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Evaluation of a Combined Therapeutic Regimen of 8-OH-DPAT and Environmental Enrichment after Experimental Traumatic Brain Injury

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Abstract

When provided individually, both the serotonin (5-HT_{1A})-receptor agonist 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) and environmental enrichment (EE) enhance behavioral outcome and reduce histopathology after experimental traumatic brain injury (TBI). The aim of this study was to determine whether combining these therapies would yield greater benefit than either used alone. Anesthetized adult male rats received a cortical impact or sham injury and then were randomly assigned to enriched or standard (STD) housing, where either 8-OH-DPAT (0.1 mg/kg) or vehicle (1.0 mL/kg) was administered intraperitoneally once daily for 3 weeks. Motor and cognitive assessments were conducted on post-injury days 1–5 and 14–19, respectively. CA1/CA3 neurons and choline acetyltransferase-positive ($ChAT^+$) medial septal cells were quantified at 3 weeks. 8-OH-DPAT and EE attenuated CA3 and $ChAT^+$ cell loss. Both therapies also enhanced motor recovery, acquisition of spatial learning, and memory retention, as verified by reduced times to traverse the beam and to locate an escape platform in the water maze, and a greater percentage of time spent searching in the target quadrant during a probe trial in the TBI + STD + 8-OH-DPAT, TBI + EE + 8-OH-DPAT, and TBI + EE + vehicle groups versus the TBI + STD + vehicle group ($p \leq 0.0016$). No statistical distinctions were revealed between the TBI + EE + 8-OH-DPAT and TBI + EE + vehicle groups in functional outcome or CA1/CA3 cell survival, but there were significantly more $ChAT^+$ cells in the former ($p = 0.003$). These data suggest that a combined therapeutic regimen of 8-OH-DPAT and EE reduces TBI-induced $ChAT^+$ cell loss, but does not enhance hippocampal cell survival or neurobehavioral performance beyond that of either treatment alone. The findings underscore the complexity of combinational therapies and of elucidating potential targets for TBI.

Key words: beam-walking; behavior; controlled cortical impact; ChAT; 5-HT_{1A} receptor agonist; functional recovery; hippocampus; learning and memory; Morris water maze; traumatic brain injury

Introduction

Often a result of motor vehicle accidents and falls (Faul et al., 2010; Summers et al., 2009), traumatic brain injury (TBI) affects approximately 1.4–2 million individuals in the United States annually (Faul et al., 2010; Goldstein, 1990; Selassie et al., 2008). Of these, 52,000 die (Faul et al., 2010; Sosin et al., 1995), and an additional 90,000 endure long-term neurobehavioral and cognitive deficits (Thurman et al., 1999). Prolonged disturbances resulting from brain trauma, most commonly memory impairment (Horneman and Emanuelson, 2009), can have permanent adverse consequences on the quality of life (Binder, 1996; Millis et al., 2001). While the emotional toll of TBI on an afflicted individual's interpersonal relationships with family, friends, and coworkers is immeasurable, the economic cost to society, which is based on long-term health care costs and loss of productivity due to the inability to return to the work force, accounts for billions of dollars each year (Max et al., 1991; Selassie et al., 2008). Consequently, if individuals sustaining TBI are to return to, or near, premorbid conditions, the development of treatment strategies capable of producing neurobehavioral and cognitive recovery after TBI is crucial.

To this end, numerous pre-clinical treatment approaches including pharmacotherapy and hypothermia, and non-invasive methods such as exercise and environmental

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enrichment, have been utilized (Bales et al., 2009; Bramlett et al., 1995; Clark et al., 1996; Clifton et al., 1991; Kline et al., 2002c, 2004b; Griesbach et al., 2008, 2009; Parton et al., 2005; Sozda et al., 2010; Will et al., 2004). While many of these strategies have produced significant motor and/or cognitive enhancement in the laboratory, the benefits have not consistently extended to the clinical setting (Doppenberg et al., 2004; Menon, 2009). The modest translational success may be due in part to inherent differences between controlled experimental manipulations and unpredictable clinical factors, such as consistent and replicable injury in animals versus variable types of trauma in humans. These disparities may affect secondary injuries and the subsequent pathophysiology differently (Greve and Zink, 2009), thus necessitating the use of combined or adjunctive therapies to additively or synergistically attenuate the deleterious effects of TBI. While not copiously studied, combinational treatment approaches after TBI have been shown to provide some benefits (Barbre and Hoane, 2006; Lyeth et al., 1993; Menkü et al., 2003; Yan et al., 2000) and thus these select but compelling studies support further evaluation of this potentially efficacious treatment paradigm for TBI.

The aim of the current study was to evaluate the potential additive benefit of two therapies that have shown significant promise in the laboratory when used independently. The first therapeutic approach is systemic administration of the 5-HT_{1A}-receptor agonist 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT). Studies from our laboratory have shown that both acute and chronic administration of 8-OH-DPAT attenuates histopathology (i.e., decreases cortical lesion volume and confers hippocampal neuron survival), and improves neurobehavioral performance after controlled cortical impact (CCI) injury (Cheng et al., 2007, 2008; Kline et al., 2002b, 2004a, 2007). The second therapy in our combination paradigm is environmental enrichment (EE). EE consists of exposing rats to exploratory, sensory, and social housing, where they interact extensively with the environment and therefore may be considered a rodent correlate of physiotherapeutic intervention (Kline et al., 2007; Sozda et al., 2010). Using the same CCI injury paradigm as in the 8-OH-DPAT studies, EE has also been shown to improve motor function and enhance the acquisition of spatial learning and memory retention (Hoffman et al., 2008b; Kline et al., 2007; Sozda et al., 2010). Furthermore, significant EE-mediated cognitive improvement and histological protection has also been observed after TBI produced by fluid percussion (Hamm et al., 1996; Hicks et al., 2002; Passineau et al., 2001). Given that both 8-OH-DPAT and EE confer similar behavioral benefits after TBI and affect corresponding targets, one of which is the cholinergic neurotransmitter system (Barnes and Sharp, 1999; Fujii et al., 1997; Krech et al., 1960; Lazaris et al., 2003; Paban et al., 2005; Park et al., 1992; Segovia et al., 2009). which is involved in learning and memory (Gibbs, 2010; Gold, 2003; Hasselmo, 2006; Sarter et al., 2003), we hypothesized that the combination of therapies would be more efficacious than either alone in attenuating brain trauma–induced choline acetyltransferase-positive ($ChAT^+$) cell loss and promoting behavioral recovery.

Methods

Subjects

Sixty-five adult male Sprague-Dawley rats (Harlan Co., Indianapolis, IN) weighing 300–325 g on the day of surgery were housed in standard steel-wire mesh cages and maintained in a temperature- ($21 \pm 1^{\circ}C$) and light-controlled (on 7:00 AM to 7:00 PM) environment with food and water available *ad libitum*. After 1 week of acclimatization, all but 5 rats (naïves) underwent a single day of beam-walk training, which consisted of 3–5 trials to traverse the beam. Following training the rats were randomly assigned to one of the following group conditions: TBI + EE + 8-OH-DPAT (0.1 mg/kg; $n = 10$), TBI + EE + vehicle (1.0 mL/kg; $n = 10$), TBI + STD + 8-OH-DPAT ($n = 10$), TBI + STD + vehicle ($n = 10$), sham + EE + 8-OH-DPAT ($n = 5$), sham + EE + vehicle ($n = 5$), sham + STD + 8-OH-DPAT ($n = 5$), or sham + STD + vehicle ($n = 5$). Additionally, as mentioned above, a group of 5 naïve rats was included as controls for ChAT immunocytochemistry. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh, and were conducted in accordance with the recommendations provided in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996). Every attempt was made to limit the number of rats used and to minimize suffering.

Surgery

A CCI injury was produced as previously described (Cheng et al., 2008; Dixon and Kline, 2009; Dixon et al., 1991; Hoffman et al., 2008a; Kline et al., 2001). Briefly, surgical anesthesia was induced and maintained with inspired concentrations of 4% and 2% isoflurane, respectively, in 2:1 N_2O:O_2. After endotracheal intubation the rats were secured in a stereotaxic frame, ventilated mechanically, and temperature was maintained at $37 \pm 0.5^{\circ}C$ with a heating blanket. Utilizing aseptic procedures a midline scalp incision was made, the skin and fascia were reflected to expose the skull, and a craniectomy (6 mm in diameter) was made in the right hemisphere (encompassing the bregma and the lambda and between the sagittal suture and the coronal ridge) with a hand-held trephine. The bone flap was removed and the craniectomy was enlarged further with cranial rongeurs. Subsequently the impacting rod was extended and the impact tip (6 mm, flat) was centered and lowered through the craniectomy until it touched the dura mater, then the rod was retracted and the impact tip was advanced 2.8 mm farther, to produce a brain injury of moderate severity (2.8 mm tissue deformation at 4 m/sec). Immediately after the CCI, anesthesia was discontinued and the incision was promptly sutured. The rats were subsequently extubated and assessed for acute neurological outcome. Sham rats underwent similar surgical procedures, but were not subjected to the impact.

Acute neurological evaluation

Hindlimb reflexive ability was assessed immediately following the cessation of anesthesia by gently squeezing the rat's paw every 5 sec and recording the time to elicit a withdrawal response. Return of the righting reflex was determined by the time required to turn from the supine to the prone position.

Drug administration

8-OH-DPAT was purchased from Sigma-Aldrich (Sigma-Aldrich, Inc., St. Louis, MO), and was prepared daily by

dissolving it in sterile saline, which also served as the vehicle. 8-OH-DPAT (0.1 mg/kg) or a comparable volume of vehicle (1.0 mL/kg) was administered intraperitoneally beginning 24 h after cortical impact or sham injury, and once daily for 3 weeks. The dose of 8-OH-DPAT and route of administration were selected based on previous data from our laboratory showing this regimen to be optimal after chronic treatment (Cheng et al., 2008).

Housing conditions: Environmental manipulation

Following surgery, and after the effects of anesthesia abated (as evidenced by free movement in the holding cage), the rats were returned to the colony where those designated for enrichment were immediately placed in specifically designed 36×30×20-inch steel-wire cages. The EE cages consisted of three levels with ladders to ambulate from one level to another, and contained various toys (e.g., balls, blocks, and tubes), nesting materials (e.g., paper towels), and *ad libitum* food and water (Kline et al., 2007; Sozda et al., 2010). To maintain novelty, the objects were rearranged every day and changed each time the cage was cleaned, which was twice per week. Ten to 12 rats, which included 8-OH-DPAT-treated and vehicle-treated TBI animals and sham controls, were housed in the EE at any given time. Rats in the STD conditions were placed in standard steel-wire mesh cages (2 rats per cage) with only food and water.

Motor performance

Established beam-balance and beam-walk tasks were used to assess motor function. The beam-balance task consists of placing the rat on an elevated beam (1.5 cm wide) and recording the time it remains on up to a maximum of 60 sec. The beam-walk task, modified from that originally devised by Feeney and colleagues (1982), consists of training/assessing rats using a negative-reinforcement paradigm to escape ambient light and white noise by traversing an elevated narrow beam (2.5×100 cm), and entering a darkened goal box situated at the opposite end. When the rat enters the goal box the adverse stimuli (light and noise) are terminated, thus serving as reinforcement (reward) for completing the task. Performance was assessed by recording both the elapsed time to traverse the beam as well as the distance traveled. The scoring criteria for distance traveled is based on a rating scale from 0–5, where 0 indicates an inability to ambulate beyond the start location, 1–4 corresponds to distal segments of 20, 40, 60, or 80 cm from the start point, and 5 corresponds to traversing the entire length of the beam (100 cm) and entering the goal box (Cheng et al., 2008). The rats were tested for beam-balance and beam-walk performance prior to surgery to establish a baseline measure, and again on post-operative days 1–5. Testing consisted of three trials (60 sec allotted time with an inter-trial interval of 30 sec) per day for each task. If the rat was unable to traverse the entire length of the beam, the maximum allowed time of 60 sec was recorded. The average daily scores for each subject were used in the statistical analyses.

Cognitive function: Acquisition of spatial learning

Spatial learning was assessed by the Morris water maze task (Morris, 1984), which has been shown to be sensitive to cognitive function after TBI (Hamm et al., 1992; Kline et al., 2002a; Scheff et al., 1997). Briefly, the maze consisted of a plastic pool (180 cm diameter and 60 cm high) filled with tap water ($26 \pm 1^{\circ}$C) to a depth of 28 cm that was situated in a room with salient visual cues that remained constant throughout the study. The platform was a clear acrylic glass stand (10 cm diameter and 26 cm high) that was positioned 26 cm from the maze wall in the southwest quadrant and held constant for each rat. Spatial learning began on post-operative day 14, and consisted of providing a block of four daily trials (4-min inter-trial interval) for five consecutive days (days 14–18), to locate the platform when it was submerged 2 cm below the water's surface (i.e., invisible to the rat). On day 19 the platform was raised 2 cm above the water's surface (i.e., visible to the rat) as a control procedure to determine the contributions of non-spatial factors (e.g., sensorimotor performance, motivation, and visual acuity) to cognitive performance. For each daily block of trials the rat was placed in the pool facing the wall at each of the four possible start locations (north, east, south, and west) in a randomized manner. Each trial lasted until the rat climbed onto the platform or until 120 sec had elapsed, whichever occurred first. Rats that failed to locate the goal within the allotted time were manually guided to it. All rats remained on the platform for 30 sec before being placed in a heated incubator between trials. The times of the four daily trials for each rat were averaged and used in the statistical analyses.

Cognitive function: Probe trial (memory retention)

One day after the final acquisition training session (i.e., day 19), all rats were given a single probe trial to measure retention. Briefly, the platform was removed from the pool and the rats were placed in the maze from the location point most distal to the quadrant where the platform was previously situated (i.e., "target quadrant"), and allowed to freely explore the pool for 30 sec. Typically, rats that have learned the specific location of the escape platform exhibit a spatial bias and spend significantly more time in the target quadrant. The percentage of time spent in the target quadrant was used in the statistical analysis.

A spontaneous motor activity recording and tracking (SMART) system (San Diego Instruments, San Diego, CA) was used to record the data, which included time to locate the platform, time in the target quadrant, and swim speed.

Histology: Quantification of hippocampal neurons

Three weeks after CCI or sham injury, half of the rats from each group were anesthetized with pentobarbital (50 mg/kg IP), and then perfused transcardially with 200 mL heparinized 0.1 M phosphate-buffered saline (pH 7.4), followed by 300 mL 10% buffered formalin. The brains were extracted, post-fixed in 10% buffered formalin for 1 week, dehydrated with alcohol solutions, and embedded in paraffin. Coronal sections 7 μm thick were cut at 1-mm intervals through the lesion on a rotary microtome and mounted on gelatin-coated glass microscope slides. After drying at room temperature, the sections were deparaffinized in xylene, rehydrated, and stained with cresyl violet. An observer blinded to experimental conditions analyzed one coronal section underlying the area of contusion (3.5 mm posterior to the bregma) from the rats in each group for determination of treatment efficacy on selectively vulnerable hippocampal CA1 and CA3 neurons. To reduce

counting errors associated with false-positive identification of dying neurons, the total number of CA1 and CA3 morphologically-intact neurons (i.e., those with a clearly defined cell body and nucleus) were counted using a Nikon Eclipse E600 microscope (Nikon Corporation, Tokyo, Japan) with a 40×objective. For consistency and replication, the data are presented as the percentage of total neurons in the ipsilateral (injured) CA1 and CA3 regions relative to the contralateral (uninjured) hippocampus, as previously reported (Cheng et al., 2007; Dixon et al., 1999; Hoffman et al., 2008a; Kline et al., 2004a, 2004b; Sozda et al., 2010).

Immunocytochemistry: Choline acetyltransferase-positive medial septal cells

Three weeks after TBI or sham surgery, the 5 naïve rats and the remaining half from each surgical group ($n = 5$) were perfused transcardially under pentobarbital (50 mg/kg IP) anesthesia with 200 mL of isotonic saline, followed by 500 mL of 4.0% paraformaldehyde/0.2% picric acid in 0.1 M phosphate buffer. After perfusion, the rostral portion of the brain containing the medial septal area was post-fixed in the same solution overnight at 4°C, followed by immersion in 30% sucrose until sunken. The cryoprotected brain was coronally cut (35 μm thick), and sections were collected in culture plates containing 0.1 M PBS. Free-floating sections were incubated in a final ChAT antibody concentration (1:50) of 1.0 μg/mL in a 0.01 M PBS solution containing 0.1% Triton X-100. The sections were incubated in the primary antibody for 24 h at room temperature. After four washes in PBS (5 min each), the sections were incubated in the secondary antibody (horse anti-mouse IgG, 1:100; Vector Laboratories, Burlingame, CA) for 1 h at 37°C (Johnson et al., 2002). After three washes in PBS (5 min each), the sections were incubated with mouse avidin-biotin-peroxidase complex (ABC; Vector Laboratories) technique for 2 h at 37°C. After three 5-min washes in PBS and one 5-min wash in 0.1 M Tris-buffered saline (TBS), free-floating sections were processed using the glucose oxidase–diaminobenzidine–nickel method (Gibbs, 2000, 2002; Shu et al., 1988). The reaction was stopped by transferring the sections into TBS (two 5-min washes). The sections were subsequently mounted on gelatin-coated glass slides, dried at room temperature, dehydrated in ascending concentrations of ethanol and xylene, and then cover-slipped with mounting medium. $ChAT^+$ cells in the medial septal area were counted using a Nikon Eclipse E600 microscope with a 40×objective.

Data analyses

Statistical analyses were performed on data collected by observers blinded to treatment conditions using StatView 5.0.1 software (Abacus Concepts, Inc., Berkeley, CA). The motor and cognitive data were analyzed by repeated-measures analysis of variance (ANOVA). The acute neurological, probe trial, swim speed, histological, and immunocytochemical data were analyzed by one-factor ANOVAs. When the ANOVA showed a significant effect, the Bonferroni/Dunn *post-hoc* test was utilized to determine specific group differences. The results are expressed as the mean ± standard error of the mean (SEM), and were considered significant when p values were ≤ 0.05, or as determined by the Bonferroni/Dunn statistic after correcting for multiple comparisons.

Results

Because there were no significant differences in any assessment among the sham control groups, regardless of treatment (8-OH-DPAT or housing), the data were pooled into one group (denoted as Sham).

Acute neurological function

No significant differences were observed among the TBI groups in time to recover the hindlimb withdrawal reflex in response to a brief paw pinch (left range = 175.3 ± 6.4 sec to 187.7 ± 4.1 sec, $p > 0.05$; right range = 167.3 ± 5.5 sec to 178.1 ± 2.5 sec, $p > 0.05$), or for return of righting ability (range 390.0 ± 9.9 sec to 414.0 ± 8.8 sec, $p > 0.05$), following the cessation of anesthesia. The lack of significant differences in these acute neurological indices suggests that all groups experienced an equivalent level of injury and anesthesia.

Motor function: Beam-balance

No pre-surgical differences were observed among groups, as all rats were capable of balancing on the beam for the allotted 60 sec (Fig. 1). Following the cortical impact all rats were markedly impaired relative to the Sham group, which was able to maintain balance for the full 60 sec. The ANOVA revealed significant group ($F_{4,55} = 6.616$, $p = 0.0002$) and day ($F_{5,275} = 43.146$, $p < 0.0001$) differences, as well as a significant group×day interaction ($F_{20,275} = 4.332$, $p < 0.0001$). A repeated-measures ANOVA on the 5 days of testing did not reveal any significant differences among the TBI groups ($p < 0.05$), but individual day analyses showed that the TBI + STD + vehicle group was significantly impaired versus all other TBI groups on post-operative days 4 and 5 ($p < 0.005$; Fig. 1).

Motor function: Beam-walk (time to traverse)

No pre-surgical differences in time to traverse the beam were revealed among the groups, as all rats were proficient and reached the goal box in approximately 5 sec (Fig. 2). After TBI, the ANOVA revealed significant group ($F_{4,55} = 25.427$, $p < 0.0001$) and day ($F_{5,275} = 133.441$, $p < 0.0001$) differences, as well as a significant group×day interaction ($F_{20,275} = 12.193$, $p < 0.0001$). All TBI groups differed from the Sham controls ($p < 0.0001$). Additionally, the TBI + EE + 8-OH-DPAT, TBI + EE + vehicle, and TBI + STD + 8-OH-DPAT groups traversed the beam significantly quicker than the TBI + STD + vehicle group ($p < 0.0001$), but did not differ significantly from one another ($p > 0.05$).

Motor function: Beam-walk (distance traveled)

No pre-surgical differences were observed among groups, as all rats were capable of traversing the entire length of the beam for a score of 5 (Fig. 3). However, after TBI, the ANOVA revealed significant group ($F_{4,55} = 22.922$, $p < 0.0001$) and day ($F_{5,275} = 82.545$, $p < 0.0001$) differences, as well as a significant group×day interaction ($F_{20,275} = 8.819$, $p < 0.0001$). No differences were observed among the TBI + EE + 8-OH-DPAT, TBI + EE + vehicle, and TBI + STD + 8-OH-DPAT groups ($p > 0.05$), as they all returned to baseline performance by the end of the 5 days of testing. However, all three groups performed significantly better than the TBI + STD + vehicle group ($p < 0.005$), which was only capable of traversing about

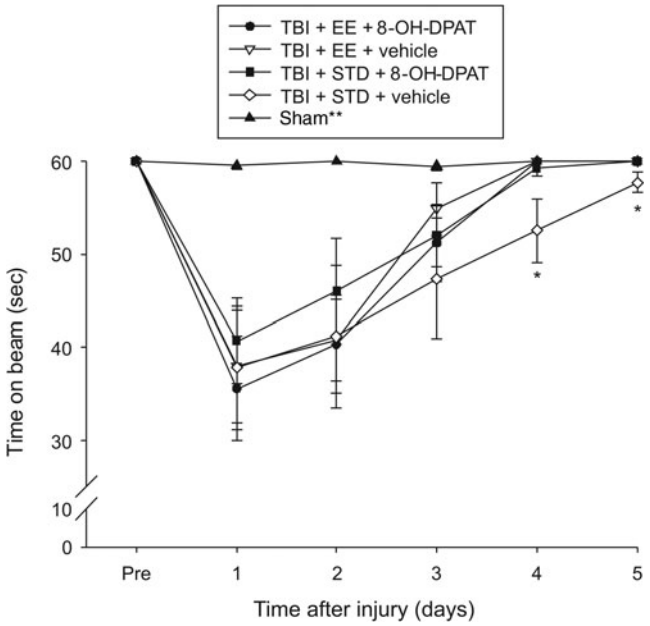

FIG. 1. Mean time (seconds) balancing on an elevated narrow beam prior to, and after, TBI or sham injury. All TBI groups were significantly impaired relative to the Sham group. While no overall difference was revealed among the TBI groups, individual day analyses showed that on post-injury days 4 and 5 the TBI + STD + vehicle group, which did not return to baseline, was significantly different from the TBI + EE + 8-OH-DPAT, TBI + EE +vehicle, and TBI + STD + 8-OH-DPAT groups (*$p < 0.005$).

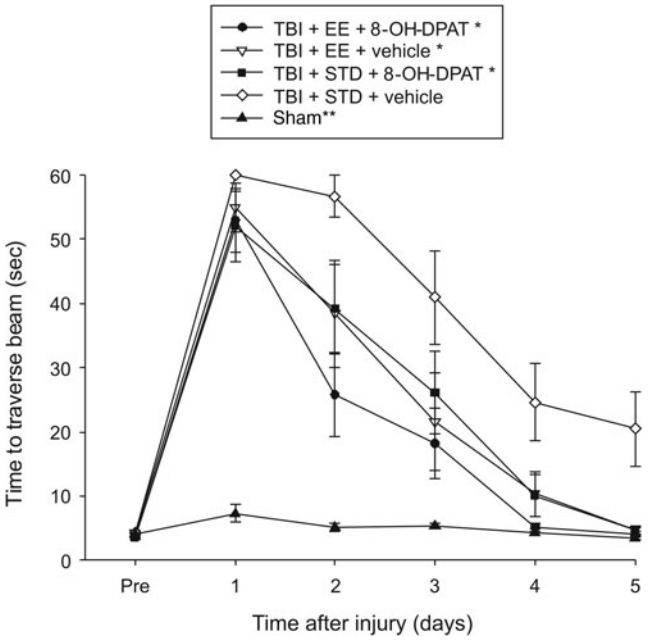

FIG. 2. Mean time (seconds) to traverse an elevated narrow beam prior to, and after, TBI or sham injury (*$p < 0.0008$ versus TBI + STD + vehicle; **$p < 0.0001$ versus all TBI groups).

60–80 cm of the beam for scores of 3–4 before the allotted time expired. The Sham group was significantly better than all TBI groups ($p < 0.0001$).

Cognitive function: Acquisition of spatial learning

Analysis of the water maze data revealed significant group ($F_{4,55} = 15.247$, $p < 0.0001$) and day ($F_{4,220} = 40.935$, $p < 0.0001$) differences. *Post-hoc* analyses indicated that the TBI + EE + 8-OH-DPAT, TBI + EE + vehicle, and TBI + STD + 8-OH-DPAT groups were able to locate the escape platform significantly quicker over time than the TBI + STD + vehicle group ($p < 0.0001$; Fig. 4). Moreover, the TBI + EE + 8-OH-DPAT group performed better than the TBI + STD + 8-OH-DPAT and TBI + STD + vehicle groups ($p = 0.0016$ and $p < 0.0001$, respectively), but it was not statistically significantly different from the TBI + EE + vehicle group ($p = 0.03$, but $p = 0.005$ is required by the Bonferroni/Dunn statistic after adjusting for multiple comparisons). The Sham group was significantly better than all TBI groups, except the TBI + EE + 8-OH-DPAT group ($p < 0.0001$). No significant differences in swim speed (range = 28.6 ± 0.8 cm/sec to 30.2 ± 1.2 cm/sec), or visible platform acquisition (Fig. 4), were observed among the groups, suggesting that neither motor impairments nor visual disparities influenced the assessment of place learning.

Cognitive function: Probe trial

Analysis of the probe data revealed significant memory retention as evidenced by a greater percentage of the 30-sec allotted time spent in the target quadrant for the TBI + EE + 8-OH-DPAT ($36.8 \pm 1.7\%$), TBI + EE + vehicle ($36.6 \pm 1.6\%$), and TBI + STD + 8-OH-DPAT ($33.6 \pm 2.1\%$) groups versus the TBI + STD + vehicle group ($25.6 \pm 1.4\%$; $p \leq 0.0009$). The Sham group spent $40.9 \pm 2.0\%$ of its time in the target quadrant,

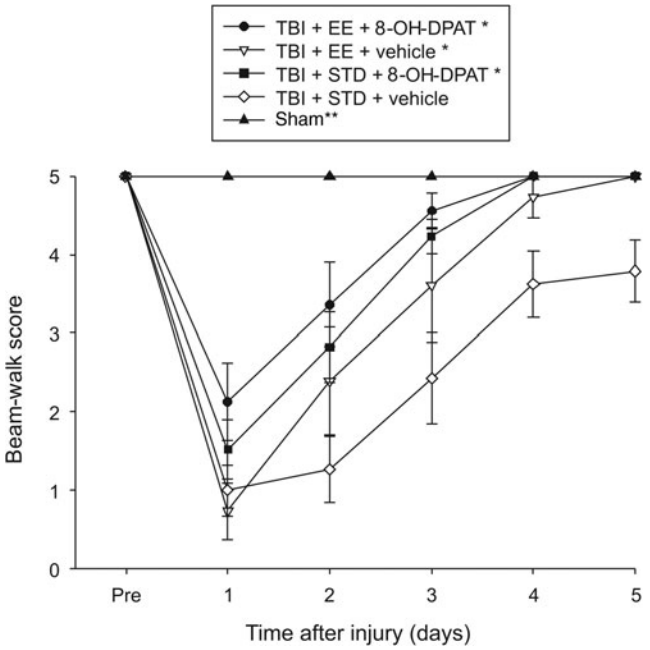

FIG. 3. Mean distance traveled along an elevated narrow beam prior to, and after, TBI or sham injury (*$p < 0.005$ versus TBI + STD + vehicle animals; **$p < 0.0003$ versus all TBI groups).

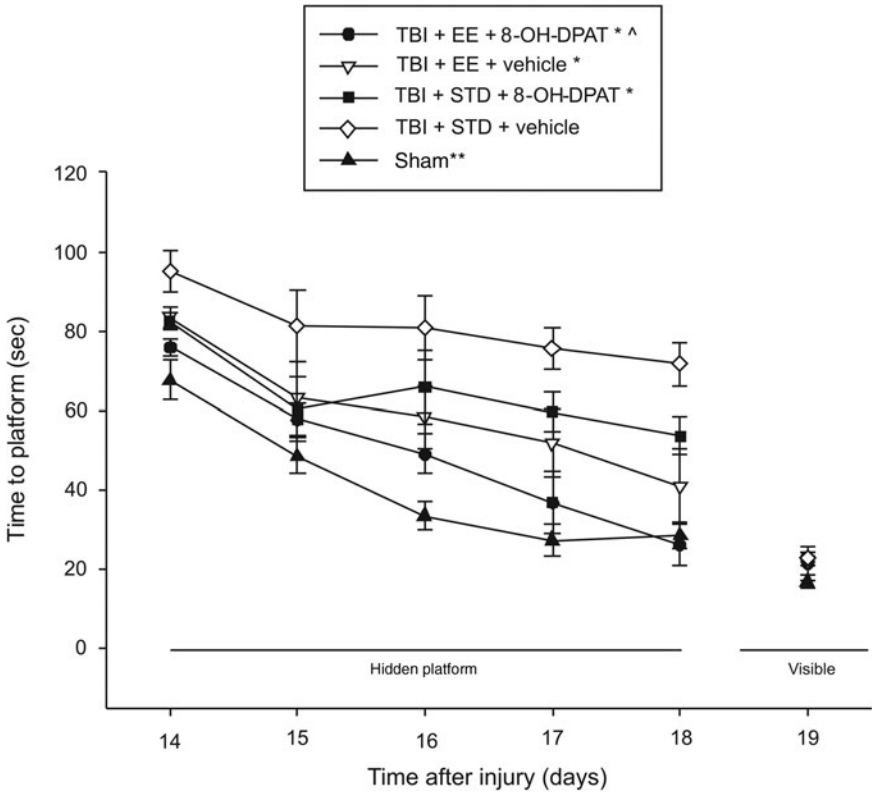

FIG. 4. Mean time (seconds) to locate either a hidden (submerged) or visible (raised) platform in a water maze (*$p < 0.0001$ versus TBI + STD + vehicle animals; **$p < 0.0001$ versus all TBI groups, except the TBI + EE + 8-OH-DPAT group [^$p < 0.0001$ and ^$p = 0.0016$ versus the TBI + STD + vehicle and TBI + STD + 8-OH-DPAT groups, respectively, but was not statistically different from the TBI + EE + vehicle group]). No significant differences in time to locate the visible platform were revealed among groups.

which was significantly better than the TBI + STD + vehicle group (Fig. 5).

Histology: Quantification of hippocampal neurons

A significant reduction in normal-appearing (i.e., morphologically intact) CA1 and CA3 neurons was observed in the hippocampus ipsilateral to the impact. Regarding the CA1 region, all TBI groups differed from the Sham control group. As depicted in Figure 6A, the percentage of normal-appearing neurons in the Sham group was $100.6 \pm 1.0\%$, while in the TBI + EE + 8-OH-DPAT, TBI + EE + vehicle, TBI + STD + 8-OH-DPAT, and TBI + STD + vehicle groups, the percentages exhibited were $53.1 \pm 3.4\%$, $46.5 \pm 3.2\%$, $48.4 \pm 5.1\%$, and $37.9 \pm 5.6\%$, respectively. While the TBI + EE + 8-OH-DPAT group exhibited a visibly greater percentage of cells than the TBI + STD + vehicle group, the *post-hoc* statistic did not reveal significance, as a corrected p value of 0.005 was required, and only a p of 0.015 was attained. Similarly to the CA1 data, all TBI groups differed from the Sham control group in the CA3 region as well. Specifically, the mean percentages of morphologically-intact neurons in the Sham group was $99.9 \pm 0.8\%$, while in the TBI + EE + 8-OH-DPAT, TBI + EE + vehicle, TBI + STD + 8-OH-DPAT, and TBI + STD + vehicle groups the percentages exhibited were $65.3 \pm 4.1\%$, $61.1 \pm 5.4\%$, $60.2 \pm 3.9\%$, and $40.3 \pm 4.4\%$ cells, respectively. Moreover, as depicted in Figure 6B, the TBI + EE + 8-OH-DPAT, TBI + EE + vehicle, and TBI + STD + 8-OH-DPAT groups revealed a greater percentage of intact cells than the TBI + STD + vehicle group ($p = 0.0003$, 0.0016, and 0.0023, respectively). The combined treatment group TBI + EE + 8-OH-DPAT did not differ from the TBI + EE + vehicle or TBI + STD + 8-OH-DPAT groups ($p = 0.473$ and $p = 0.376$, respectively).

Immunocytochemistry: ChAT$^+$ medial septal cells

As depicted in Figure 7A, E, and F, TBI induced a significant loss of ChAT$^+$ cells in the medial septum as demonstrated by substantially reduced numbers of cells in the TBI + STD + vehicle group (63.7 ± 9.2) versus naïve controls (130.0 ± 6.7). Moreover, the expression of ChAT$^+$ cells in the TBI + EE + 8-OH-DPAT group (120.0 ± 11.8) did not differ from that of the naïve animals ($p > 0.05$), but did significantly surpass the expression in the TBI + EE + vehicle (97.6 ± 5.5; $p = 0.003$), TBI + STD + 8-OH-DPAT (91.0 ± 5.8), and TBI + STD + vehicle groups ($p < 0.005$). No significant difference in ChAT$^+$ expression was revealed between the TBI + EE + vehicle and TBI + STD + 8-OH-DPAT groups ($p > 0.05$), although both did exhibit significantly more cells than the TBI + STD + vehicle group ($p < 0.005$).

Discussion

The aim of the current study was to determine whether combining two distinct therapies that have been shown to promote motor, cognitive, and histological improvement

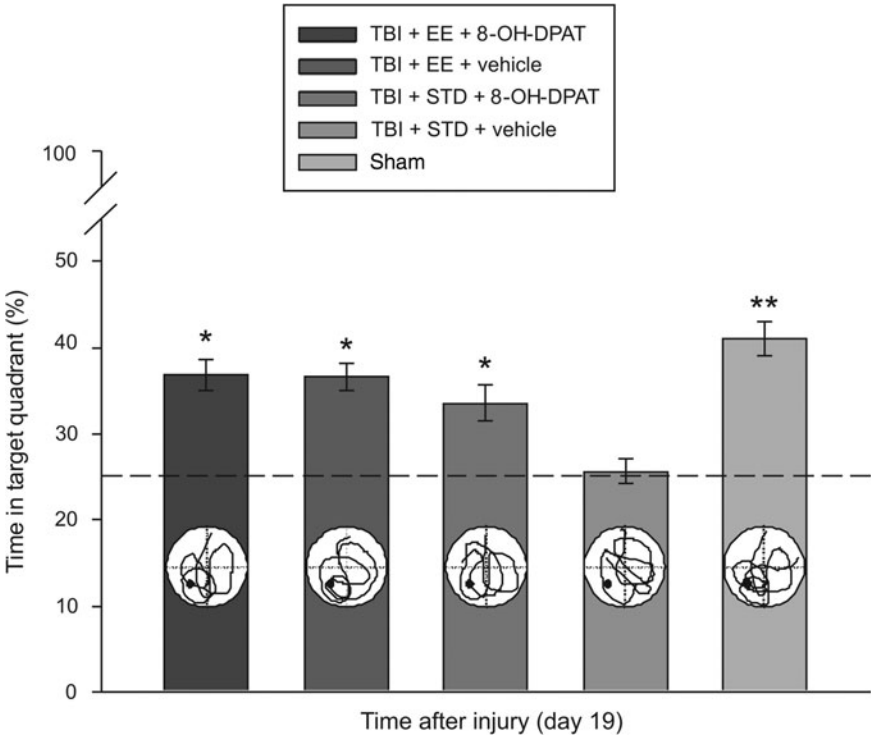

FIG. 5. Mean percentage of time spent in the target quadrant (i.e., where the platform was previously located) following a single probe trial 19 days after cortical impact or sham injury (*$p \leq 0.0009$ versus the TBI + STD + vehicle group; **$p < 0.0001$ versus the TBI + STD + vehicle group). There were no statistically significant differences among the TBI + EE + 8-OH-DPAT, TBI + EE + vehicle, TBI + STD + 8-OH-DPAT, and Sham groups. The dashed line represents performance at the chance level (25%), and the diagrams of the mazes inserted in the data bars depict the representative swim paths showing the percentage of time in each quadrant.

when provided singly after TBI would confer additional benefits when combined. Consequently, 8-OH-DPAT and EE were evaluated alone and together after CCI injury. The data revealed that chronic administration of 8-OH-DPAT facilitated beam-walking, acquisition of spatial learning, and memory retention, relative to the vehicle-treated controls when both groups were housed in STD conditions, which is consistent with data from a recent study (Cheng et al., 2008). 8-OH-DPAT also lessened TBI-induced hippocampal CA3 and $ChAT^+$ cell loss, which are new findings in this TBI paradigm. The data further showed that EE also improved motor and cognitive performance, a finding in agreement with previous reports from our laboratory (Hoffman et al., 2008b; Kline et al., 2007), and those of others (Gentile et al., 1987; Hamm et al., 1996; Held et al., 1985; Hicks et al., 2002; Passineau et al., 2001; Rose et al., 1987). EE also reduced CA3 and $ChAT^+$ cell loss relative to the STD vehicle-treated controls. Moreover, the combination paradigm of 8-OH-DPAT and EE also demonstrated improved motor, cognitive, and histological outcomes versus STD vehicle controls, but the effects were not significantly different than those seen with either treatment alone. However, despite the lack of an additive effect on behavioral improvement, the combined therapy paradigm was still effective, which is unlike other dual approaches, for which the beneficial effects of the individual treatments were lost (Faden, 1993; Griesbach et al., 2008; Guluma et al., 1999; Kline et al., 2002a). In contrast to the behavioral data, the combinational approach did confer greater protection against TBI-induced $ChAT^+$ cell loss than the individual therapies, which is a novel finding. Overall, our hypothesis that the combination of 8-OH-DPAT and EE would be more efficacious than either therapy alone in attenuating TBI-induced $ChAT^+$ cell loss and promoting enhanced cognitive performance was only partially supported.

Several lines of evidence, including the current study, indicate that TBI produces hippocampal and medial septal cell loss, as well as disturbances in cholinergic neurotransmission (Dixon et al., 1999; Schmidt et al., 2000). A time-dependent loss of ChAT enzymatic activity, the enzyme responsible for acetylcholine synthesis, and ChAT immunohistochemical staining have been reported after TBI (Donat et al., 2008; Gorman et al., 1996; Leonard et al., 1994; Schmidt and Grady, 1995; Sinson et al., 1997), and hypoxia (Row et al., 2007). Additionally, both 8-OH-DPAT and EE affect the cholinergic system (Barnes and Sharp, 1999; Paban et al., 2005; Torasdotter et al., 1998). Moreover, cholinergic projections to the hippocampus and cerebral cortex are well documented to play an important role in learning, memory, and attention (Baxter and Chiba, 1999; Everitt and Robbins, 1997; Gibbs, 2010), and there is strong evidence that impairments in basal forebrain cholinergic function contribute to the age-related cognitive decline, and to the behavioral and psychological symptoms of dementia (Lanari et al., 2006; Mufson et al., 2008; Smith et al., 1999; von Linstow and Platt, 1999). Cholinergic cell loss also contributes to the cognitive impairment associated with TBI (Dixon et al., 1996, 1997a, 1997b; Schmidt and

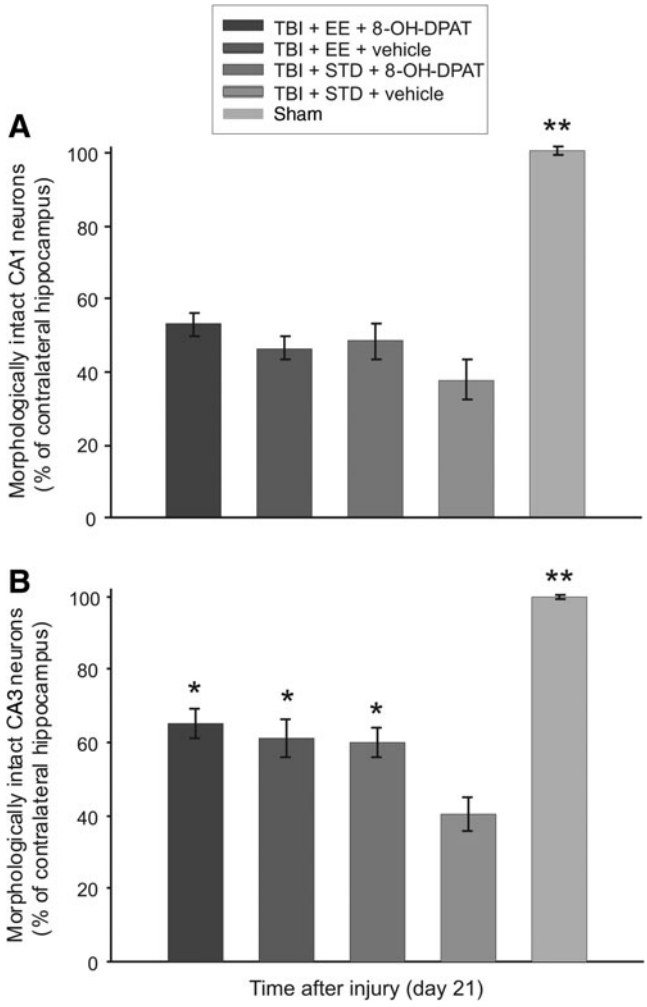

FIG. 6. Mean percentages of morphologically intact CA1 and CA3 neurons (% of the contralateral hippocampus) at 3 weeks after TBI or sham injury. No statistically significant differences were revealed among the TBI groups (**A** = $^{**}p < 0.0001$ versus all TBI groups; **B** = $^{*}p < 0.002$ versus TBI + STD + vehicle; $^{**}p < 0.0001$ versus all TBI groups).

Grady, 1995; Sinson et al., 1997), and attenuating cholinergic deficits enhances functional outcome (Dixon et al., 1997a, 1997b; Sinson et al., 1997; Verbois et al., 2003).

In the present study the number of $ChAT^+$ cells detected in the medial septum was used to assess the integrity of cholinergic neurons projecting to the hippocampus. The data show that 8-OH-DPAT and EE each had neuroprotective effects on cholinergic neurons in the medial septum following TBI, which was associated with improved performance. This finding is consistent with other studies showing that 5-HT_{1A}-receptor agonists protect basal forebrain cholinergic neurons from the neurotoxic effects of NMDA, with corresponding reductions in functional impairment (Harkany et al., 2001; Oosterink et al., 2003). Moreover, the findings suggest that the effects of EE and 8-OH-DPAT on the cholinergic neurons were additive, as indicated by the combined treatment group exhibiting greater sparing of $ChAT^+$ cells than either the EE-alone or 8-OH-DPAT-alone groups. However, the additional $ChAT^+$ cells did not translate into an additive effect on performance. One possibility is that in addition to preventing cell death, the increased numbers of $ChAT^+$ cells reflects an increase in the expression of ChAT protein within the surviving neurons. ChAT is necessary for the production of acetylcholine, and under normal conditions, levels of ChAT are in kinetic excess (Rylett and Schmidt, 1993; Wu and Hersh, 1994). However, following TBI, the levels of ChAT within the cholinergic neurons may decrease significantly and become rate-limiting for the production of acetylcholine. EE and 8-OH-DPAT may attenuate the loss of ChAT, and in so doing may increase the number of $ChAT^+$ cells detected, and thus prevent ChAT from becoming rate-limiting. This in turn helps to maintain cholinergic function and cognitive performance; however, once ChAT is no longer rate-limiting, further increases in ChAT have no added functional effect. This could account for the effects of EE and 8-OH-DPAT on $ChAT^+$ cells, and the lack of an additive effect on performance reported here.

Another possible explanation for the lack of an additive effect is that EE produced robust effects on its own, and thus the "ceiling effect" curtailed any opportunity for 8-OH-DPAT to confer additional statistically significant improvement. Indeed, a previous study from our laboratory, evaluating the potential increased efficacy of a single administration of 8-OH-DPAT plus EE treatment, was also unsuccessful in producing an additive effect, mainly because the EE-alone group reached the level of sham controls (Kline et al., 2007). If the ability to evaluate combinational therapies with the potential for additive or synergistic outcomes is limited by ceiling effects, then alterations to the current testing paradigms are required. One example may be to choose a more rigorous training approach, such that neither treatment (EE or 8-OH-DPAT) can reach the level of uninjured controls, thus affording the opportunity for additional recovery. Another change in the paradigm might be to consider producing a TBI that is more severe than the current moderate level of severity, such that the animals are more impaired and do not recover as efficiently. The use of sub-therapeutic doses of 8-OH-DPAT and/or EE is another possible alternative. The rationale is that neither therapy would be effective on its own, but when combined would produce a synergistic effect. This type of therapeutic paradigm would be appropriate for the clinic, because the potential for pharmacological side effects may be curtailed, while still conferring a beneficial effect on functional recovery. Such studies are currently ongoing in our laboratory.

In conclusion, the data show that a combined therapeutic regimen of 8-OH-DPAT and EE reduces TBI-induced $ChAT^+$ cell loss, but does not enhance hippocampal cell survival or behavioral performance beyond that of either treatment used alone. These findings underscore the complexity of combinational therapies (Margulies et al., 2009), and of elucidating potential means of treating TBI.

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Author Disclosure Statement

No competing financial interests exist.

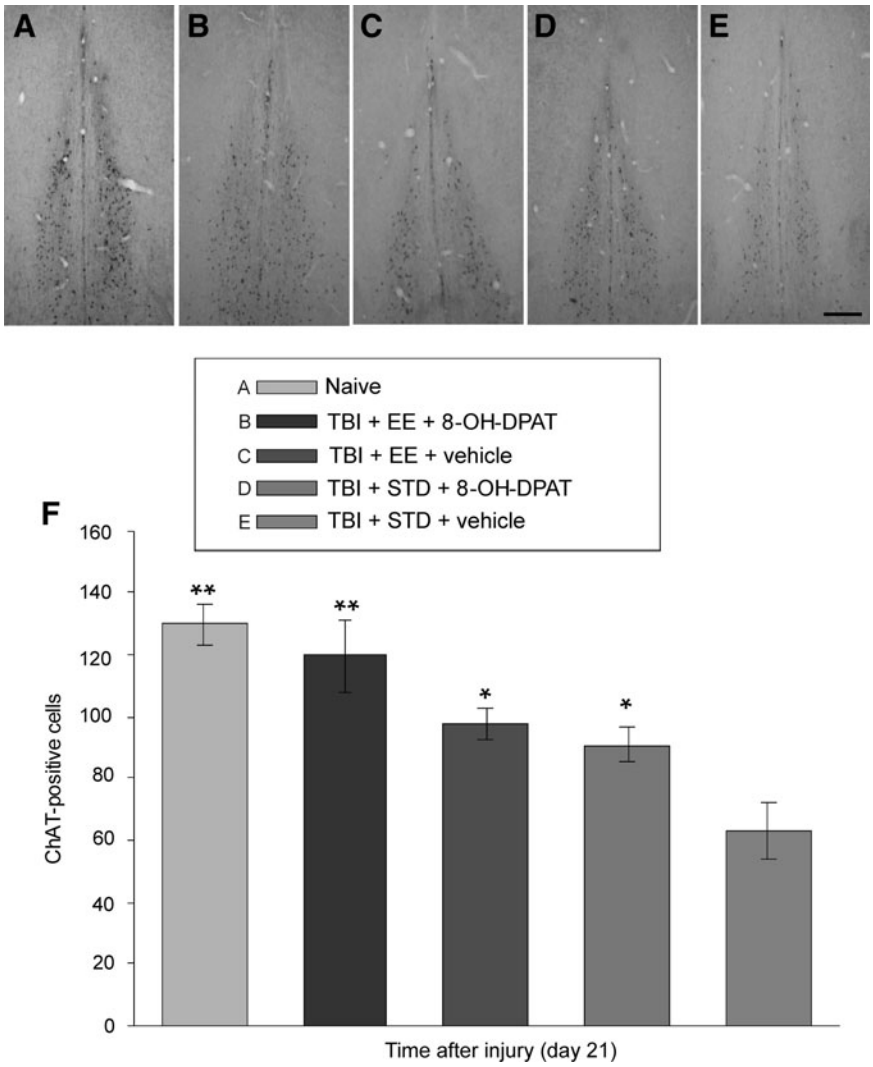

FIG. 7. Mean ChAT$^+$ medial septal neurons quantified at 3 weeks after TBI. No significant difference in ChAT$^+$ cell expression was observed between the naïve and TBI + EE + 8-OH + DPAT groups ($p > 0.05$ for **A**, **B**, and **F**), but both had significantly more than the TBI + EE + vehicle, TBI + STD + 8-OH-DPAT, and TBI + STD + vehicle groups (**$p < 0.005$ for **C**, **D**, and **E**, respectively, and **F**). Although not significantly different from each other ($p > 0.05$), both the TBI + EE + vehicle and TBI + STD + 8-OH-DPAT groups had significantly more ChAT$^+$ cells than the TBI + STD + vehicle group (*$p < 0.005$ for **C** and **D**, respectively, versus **E**; scale bar = 250 μm).

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