavior in CCI mice

Mice generally exhibit a strong thigmotactic behavior, a tendency to remain close to the walls of the testing arena due to a natural aversion to open space. Thus, the OF test (**Figure 2A**) was performed to evaluate the effects of chronic nicotine exposure on locomotor activity and anxiety-like behaviors. In the current study, while no difference was observed in all three parameters within the three Sham groups, mice from CCI-Nic group exhibited significantly increased velocity, total distance traveled, and time spent in the arena center compared to CCI-Veh and CCI-Nic Withdrawal group (**Figure 2B-D**), indicating that chronic nicotine exposure induced a transient increase in locomotor activity accompanied by suppressed thigmotaxis in CCI mice.

3.3 Chronic nicotine exposure improves long-term sensorimotor function recovery and protects against neuronal loss in the perilesion motor cortex

We next performed the nesting test to assess sensorimotor function and the general wellness of mice after chronic nicotine exposure. Nesting performance was evaluated quantitatively and qualitatively via percent nestlet usage and nesting score, respectively. After 5 weeks of exposure, no difference in nestlet usage and nesting score was observed within Sham groups (**Figure 3A-B**), suggesting that chronic nicotine exposure or withdrawal did not affect the animal's tendency and ability to construct nests. Since the current CCI model causes significant tissue loss in the motor and sensory cortices, as expected, mice from the CCI-Veh group exhibited a significant decrease in percent nestlet usage and nesting score compared to that of the Sham-Veh group. This result is consistent with a previous study using a similar chronic CCI mouse model (Ritzel et al., 2020). On the other hand, mice from CCI-Nic and CCI-Nic Withdrawal groups displayed significant improvement in nestlet usage and nesting score compared to CCI-Veh group (**Figure 3A-B)**. To further identify the time course of functional recovery, a separate cohort was used to track nesting performance biweekly. As shown in **Figure 3C**, an increasing trend in percent nestlet use ($p=0.09$) was observed as early as post-exposure week 3, and a significant enhancement in nestlet usage was detected at post-exposure week 5. Histological analyses of cortical tissue damage using Nissl stain revealed that while neuron number in the CCI-Veh group significantly decreased compared to Sham-Veh group, CCI-Nic group showed a significantly higher neuron count compared to that of CCI-Veh group (**Figure 3D-F**). No significant difference was observed between CCI-

Nic group and CCI-Nic withdrawal group. Together, our data suggest that nicotine exposure could significantly enhance long-term sensorimotor functional recovery after CCI.

3.4 Withdrawal from chronic nicotine exposure adversely affects social approach behavior in CCI mice

One of the most common symptoms observed in TBI patients is social maladjustment (Walz et al., 2009). However, baseline comparison of pre-exposure social preference showed no significant difference between Sham and CCI mice, and the social preference index for both groups fell within the normal range (**Figure 4B**) (Rein et al., 2020). The mice were then randomized to Veh, Nic and Nic withdrawal groups to ensure all groups exhibit similar interaction time with strange mice and object (**Figure 4C)**. As shown in **Figure 4D**, after exposure, both Sham-Nic and CCI-Withdrawal groups showed no difference in time interacted with strange mice and object, suggest nicotine suppressed social behavior in Sham surgerized mice and nicotine withdrawal inhibited social behavior in the CCI mice. We further compared social preference index before and after exposure (**Figure 4E**) which shows significantly decrease in preference after exposure in CCI-Withdrawal group, suggesting a compounding effect of chronic nicotine withdrawal and CCI on social approach behavior.

3.5 Chronic nicotine exposure increases mBDNF expression and activates associated downstream pro-survival signaling in the cortex

Since brain-derived neurotrophic factor (BDNF) expression is regulated by various neurotransmitter systems that are sensitive to nicotine (Knipper et al., 1994; Li et al., 2014), we assayed for BDNF expression and its associated signaling proteins in the cortex. As shown in **Figure 5A-B,** ipsilateral cortical tissue from Sham-Nic mice showed significantly increased mature BDNF (mBDNF) expression compared to both Sham-Veh group and Sham-Nic withdrawal group, findings supported by a previous study that reported nicotine treatment increased mBDNF expression in the hippocampus of an aging model (Yang et al., 2023). Corresponding to this elevated level of mBDNF expression, we detected increased phosphorylated tyrosine receptor kinase B (p-TrkB), p-Akt, and p-Erk expression in the Sham-Nic group, compared to that of Sham-Veh and Sham-Nic withdrawal groups, confirming the activation of the BDNF-TrkB signaling pathway triggered by chronic nicotine exposure. Similar to what was observed within Sham groups, ipsilateral cortical tissue from CCI-Nic mice exhibited significantly elevated mBDNF, p-TrkB, p-AKT, and p-Erk expression compared to CCI-Veh group and CCI-Nic withdrawal group (**Figures 5A-B**), suggesting a nicotine-induced upregulation in BDNF signaling despite extensive damage to the ipsilateral cortex. No difference was observed in mBDNF, p-TrkB, p-Akt, and p-Erk expression between Sham-Veh and CCI-Veh groups (**Supplemental Figure 2**). Considering that IB could not identify changes in specific subregions of the cortex nor cell types that contribute to the increased BDNF expression, we further performed immunostaining of BDNF and NeuN. As shown in **Figure 5C** (Top 3 row), while CCI-Veh and Sham-Veh presented a similar pattern of weak and sparse BDNF signals in a few cells, the CCI-Nic group showed a strong BDNF staining within multiple cells located in the perilesional area. Further examination revealed co-localization of BDNF with NeuN-positive neurons (**Figure 5C** bottom row).

Similarly, a significant increase in the expression of mBDNF and associated downstream proteins was observed in the contralateral cortex of Sham-Nic and CCI-Nic mice (**Figure 6A-B**), revealing a global increase in BDNF-TrkB signaling due to chronic nicotine exposure. Furthermore, immunostaining revealed that p-Akt was colocalized with NeuN-positive neurons in the contralateral S1 cortex of CCI-Nic mice (**Figure 6C**), indicating activation of Akt signaling in neuron population, a pro-growth and pro-survival signaling pathway that affords beneficial effects in multiple neurological disorders. IB of cortical tissue from different time points after chronic nicotine exposure revealed that the upregulated mBDNF and downstream signaling proteins were maintained at a high level even at one week after the cessation of nicotine exposure. This elevated mBDNF expression gradually returned to baseline level at two weeks post cessation of nicotine (**Supplemental Figure 3)**.

3.6 Chronic nicotine exposure attenuates microglia-mediated chronic neuroinflammation in CCI mice

Nicotine has been shown to regulate microglia activation and suppress inflammation in the CNS via interactions with α7 acetylcholine receptors (Noda & Kobayashi, 2017; Shytle et al., 2004; Zhang et al., 2017); thus, we performed Iba-1 immunolabeling to determine the effects of chronic nicotine exposure on CCI-induced chronic neuroinflammation. As shown in **Figure 7A-B**, while microglia in the Sham-Veh group maintained a ramified

morphology, reactive microglia with a distinct amoeboid shape were found in all three CCI groups, confirming persistent microglia-mediated neuroinflammation at 2 months post CCI. Quantification of the Iba1-positive cells in the perilesion cortex (**Figure 7C**) showed that the CCI-Veh group presented significantly increased microglia count compared to that of the Sham-Veh group while perilesion cortex of nicotine-exposed mice showed significantly decreased microglia count compared to CCI-Veh group. Additionally, a similar decreasing trend ($p=0.067$) in the microglial count was observed in the CCI-Nic Withdrawal group, suggesting chronic nicotine exposure via E-cig alleviated microglia-mediated neuroinflammation following CCI.

4. DISCUSSION

The current study utilized a clinically relevant full-body E-cig vapor exposure system to examine the effects of chronic nicotine exposure on traumatic brain injury. Here we report that chronic nicotine exposure via E-cig inhalation significantly improved long-term sensorimotor functional recovery in CCI mice, increased cortical expression of mBDNF, and upregulated BDNF/TrkB-associated pro-survival signaling pathways, including p-Erk and p-Akt. Importantly, increased BDNF and p-Akt signaling was observed predominantly in neurons. Furthermore, chronic nicotine exposure alleviated microglia-mediated chronic neuroinflammation following CCI. While these results support the neuroprotective effects of post-injury chronic nicotine exposure, nicotine withdrawal was shown to significantly impede social approach behavior in the CCI mice.

Individuals with severe TBI suffer from long-lasting sensorimotor deficits due to significant neuronal loss, diffuse axonal injuries, and chronic neuroinflammation. While studies using moderate and diffuse TBI rodent models showed that nesting behavior could recover to baseline level at 7 days post CCI (Muccigrosso et al., 2016), severe TBI models presented persistent nesting deficits that last for months (Ritzel et al., 2020). In this current study, both CCI-Nic and CCI-Nic Withdrawal group showed significantly enhanced function recovery, suggesting that the functional improvement is not dependent on nicotine's transient modulating effects on DA-mediated motivation change but a true improvement in long-term functional recovery after CCI. No significant difference was observed in the lesion size among the three CCI groups (**Supplemental Figure 4),** a result expected as nicotine exposure did not begin until 7 days post-injury. Together, these results suggest that the observed beneficial effect of nicotine in nesting function is possibly achieved by alleviating secondary injuries such as neurodegeneration and/or chronic neuroinflammation during the chronic phase of TBI rather than by protecting against apoptosis or BBB leakage, which peaked during the first few days after TBI (acute phase). Considering that an upward trend in nesting performance could be detected as early as 3 weeks post-exposure, future studies that examine whether a shorter nicotine treatment could achieve similar functional recovery while avoiding nicotine dependence and associated symptoms may provide valuable information for the development of nicotine-based therapy for TBI patients. Additionally, previous animal studies suggested that nicotine treatment immediately post-injury via mini-pumps could provide potential benefits for TBI-induced deficits by compensating impaired cholinergic, serotonergic, and dopaminergic signaling (Rao et al., 2022; Verbois, Hopkins, et al., 2003; Verbois, Scheff, et al., 2003), it would be interesting to investigate whether E-Cig exposure immediately after injury could also afford greater neuroprotective effects and further accelerate the timeline of functional recovery.

Behavioral characterization of the TBI mice in the OF test demonstrated hyperlocomotion and decreased thigmotaxis in mice from the CCI-Nic group compared to CCI-Veh and CCI-Nic withdrawal groups, while no significant change in all three test parameters was observed in the three Sham groups. We suspect that the greater anxiolytic effect detected in CCI-Nic mice is likely due to disruptions of the BBB. Importantly, no changes in locomotion and thigmotactic behavior were observed in Sham/CCI mice in the nicotine withdrawal group, indicating that the effects of nicotine on psychomotor behavior are likely short-lived. Together, these results could possibly explain the prevalent use of nicotine-containing products among TBI patients as a form of self-medication to cope with negative affective states caused by brain injuries (Brown, 2010; Silva et al., 2018).

BDNF, an important neurotrophin that binds to TrkB receptors, is well-established for its role in enhancing neuronal survival and synaptic plasticity in the injured CNS (Gustafsson et al., 2021; Schabitz et al., 2007; Wurzelmann et al., 2017). Previous studies on the relationship between nicotine and BDNF yielded varied results from different groups. While some studies claimed that chronic nicotine treatment could increase BDNF

mRNA or protein expression in the adult rodent brain (Kenny et al., 2000; Naha et al., 2018; Romano et al., 2014), others reported no change or decreased BDNF mRNA/protein expression after chronic nicotine treatment (Aleisa et al., 2006; Monteggia et al., 1994; Ortega et al., 2013). The current study is the first to demonstrate that, chronic nicotine exposure via E-cig vaping increased cortical mBDNF expression in both healthy (Sham) and injured (CCI) mice. Furthermore, a parallel increase in Erk and Akt activation was observed in the cortex from nicotine-treated Sham and CCI mice, suggesting that, even in the setting of TBI, which has been shown to induce significant deficits in Erk activation (Atkins et al., 2009), chronic nicotine exposure still induced strong activation of Erk and Akt, two crucial protein kinases responsible for promoting neuronal survival (Noshita et al., 2002). Importantly, immunostaining confirmed the upregulated survival signals p-Akt in the perilesion area mostly localized within the neuronal population, which could explain the greater functional recovery observed in nicotine-exposed mice.

Microglia activation is another important pathological hallmark of TBI. Mounting evidence has shown that microglia activation can persist beyond months to years in the rodent TBI model, contributing to accelerated neurodegeneration and encephalopathy (Loane et al., 2014; Simon et al., 2017). The cholinergic anti-inflammatory pathway, which depends on the activation of α7 nAchRs on immune cells, plays an important role in regulating systemic and CNS inflammation (Mizrachi et al., 2021; Patel et al., 2017; Shytle et al., 2004). The present study demonstrates that post-injury chronic nicotine exposure could attenuate neuroinflammation by suppressing microgliosis in the perilesional cortex of CCI mice which may in part contribute to the preserved neuronal population and enhanced sensorimotor function.

Although nicotine treatment affords beneficial effects on sensorimotor recovery after TBI, the current study also demonstrated significant inhibition of SA behavior in CCI mice as a result of nicotine withdrawal. Such inhibition may reflect anhedonia and/or a disrupted mental state, symptoms that are closely associated with nicotine withdrawal (Mayer et al., 2001). The unaltered social behavior observed after CCI injury in CCI-Veh group is likely because the CCI model used in the current setting does not directly damage the medial prefrontal cortex and amygdala, two brain regions that are primarily responsible for modulating social behavior (Adolphs, 2009; Franklin et al., 2017). Interestingly, the absence of nicotine's inhibitory effect in the CCI-Nic group may indicate that, in the injured CNS, nicotine-mediated activation of native cholinergic signaling is disrupted due to extensive damage to the cortex and the hippocampus, both of which receive dense cholinergic projections (Schmidt & Grady, 1995; Shin & Dixon, 2015). Together, these findings suggest that while nicotine provides neuroprotective effect in the treatment of TBI, withdrawal from chronic nicotine use could induce adversely affect social behavior in TBI patients.

One limitation of this study is that we exclusively investigated the biochemistry change in perilesion cortical tissue, but the nicotine exposure model utilized in our current study affects the whole CNS, particularly structures that rely on DA signaling such as the basal ganglia circuitry (Shin et al., 2012). Further investigations on the effects of chronic nicotine exposure on the dopaminergic pathway in the injured brain may provide additional insights into the therapeutic potential of nicotine. Another limitation of the current study is that, despite containing fewer toxic chemicals than traditional cigarettes, the heavy metals present in E-cig aerosol have been shown to compromise the integrity of the BBB and promote neuroinflammation (Heldt et al., 2020). Thus, exposure to vehicle vapor may induce additional pathological changes in CCI mice. An air exposure group should be included in future studies to assess the effect of post-injury E-cig constituent exposure on TBI.

In conclusion, the present study showed that post-injury chronic nicotine exposure via E-cig facilitated long-term functional recovery from CCI-induced sensorimotor deficits by upregulating neuroprotective BDNF-TrkB signaling and alleviated microglia-mediated neuroinflammation. However, nicotine withdrawal induced abnormal social behaviors in CCI mice. Further investigation into specific treatment regimens (delivery route, dose, and duration) of nicotine and selective α7 nAchRs agonist in the setting of TBI could provide valuable information for potential therapeutic target for traumatic brain injury and other forms of neurodegeneration.

Abbreviations

AD: Alzheimer's Disease; AP: Anterior-posterior; ML: Medial-lateral; BBB: Blood-Brain-Barrier; mBDNF: mature Brain-derived neurotrophic factor; CCI: Controlled Cortical Impact; CCI-Nic: CCI surgery group exposed to nicotine vapor; CCI-Veh: CCI surgery group exposed to vehicle vapor; CDC: Centers for Disease Control and Prevention; *DA*: Dopamine; E-cig: E-cigarette; EB: Evans Blue Dye; Em: Emission; Ex: Excitation; Iba-1: Ionized calcium-binding adaptor molecule 1; IACUC: Institutional Animal Care and Use Committee; IVIS: In Vivo Imaging System; MS: Multiple Sclerosis; NeuN: Neuronal nuclear protein; nAchRs: Nicotinic acetylcholine receptors; OF: Open field test; PFA: Paraformaldehyde; PVC: Polyvinyl Chloride; S1: The primary somatosensory cortex; SA: Social approach; Sham-Nic: Sham-surgerized mice exposed to nicotine vapor; Sham-Veh: Sham-surgerized mice exposed to vehicle vapor; TBS-T: Tris-*buffered* saline with added Triton; TBI: Traumatic brain injury; TrkB: Tropomyosin-related kinase receptor type B; p-TrkB: Phosphorylated tropomyosin-related kinase receptor type B.

Author contribution

Conceptualization: Brian Head, Shanshan Wang. Methodology for E-cig exposure and SA: Ellen Breen, Susan Powell. Behavior test: Natalia Kleschevnikova, Tiffany Duong. Immunoblot and Immunofluorescence: Dongsheng Wang, Hongxia Wang, Xiaojing Li, Wenxi Li. Formal analysis: Shanshan Wang, Dongsheng Wang, Susan Powell. Original writing: Dongsheng Wang, Shanshan Wang. Review and editing: Shanshan Wang, Susan Powell, Brian Head and Hemal Patel. All authors have read and approved the final version of the manuscript.

Declarations

H.H.P. and B.P.H. hold equity and are non-paid consultants with Eikonoklastes Therapeutics LLC.

Ethics approval

All procedures are approved by IACUC (#20-021)

Consent for publication

Not applicable

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