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Exercise leads to the re-emergence of the cholinergic/nestin neuronal phenotype within the medial septum/diagonal band and subsequent rescue of both hippocampal ACh efflux and spatial behavior

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Abstract

Exercise has been shown to improve cognitive functioning in a range of species, presumably through an increase in neurotrophins throughout the brain, but in particular the hippocampus. The current study assessed the ability of exercise to restore septohippocampal cholinergic functioning in the pyrithiamine-induced thiamine deficiency (PTD) rat model of the amnesic disorder Korsakoff Syndrome. After voluntary wheel running or sedentary control conditions (stationary wheel attached to the home cage), PTD and control rats were behaviorally tested with concurrent in vivo microdialysis, at one of two time points: 24-hrs or 2-wks post-exercise. It was found that only after the 2-wk adaption period did exercise lead to an interrelated sequence of events in PTD rats that included: (1) restored spatial working memory; (2) rescued behaviorally-stimulated hippocampal acetylcholine efflux; and (3) within the medial septum/diagonal band, the reemergence of the cholinergic (choline acetyltransferase [ChAT+]) phenotype, with the greatest change occurring in the ChAT+/nestin+ neurons. Furthermore, in control rats, exercise followed by a 2-wk adaption period improved hippocampal acetylcholine efflux and increased the number of neurons co-expressing the ChAT and nestin phenotype. These findings demonstrate a novel mechanism by which exercise can modulate the mature cholinergic/nestin neuronal phenotype leading to improved neurotransmitter function as well as enhanced learning and memory.

Graphical abstract

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Keywords

Exercise; Acetylcholine; Nestin; Medial Septum; Diagonal band; BDNF; NGF

1. Introduction

Changes in cholinergic function are associated with the pathogenesis of memory and cognitive dysfunction across a range of neurological disorders. The anatomical organization of the cholinergic forebrain system positions acetylcholine (ACh) to modulate neuronal activity within, as well as across, the cortex and hippocampus (Hasselmo and Sarter, 2011; Woolf et al., 1984). The loss of cholinergic neurons in aging and disease reduces behaviorally relevant ACh efflux in both the hippocampus and cortex (see Pepeu and Giovannini, 2004). The loss of central cholinergic functioning in several neurological diseases has been linked to impaired neurotrophic signaling.

Cholinergic neurons are very responsive to changes in neurotrophin concentrations. Reductions in nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) lead to hypotrophy of cholinergic neurons, whereas sustained increases in these neurotrophins rescues degenerating forebrain cholinergic neurons (Burke et al., 1994; Dekker et al., 1994; Tuszynski et al., 1990). Furthermore, exogenous application of NGF and BDNF increase ACh release (Auld et al., 2001; Huh et al., 2008; Knipper et al., 1994) and recovers memory performance following the loss of basal forebrain neurons (Fischer et al., 1987; Frick et al., 1997; Higgins and Mufson, 1989; Markowska et al., 1996; Nagahara et al., 2009). Although these seminal works were conducted using exogenous delivery of neurotrophins, voluntary exercise robustly increases both BDNF and NGF for weeks within the hippocampus as well as the forebrain regions (Berchtold et al., 2010; Neeper et al., 1996).

Thus, it is somewhat surprising that changes in cholinergic phenotype expression and in vivo ACh efflux, relative to behavioral recovery, have not been examined following voluntary exercise. Cognitive recovery after cholinergic cell loss and following neurotrophin delivery requires weeks to emerge, suggesting that improved behavioral effects are mediated

by structural changes in cholinergic neurons (Gustilo et al., 1999; Morse et al., 1993). Such remodeling could be mediated by the intermediate filament protein nestin, which is co-localized within 30–40% of cholinergic neurons in the medial septum/diagonal band ([MS/DB]; (Gu et al., 2006). This unique population of choline acetyltransferase (ChAT+)/nestin+ neurons has been documented in both the human and rat brain (Hendrickson et al., 2011). The distinctive role of these ChAT+/nestin+ neurons is unknown, but nestin may exert a cytoprotective function in the adult nervous system (Guo et al., 2010; Yu et al., 2011).

Several human disorders of amnesia and dementia, as well as models of those disorders, have cholinergic cell loss as a key neuropathological feature (Schliebs and Arendt, 2011). Alcohol-related brain damage is a significant contributor to cognitive and memory decline. There is both a high prevalence of alcohol abuse (9%–22%) in patients with dementia as well as high rates of dementia (10%–24%) and cognitive decline (50%) in alcohol abusers (Richie and Villebrun, 2008). A history of alcohol abuse at middle age more than doubles the probability of diagnosis of a severe memory disorder as one ages (Kuzma et al., 2014). The toxic effects of alcohol, metabolic changes, as well as nutritional deficiency all contribute to alcohol-related brain damage. Thiamine deficiency is most commonly diagnosed in alcoholics, and if left untreated progresses into Korsakoff syndrome (KS), the most severe spectrum of memory dysfunction associated with alcoholism. We employed the pyridoxamine-induced thiamine deficiency (PTD) rat model of the amnesic disorder KS because of its high face and construct validity in replicating neuropathology and behavioral impairment (Savage et al., 2012). Although the hallmark neuropathology in both the PTD model, and KS, is neuronal loss in the anterior and midline thalamus as well as the mammillary bodies, there is a significant loss (30–40%) loss of MS/DB cholinergic neurons with concomitant reductions in cholinergic innervation of the hippocampus that leads to blunted hippocampal behaviorally-stimulated ACh efflux (Anzalone et al., 2010; Savage et al., 2007; Schliebs and Arendt, 2011). Furthermore, the PTD model is responsive to cholinergic modulation: increasing cholinergic tone within the septohippocampal circuit reduces or eliminates the amnesic profile of the PTD model (Roland et al., 2010; Roland et al., 2008). Thus, as in other models of memory dysfunction, the cholinergic system is critical in modulating the recovery of learning and memory.

Previous research has demonstrated that voluntary exercise is able to restore the spatial working memory deficit in the PTD model (Hall et al., 2014). In the current set of experiments, we test the premise that voluntary exercise has the capacity to rescue degenerating MS/DB cholinergic neurons and restore both behaviorally-stimulated hippocampal ACh efflux and spatial memory. Specifically, we hypothesize that the recovery of the ChAT+/nestin+ phenotype, in vivo ACh efflux and spatial working memory will require an extended period of neurotrophin exposure. The duration of 2-wks for the length of exercise exposure was chosen because this period is sufficient to reverse hippocampal-based cognitive deficits in several other models of cognitive dysfunction (Hall et al., 2014; van Praag et al., 2005). Given that cholinergic remodeling takes time (Gustilo et al., 1999; Naumann et al., 1997), we assessed the recovery of septohippocampal functioning at two time periods: shortly after exercise (24-hrs post exercise) and after an extended adaption period (2-wks post exercise). This design permits the detection of the critical period needed for exercise to modulate the cholinergic septohippocampal system.

2. Methods

2.1. Animals and Treatment

Adult male Sprague-Dawley rats (N=64; Harlan-Teklad Corp., IN), weighing between 300–350 g (9–11 wks of age at the start of the experiment) served as subjects. Rats were pair-housed, placed in a temperature-controlled vivarium (20–22° C), and maintained on a 12-hr light/dark cycle with light onset at 07:00 hr. Procedures were in full accordance with the Institutional Animal Care and Use Committee of Binghamton University and the National Institute of Health: Guide for the Care and Use of Laboratory Animals (2011).

Initially, rats were randomly assigned to one of two treatment conditions: (1) pyriethamine-induced thiamine deficiency (PTD; n=32) or (2) pair-fed controls (PF; n=32). Figure 1 displays the experimental timeline and design. Rats in the PTD condition received daily intraperitoneal (i.p.) pyriethamine HBr injections (0.25 mg/kg; Sigma-Aldrich Corp., St. Louis, MO) in conjunction with *ad libitum* access to thiamine-deficient rat chow (Harlan-Teklad Laboratories, Indianapolis, IN). For the PF control condition, in order to replicate both dietary changes and injection procedures, rats were given a thiamine deficient chow equivalent to the amount consumed by animals in the PTD condition and were administered daily i.p. (0.4 mg/kg) injections of thiamine hydrochloride (Sigma-Aldrich Corp) to replace thiamine. Treatment continued for a period of 12–15 days until the onset of the prototypical neurological symptoms of thiamine deficiency (ataxia, loss of righting reflexes and ultimately tonic-clonic seizure activity). Subsequently, rats were closely monitored for seizure-like activity, and a thiamine bolus (100 mg/kg) was administered 4.5-hrs following onset of opisthotonus. The same dosage of thiamine was delivered 24-hrs later. Following treatment, PTD and PF rodents were placed back onto a normal diet consisting of Purina rat chow for a seven-day recovery period prior to surgery. During this time frame, rats recovered from the weight lost related to treatment.

2.2. Cannulation Implantation Surgery

Hippocampal cannulations were performed on all rats 7 days after PTD/PF treatment. Prior to surgery, administration of a ketamine cocktail (10 mL)/xylazine (1.43 mL) mixture at a dosage of 50 mg/kg (i.p.) was administered. Rodents were placed into a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) and the incisor bar set to 3.00 mm below the interaural line. Guide cannula (8 mm; Synaptech Technology Inc., Marquette, MI) were placed into the following ventral hippocampal coordinates [(AP) = –5.3 mm posterior to Bregma, (ML) = –5.1 mm lateral to the midline, (DV) = –4.2 mm] relative to Bregma (Paxinos and Watson, 2014). Dental acrylic cement with anchor bone screws secured guide cannula to the skull. Administration of 0.3 cc of 0.05 mg/mL buprenorphine (buprenorphine hydrochloride, Hospira Inc., Lake Forest, IL) was administered pre- and post-operatively as an analgesic. Rodents were given a 7-day recovery period with *ad libitum* access to food prior to exercise exposure to ensure sufficient recuperation.

2.3. Exercise Paradigm

Following recovery from treatment (14 days post treatment, 7-days post surgery), PTD-treated and PF-treated rats were randomly assigned into one of two conditions: (a) a

voluntary exercise condition (VEx; PTD=16, PF=16) or (b) a stationary condition (Stat; PTD=16, PF=16). The exercise wheel (35.56 cm diameter; turning resistance < 6 gms) was attached to a clear polycarbonate home cage (Lafayette Instruments, Lafayette, IN, 48.3 × 26.7 × 20.3 cm) for each pair of rats. To control for the environmental context, rats in the Stat condition had immobilized wheels attached to their cages. In the VEx condition wheels had a counter to measure daily wheel revolutions via a Dell Laptop equipped with AWM software (Lafayette Instruments). Throughout the duration of this procedure, all rats were slightly food restricted to 95% of free-feeding weight, to increase running distance (see Sherwin, 1998; Lee et al., 2002).

2.4 Behavioral testing

2.4.1. Spontaneous alternation with concurrent *in vivo* microdialysis—Rats were behaviorally tested either 24-hrs or 2-wks following respective Stat/VEx conditions in order to determine the unique temporal profile of cholinergic recovery. Microdialysis procedure parameters were followed as previously described (see Savage et al., 2003). On the day of testing, the microdialysis probe (S-8020; 2 mm; Synaptech Inc.) was inserted into the hippocampal guide cannula, and the rat was placed into the opaque chamber (30 cm × 40 cm × 35 cm) to acclimate for a period of 60-min prior to maze testing. The probe was connected to a CMA microinfusion pump (CMA/400 pump; Holliston, MA) and artificial cerebrospinal fluid (7.4 pH solution: 127.6 mM NaCl, 0.9 mM NaH₂PO₄, 2 mM Na₂HPO₄, 4 mM KCl, 1.3 mM CaCl₂ dihydrate, 1.0 mM glucose, and 0.9 mM MgCl₂) with 500 nM neostigmine hydrobromide (Sigma-Aldrich Corp.) solution was perfused continuously at a rate of 2.0 µL/min.

Baseline dialysate collection began after a 60-min habituation period, and samples were collected at 6-min intervals. Three samples were collected prior to maze testing in order to determine basal levels of ACh. During spontaneous alternation testing, the rat was placed into the center of the plus maze that was elevated 80 cm from the floor. The arms of the plus maze were 55 cm equidistant lengths with 12 cm clear acrylic sidewalls. The behavioral testing room had numerous extra-maze cues on the walls, in addition to cues located closer to the maze. The rat was allowed to freely transverse all arms for a period of 18-min and during this time microdialysis collection continued. All activity was recorded regarding sequence and number of arms entered. Whereas an arm entry was defined as all four paws of a rat being placed within an arm, an alternation was defined as entry into four different arms in overlapping successive sequences of 4 arm entries. (Example: The successive arm entries of A, D, C, B, D, A, C, D, B, D, A, C, D, A, C; the first sequence of ADCB was an alternation, but the following sequence of 4 arm entries DCBD was not.) The percent alternation score is equal to the ratio of (actual alternations/possible alternations [trial number-3]) and multiplied by 100. (For the above hypothetical data set: 5/(15-3) = .416 × 100 = 41.6%.) This criterion was based upon previous experiments (Anzalone et al, 2010; Hall et al, 2014; Hughes, 2004; Ragozzino et al, 2012). Following maze testing, the rat was placed back into the opaque chamber with continued dialysate collection for 18-min (post-baseline).

2.4.2. Novel Object Recognition—Parameters for Novel Object Recognition (NOR) were adapted from Bevins and Besheer (2006). An opaque acrylic box served as the arena (41 cm × 41 cm × 30.5 cm). Briefly, on the same day following microdialysis testing, rats were habituated to the arena for a period of 20-min. A tripod with a camcorder (JVC, GZ-MG27U) recorded all activity as well as object interaction on the training and testing sessions. Following this initial habituation to the arena, rats were placed back into respective home cages overnight.

The next day, two identical objects were placed into the left and right corners (6 cm from walls) of the apparatus equidistant from the walls and each other. Two types of objects were used for NOR training and testing: (1) a Duplo Lego® object created by combining four rectangular Lego® blocks (7.2 × 10 cm) with a center hole, and (2) a 10.16 cm square wooden block containing 4 equidistant drilled holes (1 cm diameter). Importantly, we initially ran a pilot study on a separate cohort of animals, which revealed that rats displayed no initial preference for either of these objects when presented together (determined by interaction time; $p > 0.10$). The training/testing object was counterbalanced across Treatment and Exercise conditions. In the training phase, each rat was placed facing the midpoint of the wall opposite to the two identical sample objects and was allowed to explore the objects and arena for 10-min.

The testing delay for NOR was 24-hrs following the training phase. The apparatus was set up similar to the training phase, except one object was the previously utilized familiar object, while the other object was novel. Behavior was recorded during the testing phase for 5-min. The time (s) spent at the novel and familiar objects were determined by an individual blind to Treatment, Exercise condition and novelty of the object. Data were expressed as percent of time spent at the novel object. Importantly, only time spent actively sniffing or investigating the object was considered exploratory behavior.

2.5. High Performance Liquid Chromatography

Dialysate samples were assayed for ACh using HPLC with electrochemical detection (Eicom USA, San Diego, CA). ACh peaks were quantified by comparison to peak heights of standard solutions and corrected for in vitro recovery of the probe. The system detection limit is reliably 5 femtomole of ACh. Chromatographs obtained every 15 min/sample were analyzed using the software program Envision (provided by Eicom, USA).

2.6. Tissue Preparation and Immunohistochemistry

The day following the completion of behavioral testing all rats were deeply anesthetized (Sleep-Away, Fort Dodge Animal Health, Fort Dodge, IA) and then transcardially perfused with ice-cold (4°C) phosphate buffered saline (PBS) followed by ice-cold (4°C) filtered 4% paraformaldehyde (Electron Microscopy Services, Hatfield, PA). Whole brains were extracted, kept in paraformaldehyde (4%) for 24-hr, post-fixed in a 30% sucrose solution made in 0.1M PBS and coronally sliced at 40 µm on a freezing sliding microtome (Sm2000r; Leica Instruments, Wetzlar, Germany). Tissue sections were sequentially collected individually in a 96 well plate containing a cryoprotectant solution (62.8 mg NaH₂PO₄, 2.18 g Na₂HPO₄, 160 mL dH₂O, 120 mL ethylene glycol and 120 mL glycerol;

pH: 7.4) and stored at -20°C until immunohistochemistry. All tissues were run in cohorts that include subjects from all treatment conditions to control for specimen preparation procedures.

2.6.1. Immunocytochemistry—ChAT/DAB—For ChAT immunohistochemistry, a total of six sections per subject were used. Sections were first washed in 0.1M phosphate buffer (PB). This was followed by a 30-min incubation in 0.3% H_2O_2 in 0.1M PB. Next, sections were placed into a blocking solution consisting of 3% normal rabbit serum, 0.1% Triton-X 100 and 5% sodium azide in 0.1 M PB for a 60-min incubation. Primary antibody (1:100 dilution goat monoclonal anti-ChAT; AB144P; Millipore EMD, Billerica, MA) incubation occurred at 4°C overnight. Subsequently, following a brief 15-min wash in 0.1 M PB, sections were placed in a secondary antibody solution (1:100 anti-goat IgG, Vector Laboratories, Burlingame, CA) in the blocking buffer for 2-hrs. Next, sections were incubated in an avidin/biotin complex (Elite VECTASTAIN ABC system, Vector Laboratories) for a period of 2-hrs in 0.1 M PB. Lastly, sections were placed in a chromogenic agent containing 0.005% 3,3' diaminobenzidine (Sigma-Aldrich Corp.). Tissue was subsequently mounted, dried and coverslipped with the mounting agent Permount® (Fisher Scientific, Waltham, MA).

2.6.2. ChAT+/nestin+ immunofluorescence double labeling—Three sections per subject were chosen order to determine rates of ChAT+/ nestin–, ChAT+/nestin+ and ChAT –/nestin+ expression within the MS/DB. Free-floating sections were washed using a standard 0.1 M Tris-buffered saline (TBS) solution (pH: 7.4). Next, sections were permeabilized using a 0.2% Triton-X 100 solution in 0.1 M TBS for 15-min, followed by an incubation in blocking solution consisting of 5% normal donkey serum and 0.1% Triton-X 100 in a 0.1 M TBS solution. Similarly, tissue was incubated into a cocktail of the ChAT (AP144P; Millipore EMD) and nestin (MAB353; Millipore EMD) primary antibodies (1:200 dilution) made in blocking solution for 48-hrs at 4°C . Tissue was briefly washed in 0.1 M TBS (15 min) and incubated into a fluorescent-tagged secondary antibody cocktail containing blocking solution with a 1:250 dilution of Cy2 AffiniPure anti-goat IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and Cy3 AffiniPure anti-mouse for 2-hrs. Finally, after a brief rinse in 0.1 M TBS, sections were mounted, coverslipped with Prolong Diamond Antifade agent (Life Technologies, Carlsbad, CA) and stored at -20°C until phenotypic investigation.

2.7. Unbiased Stereological Cellular Quantification of ChAT+ Neurons

Unbiased stereology was employed to estimate the total ChAT+ cell populations in the MS/DB. The parameters employed were based on previously published studies (Li et al., 2008; Roland and Savage, 2009; Savage et al., 2007; Yoder and Pang, 2005). An experimenter blind to both the Treatment, Exercise and Time point conditions performed cell counts. A Zeiss Microscope (Axioscope 2-Plus, Thornwood, NY) with an attached digital camera (DVC-1310; DVC Company, Austin, TX) containing a motorized stage in the x, y and z planes was used in conjunction with StereoInvestigator software (MicroBrightField Bioscience, Williston, VT) on a computer containing a Windows XP operating system. Using 5X magnification, contours were drawn around the specific regions

of interest (ROIs) that included the MS and horizontal and vertical limbs of the DB. For neuronal counting, sufficient staining within the soma was necessary, and counting was performed using the optical fractionator function within the software using a 40× dry-objective lens. Soma size was determined using the nucleator software function with the number of rays set to 5 (Schmitz et al., 1999). The software used a fractionator sampling approach that entailed a section-sampling fraction (ssf=1/6; every 6th section), in addition to an area-sampling fraction which represented the ratio between the counting frame and grid sizes (asf=50μm × 50μm/100μm × 100μm), and lastly, the height sampling fraction (hsf=20μm/40μm). The following equation was used for determining the regional ChAT population: $N = \Sigma \sigma Q^- \times 1/ssf \times 1/asf \times 1/hsf \times 1/tsf$, in which σQ^- constitutes the number of cells counted in a specimen and N represents the total cell estimate. The section thickness was calculated and included to estimate the total number of neurons in the population (thickness sampling fraction, tsf= 33 μm/section). Importantly, we ensured that the Gunderson-Jensen estimator of the error coefficient was lower than 0.10 (smoothness factor=1.0), since this is one of the critical assumptions of the optical fractionator approach for analyses. Table 1 lists the stereologic parameters for cell count estimations (See West, 2013).

2.8. Confocal Microscopy

In order to determine co-localization rates between ChAT+ and nestin+ immunoreactive neurons, confocal microscopy was used. This was performed using coded slides to ensure that the investigator was blind to treatment parameters. A Leica SP5 scanning confocal microscope was used for capturing z-stacks along with the Leica LAS AF software suite on a computer operating with Microsoft Windows XP. ChAT+ neurons were sampled equally across the MS/DB and across the 3 tissue sections. Cells were then assessed for co-localization with nestin using a 40×/1.3 oil immersion lens. Single optical sections (Z-stacks [1 μm resolution]; 1-airy unit) were collected throughout the section using lasers with excitation and emission wavelengths of 478 nm/510 nm (Cy2, green) and 550 nm/570 nm (Cy3, red). Co-localization rate percentages for each subject were determined by the following equation: ([total number of ChAT+/nestin+ co-localized neurons/total number of ChAT+ cells] × 100%).

A z-stack of the entire MS/DB ROI was taken with a 5X/dry lens for each subject throughout the MS/DB. From these sections, the number of cells per mm² was calculated using the NIH software package Image J. Briefly, an outlined ROI was selected and the total number of cells (ChAT+/nestin−, ChAT+/nestin+, ChAT−/nestin+) was divided by the total ROI area (mm²).

2.9. Histological staining

2.9.1. Pyronin Y staining—Pyronin Y staining, which stains RNA red, was performed to quantify thalamic loss and verify correct placement of cannula in the ventral hippocampus. Three sections per subject were chosen, from the fornix region through the dorsal hippocampus (from Bregma: AP coordinates: −1.80 mm to −3.30 mm; Paxinos and Watson, 2014), for assessment.

2.9.2. Intraventricular Distance—Intraventricular distance (IVD) is a measure employed to quantify thalamic loss in PTD-treated rats (see Robinson and Mair, 1992; Vetreno et al., 2011). IVD (in mm) was measured as a function of the distance between the dorsal and ventral portions of the third ventricle (−1.80 mm to −3.30 mm from Bregma; Paxinos and Watson, 2014). Section images were captured using a Nikon light microscope (Nikon Eclipse E400; Nikon Instruments, Melville, NY, USA) via a Scion 1394 camera JAVA module (Scion Corp; Fredrick, MD, USA). IVDs were then measured using the NIH-based program Image-J (v.1.48, NIH, Bethesda, MD, USA) on a MacBook Pro computer (Apple, Cupertino, CA).

2. 10. Enzyme-linked immunosorbent assays (ELISA) for Neurotrophins

ELISAs were employed in order to measure protein levels for mBDNF and β -NGF within the hippocampus. Neurotrophin levels were determined on a separate cohort of rodents with identical treatment parameters and group size. In this experiment, rats were immediately sacrificed following behavioral testing. Brains were then excised and neural structures were rapidly dissected out, placed on ice, and stored at -80°C until tissue preparation for protein quantification.

In order to prepare tissue lysates for use on both mBDNF and β -NGF ELISAs, flash frozen tissue was weighed and sonicated in a lysis buffer containing RIPA buffer, protease inhibitor cocktail, phosphatase inhibitor cocktail #2, phosphatase inhibitor cocktail #3 (all from Sigma-Aldrich) and PMSF at a ratio of 10 μL lysis buffer/mg tissue. Next, tissue was centrifuged for 30 min at 14,000 rpm at 4°C . Subsequently, supernatant was removed and stored at -80°C until total protein and neurotrophin ELISAs were performed.

2.10.1. Total Protein—Total protein levels were determined in order to quantify levels mBDNF and β -NGF. A Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) was used in order to estimate total protein in samples. Briefly, supplied bovine serum albumin (BSA) protein standards were diluted to form the standard curve from 0–2000 $\mu\text{g/mL}$. The working reagent was prepared from 50 parts of the supplied BCA reagent A to 1 part of the BCA reagent B. Using a 96-well plate, 100 μL of each standard and experimental sample was added to 2.0 mL of working reagent, mixed, plated and incubated at 37°C for 30-min. Plates were read at a wavelength of 562 nm and total protein ($\mu\text{g/mg}$) were determined from the standard curve for subsequent analyses.

2.10.2. BDNF ELISA—The BDNF Emax® ImmunoAssay System ELISA kit (Promega Corp., Madison, WI) was used to quantify mBDNF protein levels. Samples and standards were added to the plate in duplicate to ensure reliability in 1X block and sample buffer at a ratio of 1:5. Serial dilutions were performed on the supplied BDNF standard to create an eight-point standard curve ranging from 0–500 pg/mL in order to derive sample values. Plates (96 well) were subsequently read following the colorimetric reaction immediately on a ELISA plate reader (Dynex, Chantilly, VA) at 450 nm and optical density values were obtained.

2.10.3. NGF ELISA— β -NGF was measured using a rat β -NGF DuoSet ELISA kit (R&D Systems, Minneapolis, MN) according to manufacturer specifications. Briefly, 96-well plates were incubated overnight with supplied anti-rat β -NGF capture antibody. Plates were next washed and blocked with reagent diluent. Samples and standards were added to the plate in duplicate to ensure validity and reliability. Samples were diluted in reagent diluent at a ratio of 1:5. Serial dilutions were performed on standards to create an eight-point standard curve ranging from 0–1000 pg/mL in order to derive sample values. Plates were next incubated in detection antibody, washed and incubated in horseradish-peroxidase substrate solution. Finally, stop solution was added to each well, and using a Dynex ELISA plate reader, optical density values were obtained by reading the plate at a wavelength of 450 nm.

2.11. Statistical Analyses

The statistical package SPSS version 22.0 for Mac OS X Yosemite (IBM, Armonk, NY) was used to analyze all data. Cumulative distance data for exercise exposure was analyzed as a mixed model repeated measures analysis of variance (ANOVA), with the two-between subjects factors Treatment (PF vs. PTD) and Time Point (24-hr vs. 2-wk) across the within subjects factor of Day (14 total). For hippocampal ACh efflux data, a mixed model repeated measures ANOVA was performed that had three-between subjects factors of Treatment, Exercise (VEx vs. Stat) and Time and two-within subjects factors of Sample Time (block 1–3) collapsed within Phase (baseline, maze, after). For spontaneous alternation, ChAT population, ChAT/nestin somatic area, ChAT/nestin phenotyping data, β -NGF levels, mBDNF levels and thalamic pathology data, a three-factor ANOVA design was performed (Treatment, X Exercise X Time). All data are expressed as means $\pm$ S.E.M. A $p < 0.05$ was denoted as the acceptable significance level for all analyses. Follow up analyses were performed for any overall significant main effects and/or interactions using Fisher's LSD. In addition, Pearson Correlations were used to assess associations between measures of neuroanatomy, ACh efflux and behavior.

3. Results

3.1. PF-treated control rats ran greater distances than PTD-treated amnesic rats

Given that running distances did not differ as a function of Time point ($F[1, 28] < 1.0$), data were collapsed across 24-hr and 2-wk periods. Similar to previously published laboratory data (Hall et al., 2014), PTD-treated rats ran less than PF-treated control rats ($F[1, 28] = 18.54$, $p < 0.01$). This was most prevalent towards the end of exercise exposure (Treatment $\times$ Day = ($F[13, 364] = 16.73$, $p < 0.01$). Although PTD rats were less active (total cumulative distance traveled = $16,075.95 \pm 1929.08$) than PF rats ($35,005.8d \pm 3581.27$) their running was still sufficient to reveal the benefits of exercise (see below).

3.2. Exercise improved (24-hrs) and recovered (2-wk) spontaneous alternation performance in PTD-treated rats. Furthermore, following the 2-wk adaptation period, exercise also restored/enhanced behaviorally-stimulated ACh efflux in the hippocampus

As shown in Figure 2 (panels A–B), the benefits of exercise on spontaneous alternation performance were evident only in PTD rats (Treatment X Exercise interaction: $F[1,$

56]=26.61, $p<0.001$). We assessed behavior separately at 24-hr and 2-wk since there was a main effect of Time Point ($F[1, 56]=4.56$, $p<0.05$). At the 24-hr time point, PTD-treated rats alternated significantly less than PF-treated rats ($F[1, 28]=24.22$, $p<0.001$). However, there was a significant improvement in the alternation scores of PTD rats that exercised ($F[1, 14]=13.08$, $p<0.01$). It should be noted that there was not complete recovery of alternation behavior as PF rats still had higher alternation scores than PTD-VEx rats ($F[1, 14]=7.03$, $p<0.05$). In contrast to the benefits of exercise in the PTD rats, PF rats did not improve on alternation performance as a function of exercise ($F[1, 14]=2.8$, $p>0.11$).

After the 2-wk adaption period the exercise-induced improvement in alternation behavior were still only evident seen in the PTD rats (Treatment X Exercise: ($F[1, 28]=15.49$, $p<0.01$). Notably, the effects of exercise on PTD rats tested at the 2-wk period indicated that there was complete recovery of alternation behavior, as the PTD-VEx group was not different from either PF group (both p 's >0.25).

Assessment of basal femtomole hippocampal ACh levels (PF=56.93 $\pm$ 9.14; PTD=55.99 $\pm$ 9.17) revealed that these levels did not differ between PF- and PTD-treated rats at either Time point ($F[1, 56]<1$), which replicates our previous findings (see Vetreno, et al., 2008). However, changes in ACh efflux during behavioral testing as a function of Treatment and Exercise were observed. There were time-dependent differences in the exercise-induced improvement in behaviorally-stimulated ACh efflux (Exercise X Time-Point X Phase interaction ($F[2, 112]=6.69$, $p<0.01$; see Figure 2, panels C–D). Due to the significant interaction, the hippocampal ACh efflux data were analyzed separately for the 24-hr and 2-wk time points. At the 24-hr time point, during the behavioral testing phase, PTD-treated rats had blunted hippocampal ACh efflux compared to PF-treated control rats ($F[2, 56]=6.09$, $p<0.01$). It is important to note that at the 24-hr time point there was no effect of exercise on ACh efflux in either treatment condition (all p 's >0.25).

In contrast, after the 2-wk adaption period, exercise had a remarkable effect on behaviorally-stimulated hippocampal ACh efflux (Exercise X Phase: $F[2, 56]=12.04$, $p<0.001$), and in particular, in PTD rats (Treatment X Phase: $F[2, 56]=4.88$, $p<0.01$). Behaviorally-stimulated ACh efflux was blunted in PTD rats that were not exposed to exercise, compared to PF rats ($F[2, 28]=8.36$, $p<0.01$), but this dysfunction in ACh efflux was eliminated after exercise ($F[2, 28]=14.44$, $p<0.01$). Analysis at each sample time during behavioral testing revealed that the effects of exercise were prevalent at all sample times for PTD rats (all F 's[1, 14] >9.0 , all p 's <0.01). There was also an increase in the ACh efflux of PF rats, but the enhancement was only evident only at sample time 3 ($F[1, 14]=5.34$, $p<0.05$).

Overall activity levels during spontaneous alternation were not affected by treatment, exercise or time. Neither an effect of Treatment nor Exercise influenced activity rates, as assessed by number of arm entries (all p 's >0.27 ; PF arm entries: VEx: 29.56 $\pm$ 1.48, Stat: 29.75 $\pm$ 1.65; PTD arm entries: VEx: 29.70 $\pm$ 1.77, Stat: 32.63 $\pm$ 2.08). However, the 3-way ANOVA revealed an unexpected significant effect of Time point ($F[1, 56]=17.33$, $p<0.01$), such that the cohort of animals behaviorally tested at 2-wk displayed slightly greater overall activity in the number of arms entered (by approximately 20%) than rats at the 24-hr time

point. This was a small effect size (Cohen's $d=0.26$ or $r_{YT}=0.13$) and food restriction level did not differ significantly among the time points ($F[1, 56]<1$).

3.3. PTD-treated rats are not impaired on a novel object recognition task, but exercise has a time-dependent effect on improving novel object discrimination

PTD rats, relative to PF rats, did not display impairment on the NOR task at either Time point (both F 's <1.3 , $p>0.20$). Furthermore, the Treatment conditions (PTD= 55.9 ± 5.8 s [VEX= 59.4 s; Stat= 52.4 s]; and PF= 62.4 ± 6.02 s [VEX= 67.8 s; Stat= 57.0 s]) did not differ in the total amount of time spent exploring objects in the 5-min test period ($F<1$, $p>0.40$). However, exercise improved performance, regardless of treatment condition, at the 24-hr time point. Rats exposed to exercise spent a greater amount of time at the novel object, compared to sedentary rats, demonstrating successful discrimination ($F[1, 56]=4.29$, $p<0.05$; Figure 4). In contrast, at the 2-wk time point, the beneficial effect of exercise was no longer significant ($F[1, 28]<1$).

3.4. Neurotrophin levels

All ELISA data were analyzed as change from 24-hr percent stationary control (PF), but mean protein values are also reported for each condition in Figure 5.

3.4.1. mBDNF levels in the hippocampus are reduced in PTD rats, but exercise acutely and persistently increased mBDNF protein levels in all rats—Overall, PTD rats had attenuated levels of mBDNF in the hippocampus compared to PF rats (Figure 5, panels C–D; Treatment; $F[1, 56]=18.07$, $p<0.01$). However, exercise increased mBDNF levels (60%) in all rats ($F[1, 56]=45.68$, $p<0.001$). As expected, these exercise-induced increases in mBDNF faded ($\approx 20\%$) across the 2-wk period, but were still elevated in all rats exposed to exercise ($F[1, 56]=6.66$, $p<0.05$). Thus, we further parsed out these effects at each time point.

As shown in Figure 5-C, after 24-hrs there was a robust increase in mBDNF as a function of exercise, regardless of treatment conditions ($F[1, 28]=35.28$, $p<0.01$); although PTD rats had lower levels of mBDNF compared to PF rats ($F[1, 28]=4.78$, $p<0.05$). Importantly, in response to exercise, PTD rats had an increase in BDNF levels ($F[1, 14]=22.63$, $p<0.01$) that were restored to the level of PF Stat rats. Additionally, exercise increased mBDNF levels in PF rats ($F[1, 14]=14.05$, $p<0.01$).

Panel B of Figure 5 shows that there was a similar phenomenon after the 2-wk adaption period: Exercise produced a protracted increase in mBDNF levels among both PF rats and PTD rats ($F[1, 28]=13.99$, $p<0.01$). Although hippocampal mBDNF levels were decreased by PTD treatment ($F[1, 28]=14.09$, $p<0.01$), exercise increased the level of mBDNF in PTD rats such that it was now comparable to PF rats that did not exercise ($F[1, 14]<1$, $p>0.98$).

3.4.2. Hippocampal β -NGF levels are reduced in PTD rats compared to PF rats, but exercise led to a persistent increase in β -NGF levels in both treatment conditions—Irrespective of time point, overall analyses indicated that hippocampal β -NGF levels were reduced in PTD rats compared to PF rats ($F[1, 56]=11.95$, $p<0.01$; see

Figure 5-EF). Furthermore, hippocampal β -NGF levels were increased by exercise ($F[1, 56]=23.11$, $p<0.01$). Given that there was an approximate 20% reduction in hippocampal β -NGF from 24-hrs to 2-wks post exercise ($F[1, 56]=4.44$, $p<0.05$), we parsed these differences in β -NGF levels separately at both 24-hrs and 2-wks.

At the 24-hr time point, rats exposed to exercise, regardless of treatment condition, had higher β -NGF levels compared to stationary rats ($F[1, 28]=16.48$, $p<0.01$). Alternatively, there was a decrease (24%) in the levels of hippocampal β -NGF in PTD rats, relative to control PF rats ($F[1, 28]=4.05$, $p=0.05$). However, exercise improved β -NGF levels by 40% in PTD-rats ($F[1, 14]=15.70$, $p<0.01$), making the level indistinguishable from Stat PF rats ($F[1, 14]<1$, $p>0.49$). It should be noted that in PF-rats there was a 38% increase in β -NGF levels 24-hr post exercise ($F[1, 14]=4.72$, $p<0.05$). Following the 2-wk adaption period, hippocampal β -NGF levels in both PF and PTD rats were still elevated as a function of exercise ($F[1, 28]=7.17$, $p<0.01$).

3.5. Exercise did not alter thalamic pathology

Given that thalamic pathology is critical to the behavioral impairment in PTD model, we assessed whether there was any recovery of thalamic tissue loss as a function of exercise or recovery time. The data displayed in Figure 6 reveals that PTD-treated rats showed an approximate 20% reduction in intraventricular distance (IVD) compared to PF-treated rats ($F[1, 56]=30.21$, $p<0.001$). This did not change across time from 24-hr to 2-wks post exercise ($F<1$). Importantly, exercise did not alter thalamic mass ($F<1$).

3.6 Unbiased Stereological Cellular Quantification of ChAT+ Neurons: Changes are observed in the expression of the total number of cholinergic (ChAT+) neurons that reside within the MS/DB as a function of thiamine deficiency and exercise

Stereological estimates of the total number of ChAT+ neurons within the of MS/DB replicated previous findings that PTD treatment reduces ($\approx 25\%$) the number of ChAT+ neurons that reside within the MS/DB (Roland and Savage, 2009; Savage et al., 2007), compared to PF control ($F[1, 56]=18.42$, $p<0.01$). A novel finding was that exercise ($F[1, 56]=4.64$, $p<0.05$) led to an increase in the number of cells that expressed the ChAT+ phenotype in the MS/DB (Figure 7, Panels A–B). However, this effect was exclusive to the 2-wks post exercise time point (Exercise $\times$ Time: $F[1, 56]=6.41$, $p<0.05$). Specifically, at the 2-wk time period, PTD-treated rats showed a 36% increase in ChAT expression in response to exercise ($F[1, 14]=12.37$, $p<0.01$), whereas PF rats showed a 25% increase in ChAT expression ($F[1, 14]=6.67$, $p<0.05$).

3.7. Confocal Microscopy. Both the decrease in cholinergic phenotype due to PTD treatment and the increase in cholinergic phenotype due to exercise were primarily driven by changes in the ChAT+/nestin+ phenotype

Similar to the stereological estimates, the overall ANOVA on the profile counts of the immunofluorescence positive neurons of both ChAT phenotypes (ChAT+/nestin– and ChAT+/nestin+) revealed a main effects of Treatment ($F[1, 56]=9.17$, $p<0.01$), Time ($F[1, 56]=5.57$, $p<0.05$), Exercise ($F[1, 56]=7.39$, $p<0.01$), in addition to a Time point $\times$ Exercise

interaction ($F[1, 56]=4.99, p<0.05$). Thus, overall there was a recovery of the cholinergic phenotypes with exercise.

In order to a clearer picture of the role of these two distinct phenotypes in neuronal remodeling as a function of exercise and thiamine deficiency, separate analyses were conducted for the ChAT+/nestin- and ChAT+/nestin+ cells. There were very few Nestin+ cells that did not co-localize with ChAT+ cells at either time point ($<1/\text{mm}^2$), and these neurons did not change as a function of time, treatment or exercise (all p 's >0.17). As shown in Figure 8, PTD treatment decreased the number of ChAT+/nestin+ cells by 25%, but exercise had a time dependent effect of restoring the number of ChAT+/nestin+ cells (Treatment $\times$ Exercise $\times$ Time interaction, $F[1, 56]=4.22, p<0.05$). At the 24-hr time point, there was a reduction in ChAT+/nestin+ cells as a function of PTD, and although there was a trend for exercise, regardless of treatment condition, to increase the number of ChAT+/nestin+ cells, the effect did not reach significance ($F[1, 28]=3.54, p=0.07$). However, at the 2-wk post exercise time period, exercise produced a complete recovery of ChAT+/nestin+ cells in the PTD rats—as well as an increase in the ChAT+/nestin+ phenotype in PF rats (Treatment $\times$ Exercise interaction: $F[1, 28]=4.14, p=0.05$).

Interestingly, the greatest change in exercise-induced increases in the ChAT+ phenotype were seen in ChAT+ cells that had nestin as a co-factor. Cholinergic neurons without nestin (ChAT+/nestin-) did not significantly change as a function of exercise or PTD treatment (all F 's $<1.83, p$'s $>.18$). This demonstrates that the nestin is critical for cholinergic cell reorganization after trauma and during recovery. We further assessed regional volumes from the MS/DB obtained from the sampling regions to ensure that there were no differences as a function of treatment nor exercise. Structural atrophy was not evident as there were no main effects of Treatment or Exercise (both F 's $<1, p$'s >0.50).

Table 1-A displays the rate of ChAT+/nestin+ co-localization as well as estimated soma size. The overall ANOVA revealed an effect of Exercise ($F[1, 63]=8.47, p<0.01$). Follow-up analyses revealed that 24-hrs post-exercise there was a medium effect size of exercise increasing the rate of co-localization (Cohen's $d=0.39$ or $r_{YI}=0.19$), but it was not significant ($F[1,28]=1.19, p>0.20$). Thus, variance was contributing to the lack of effect at 24 hrs. However, as time progresses to the 2-wk interval, the effect size of exercise on the co-localization rate increased into the range of large (Cohen's $d=1.13$ or $r_{YI}=0.49$) and was significant ($F[1,28]=10.31, p<0.01$). We assessed ChAT somatic area (Table 1-B) as another measure of cholinergic neuron health (see Higgins et al., 1989). Since we observed a unique temporal profile of the ChAT neuronal population, we assessed each time point separately. Somatic area of ChAT+ neurons was affected in a time-dependent fashion. At 24-hrs post exercise, we observed a reduction in somatic area as a function of PTD treatment ($F[1, 28]=4.61, p<0.05$), but no effect of exercise was evident. In contrast, at the 2-wk time period, exercise did increase ChAT+ soma size selectively in the PTD rats ($F[1, 14]=8.03, p<0.05$). Although soma size of nestin+ neurons was not affected by PTD (both F 's <1), there was a hypertrophic response in nestin soma size to exercise in both treatment conditions (Table 1-C) that took time to emerge, as the effect was only significant at 2-wk period ($F[1, 28]=4.21, p=0.05$). No differences in nestin soma size were seen 24-hrs post exercise ($F[1, 28]=2.59, p>0.10$).

3.8. Correlations: At the 2-wk time point, the number of ChAT+/nestin+ neurons correlated with behaviorally-stimulated hippocampal ACh efflux, and ACh efflux correlated with spontaneous alternation behavior

Correlations between overall ChAT cell estimates and ACh efflux, specific cell phenotypes and ACh efflux, as well as between alternation behavior and behaviorally-stimulated hippocampal ACh efflux were conducted, but only significant correlation were graphed (see Figure 3-AB). The stereological estimate of the total ChAT neuronal population, at either 24-hr or 2-wk periods, did not correlate with ACh efflux (24-hr: $r=0.22$, $p>0.22$; 2-wk: $r=0.17$, $p>0.35$). Furthermore, there were no significant correlations between ChAT+/nestin- phenotype expression and ACh efflux at the 24-hr ($r=0.09$, $p>0.63$) or the 2-wk ($r=0.26$, $p>0.15$) time periods. While there was an overall positive correlation between the ChAT+/nestin+ phenotype and ACh efflux ($r=0.34$, $p<0.01$), follow-up analyses revealed that this was driven by a significant positive correlation at the 2-wk time period ($r=0.59$, $p<0.001$). At 24-hr time point, the correlation between ChAT+/nestin+ phenotype expression and ACh efflux was not significant ($r=0.13$, $p>0.47$). In addition, the average percent rise in ACh efflux at both the 2-wk time period ($r=0.52$, $p<0.01$) and the 24-hr period ($r=0.42$, $p<0.025$), had a positive relationship with the rate of spontaneous alternation. Standard error of the regression (S) was assessed as goodness of fit statistic for the significant correlations. The average distances from the regression lines were relatively small for spontaneous alternation performance and ACh efflux ($S=9.44$), as well as for the expression of ChAT+/nestin+ neurons and ACh efflux and the ($S=14.40$).

4. Discussion

The findings from this study are the first to demonstrate that the protracted cognitive improvement associated with exercise parallels both structural and functional remodeling of the cholinergic forebrain system. The major findings from this series of experiments are that, following a protracted adaption period, exercise leads to: (1) the amelioration of the spatial working memory impairment in PTD rats, (2) a recovery and enhancement of behaviorally-stimulated hippocampal ACh efflux, and (3) a re-emergence of the ChAT phenotype within the MS/DB, with the greatest change of expression occurring in the ChAT+/nestin+ subpopulation, as well as an increase in the somatic area of those unique neurons.

Although nestin+ neurons in the basal forebrain were detected over a decade ago (Gu et al., 2002; Guo et al., 2010), a complete understanding of the functional importance of these neurons remained elusive. Our phenotyping analysis, double fluorescent staining and confocal review, revealed that the ChAT+/nestin+ neuronal phenotype is the more dynamic cholinergic population that responds to both neurodegeneration and neurorecovery. The ChAT+/nestin- phenotype was minimally affected by thiamine deficiency or exercise. Thus, our data demonstrate that the reduction in medial septal cholinergic neurons after PTD treatment is due primarily to the initial loss of the expression of the ChAT+/nestin+ phenotype, rather than rapid cell death. This is plausible as these neurons re-emerge after the prolonged exposure to neurotrophins produced by exercise. These data mirror the loss of ChAT+ cells in the medial septum following fornix axotomy, which is reversible following prolonged infusion of NGF (Hagg et al., 1988). These converging lines of evidence suggest

that following a neurotoxic event, cholinergic neurons, in particular those that have nestin as a cofactor, do not immediately die, but rather enter an atrophic quiescent state in which they do not express the enzymes required to maintain cholinergic transmission. In contrast, a significant portion of cholinergic neurons (30–40%) can be rescued from a pathological state with timely and repeated exposure to NGF (Nagahara et al., 2009; Naumann et al., 1997; Tuszynski and Gage, 1995) or BDNF (Morse et al., 1993). It should be noted that this is the percentage of ChAT+ neurons within the basal forebrain that express nestin. However, there is point after neurotrauma in which ChAT+ neurons decrease TrkA receptors and are no longer responsive to the survival benefits of neurotrophins (Hagg et al., 1988; Lazo et al., 2010).

In the uncompromised brain, human or rodent, ChAT+/nestin+ neurons account for about 30% of the total forebrain cholinergic neuronal population (Hendrickson et al, 2011). Our data also demonstrate that in the healthy brain (control PF rats), exercise increases the soma size, number and co-localization rate of ChAT+/nestin+ neurons within the MS/DB. Thus, the nestin phenotype is not just marking degenerating neurons, but the population has the potential to remodel and influence cholinergic function.

We proposed that structural changes in cholinergic neurons observed after exposure to neurotrophins are mediated by the intermediate filament protein nestin. Nestin participates in the dynamic remodeling of cells during development (Ruan et al., 2001) and there is evidence that nestin exerts a cytoprotective function in the adult nervous system (Yu et al., 2011). Within the MS/DB, nestin+ cells only co-localize with ChAT neurons; no co-localization is seen in glial cells or other neuronal populations (GABAergic or glutamatergic; see (Guo et al., 2010; Hendrickson et al., 2011). Similar to previous studies, our results demonstrate that about 35% of ChAT+ neurons within the MS/DB co-express nestin. As shown by Zhu and colleagues (2011), these nestin-expressing cholinergic neurons are mature neurons that have a higher excitability and receive stronger spontaneous excitatory synaptic inputs than ChAT+ neurons that do not express nestin (ChAT+/nestin-). Stronger spontaneous excitatory activity of ChAT+/nestin+ neurons can result in higher synaptic transmission efficacy. It is well documented that after NGF or BDNF exposure, there is enhanced synaptic transmission (Auld et al., 2001; Huh et al., 2008).

Our data revealed that the re-emergence of the ChAT+/nestin+ phenotype after exercise takes time to occur: it started to emerge 24-hrs following a 2-wk bout of exercise and was fully evident with a protracted adaption period of 2-weeks post exercise. Levels of neurotrophin decline at a progressive rate following exercise. Berchold et al. (2011) ran a time-course analysis of BDNF protein decay 1, 2, 3 or 4- wks following the termination of exercise and found, similar to our data, that BDNF levels remain elevated for 2-wks. It has also been shown that mRNA for BDNF is increased 3 days into the exercise protocol and persists through out running (Vaynman, et al., 2003). Although neurotrophin levels declined (BDNF ~23%; NGF~17%) in our study 2-wks post exercise, they were still significantly elevated. Thus, given our paradigm, BDNF levels were likely increased, albeit at ascending and descending levels, for approximately 4 weeks. This time frame is comparable to the early neurotrophin delivery studies (see Dekker et al., 1994; Tuszynski et al., 1990; Frick et al., 1997), which produce similar changes in the cholinergic functioning. Furthermore, the

recovery/enhancement of behaviorally stimulated ACh-efflux required a similar time frame. This time frame is similar to that observed for the NGF-induced recovery of cholinergic neurons after fornix axotomy (Fischer and Björklund et al., 1991), as well as NGF-induced cognitive recovery in aged rats (Frick et al., 1997; Gustilo et al., 1999; Markowska et al., 1996), which suggests the behavioral effects are likely mediated by structural changes in cholinergic neurons.

The induction of plasticity-related genes and increased neurotrophin expression have various temporal patterns in response to exercise (Cotman and Berchtold, 2002; Neeper et al., 1996). It has been well established that exercise increases neurotrophin levels and this enhancement can persist for weeks (Berchtold et al., 2010). Although several growth factors (BDNF, VEGF, NGF) are up-regulated within the hippocampus and other regions, such as MS/DB, after exercise (Fabel et al., 2003; Griesbach et al., 2009; Neeper et al., 1996; Tong et al., 2012; Vivar et al., 2012), BDNF has emerged as the key player modulating memory improvement (Cotman et al., 2007; Gomez-Pinilla et al., 2008; Vaynman et al., 2004). Our data demonstrate that in the hippocampus both β -NGF and mBDNF are increased immediately proceeding exercise and stay elevated, albeit at descending levels, through out the 2-wk adaption period. Although the enhancement of the ChAT+/nestin+ cholinergic phenotype and elevated ACh efflux takes time to emerge, we do not think that the window for cholinergic plasticity after exercise needs to be void of exercise; rather, we propose that the window requires approximately 4-weeks of exposure to increased neurotrophin levels. Neurotrophins are transported to the MS/DB from the hippocampus via both anterograde and retrograde mechanisms (Morse et al., 1993; Schwab et al., 1979; Seiler and Schwab, 1984). It should be noted that when BDNF is effectively delivered to the MS/DB, it has a capacity comparable to that of NGF to rescue cholinergic neurons (Morse et al., 1993). However, there is a time sensitive period after neurotrauma, in which cholinergic neurons down regulate NGF receptors, reducing the probability of a recovery response to NGF (Lazo et al., 2010). Whether the window of cholinergic recovery for BDNF is more or less protracted than that observed for NGF, is unknown.

What is known is that different tasks differ in time sensitivity to exercise: Although some exercised-induced enhancements of behavior (delayed object recognition, retention of spatial location) are more robust immediately proceeding exercise (Berchtold et al., 2010; Hopkins et al., 2011), certain measures of memory are better following a delay from exercise (Berchtold et al., 2010). Such results demonstrate that exercise can modulate cognitive function and brain health through multiple molecular/cellular pathways. A complete understanding of the different avenues for brain enhancement and recovery after exercise needs further exploration.

A prevailing view has been that exercise-induced improvements in cognitive functioning are primarily driven by neurotrophin/neurogenesis interactions (van Praag et al., 2005; van Praag et al., 1999). Although neurogenesis is a critical part of the story, our data demonstrate that adaptations in mature neuronal protein expression, morphology, and survival contribute to improved cognitive processing after exercise. Although hippocampal neurogenesis is decreased (20%) in the PTD model (Vetreno et al., 2011), and could contribute to behavioral alterations, improved neurogenesis cannot fully account for the recovery/enhancement of

behaviorally-stimulated hippocampal ACh. Rather, there is evidence that cholinergic stimulation leads to improved neurogenesis (Kotani et al., 2006; Mohapel et al., 2005). Thus, increased neurogenesis along with improved septohippocampal cholinergic function may work in concert to lead to improved cognitive performance following exercise.

Conclusions

Our data demonstrate that the mature neuronal ChAT+/nestin+ phenotype is the most responsive cholinergic phenotype to environmental changes, such as neurotoxicity and exercise, demonstrating that these neurons show enhanced plasticity as measured by phenotypic differentiation and soma size. Upregulation of BDNF and/or NGF are the likely candidates for the exercise-induced remodeling of the septohippocampal pathway. However, it is uncertain whether exercise is altering cholinergic plasticity through the modulation of selective or combined activation of BDNF and/or NGF receptors. Data from exogenous neurotrophin studies point towards both compounds influencing cholinergic phenotypic expression (Morse et al., 1993). A clinical issue with neurotrophic-based therapies is effective delivery of such protein within in the CNS, although viral vector-mediated gene delivery has recently been promoted (Lim et al., 2010; Mufson et al., 2008; Tuszynski et al., 2015). Our novel data clearly demonstrate that exercise has the capacity to rescue degenerating cholinergic neurons, particularly those that co-express nestin, and thus has the capacity to assist with neural recovery for disorders in which cholinergic atrophy play a role in cognitive dysfunction, such as chronic alcoholism, mild cognitive impairment and Alzheimer's disease.

Exercise is more commonly being prescribed as part of a treatment regime for memory and cognitive dysfunction (Ngandu et al., 2015), and our data provides direct evidence that exercise modulates the phenotypic expression of cholinergic forebrain neurons, as well as increases behaviorally-relevant ACh efflux. Thus, exercise appears to keep cholinergic neurons vital in control subjects as well as in subjects with cognitive/memory decline. The possibility exists that under exercise conditions, patients with cholinergic abnormalities (forebrain cell loss as well as reduce innervation of the hippocampus and cortex) may be more responsive to cholinergic drug therapy. As stated by Hasselmo and Sarter (2011), treatments of cognitive disorders are commonly expected to consist solely a pharmacological 'magic bullet', but the full benefit of pharmacotherapy will likely require conjunctive behavioral interventions. Our data indicate that exercise is a critical modulator in shaping and maintaining cholinergic forebrain circuitry, even under pathological conditions.

What remains to be determined is how long do the effects of exercise on enhancing/recovering the cholinergic phenotype persist. The optimal dose and duration of a therapeutic exercise regimen for the stability/recovery of cognitive function remains to be determined, but similar to our lower running PTD rats, even moderate exercise levels, lead to improved cognitive outcome in aged humans (Vidoni et al., 2015). Future research is needed to determine whether exercise modulates the effectiveness of cholinergic therapy in disorders of cognitive and memory dysfunction.

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Highlights

- Exercise rescues medial septal/diagonal band neurons that express the ChAT+/nestin+ phenotype
- Exercise restores/increases behaviorally-relevant hippocampal ACh efflux
- The exercise-induced modulation of the cholinergic forebrain requires weeks to emerge

Figure 1. Experimental design

First, all subjects were randomly assigned to either PTD (n=32) or PF (n=32) treatment. Following recovery, all rats had a cannula placed in the ventral hippocampus for later *in vivo* microdialysis. After surgery, rats were pair-housed and allowed to recover and were then transferred to cages that had either an active running wheel (voluntary exercise [VEx]) or an inactive running wheel (Stationary Controls [Stat]) attached to the home cage for a 2-wk duration. Rats were subsequently tested either 24-hrs or 2-wks later on spontaneous alternation (SA) combined with *in vivo* microdialysis followed by a novel object recognition (NOR) test. Cellular morphological assessment from the MS/DB was performed in all subjects. In a different cohort of animals, BDNF and NGF levels were obtained 24-hrs or 2-wks after the cessation of VEx and Stat conditions.

Figure 2. Spontaneous alternation behavioral performance (panels A–B) is expressed as mean percent alternation $\pm$ S.E.M, while hippocampal acetylcholine (ACh) efflux (panels C–D) is displayed as percent change relative to baseline $\pm$ S.E.M

At the 24-hr time point (A), a significant interaction revealed that PTD rats show impaired performance, relative to PF rats, and exercise modestly improved performance, while control PF rats showed no change in spontaneous alternation in response to exercise. Two-weeks post exercise (B), PTD rats that exercised showed a robust recovery in performance and at this time point. Twenty-four hrs post exercise there was a decrease in ACh levels in PTD rats during behavioral testing (M1–M3) compared to PF (C), but no effect of exercise. However, 2-weeks post-exercise, both PTD and PF rats showed an increase in ACh efflux during behavioral testing (D).

* indicates a significant effect of Treatment, ^ indicates a significant effect of Exercise, and # indicates a significant Treatment $\times$ Exercise interaction

Figure 3. Correlations between cell counts, ACh efflux and behavior

Panel A. At 2-wks post-exercise a positive correlation was found between Nestin+/ChAT+ cell number and hippocampal ACh efflux. This was primarily driven by the PTD group. Panel B. There was also a positive correlation between spontaneous alternation performance and hippocampal ACh efflux after the 2-wk restoration period.

Triangles indicated PF control data points: filled triangles containing represented the PF VEx group and open triangles represented the PF Stat group. Circles denoted PTD rats: filled circles represented PTD VEx rats and open circles represented PTD Stat rats.

Novel Object Recognition

Figure 4. Novel object recognition (NOR) performance obtained from both 24-hrs (A) and 2-wks (B) post exercise

Data are expressed as percent of overall time spent exploring the novel object, where 50% is indicative of chance performance. PTD rats showed no impairments on this task at either time point. However, an overall effect of exercise was observed at the 24-hr time point. ^ indicates a significant effect of Exercise

Figure 5. Changes in hippocampal mBDNF and β -NGF levels at 24-hrs and 2-wks post exercise
 In the hippocampus, there was a decrease in mBDNF in PTD rats that was rescued by exercise at both time points (**AB**). Exercise also increased mBDNF levels in PF rats at both time points. β -NGF levels in the hippocampus (**CD**) were also decreased by PTD, but increased by exercise in both PTD and PF rats. Numbers within the bars are the actual mean protein values (pg/ml) for the neurotrophins each condition. * indicates a significant effect of Treatment, ^ indicates a significant effect of Exercise, and # indicates a significant Treatment $\times$ Exercise interaction

Figure 6. Thalamic damage and hippocampal cannula placement

Intraventricular distance (IVD) data (A) were collapsed across Time point and Exercise conditions, since there were no significant differences among these variables. Group means are reported and plotted for PF (triangles) and PTD (circles) rats, as shown in the legend. Overall, there is a decrease in the IVD in PTD rats, relative to PF control rats. Exercise did not affect IVD. Representative images (B) of the midline thalamus were taken from a PF and PTD rat. A characteristic image (C) of the hippocampal cannula placement for microdialysis. * indicates a significant effect of Treatment.

Figure 7. The overall estimated number of ChAT+ cells within the MS/DB were decreased by PTD treatment at 24-hrs (A) and 2-wks (B) post exercise

Although exercise did not influence total ChAT+ cell numbers 24-hrs post exercise, 2-wks post exercise there was an increase in the number of neurons that expressed the ChAT+ phenotype in both PF and PTD rats. * indicates a significant effect of Treatment, ^ indicates a significant effect of Exercise

Figure 8. Changes in cholinergic phenotype after thiamine deficiency and exercise

Double immunofluorescent staining for ChAT+ and nestin+ cells within the MS/DB of a stationary PTD rat (A) and PTD rat that exercised (B). Detailed representations of the ChAT+ and nestin+ cells and merged phenotypes within the MS/DB (C). The number of neurons (per mm²) within the MS/DB that have the overall ChAT phenotype, and the subcategories of cholinergic neurons that display the ChAT+/Nestin- phenotype and the Nestin+/ChAT+ phenotype (D). PTD treatment significantly decreased the ChAT+/nestin+ phenotype at both time points. Exercise increased ChAT+/nestin+ phenotype only after the 2-wk adaption

period in PTD and PF control rats. The ChAT^{+/nestin-} phenotype was unchanged by pathology or exercise.* indicates a significant effect of Treatment, ^ indicates a significant effect of Exercise

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Table 1

Stereological experimental parameters used to estimate the overall neuronal ChAT population

Stereological Variable Components	Value					
	24-hr	PF	PTD	2-wk	PF	PTD
Observed group mean (Mean N)	VEx	13591.1	9168.1	VEx	13931.2	14032.3
	Stat	12209.2	9003.0	Stat	11739.0	10130.8
Observed coefficient of variation from Mean N (OCV)	24-hr	PF	PTD	2-wk	PF	PTD
	VEx	841.8	647.9	VEx	1512.0	2253.7
	Stat	1201.6	1048.7	Stat	1043.2	664.6
Number of subjects/group (n)	8					
Section sampling fraction (ssf)	1/5					
Fraction of area of section sampled (asf)	(50 μ m x 50 μ m)/(100 μ m x 100 μ m)					
Thickness sampling fraction (h/t; tsf)	33 μ m/section					
Disector volume (h x A _{frame}) – μ m ³	65000 μ m ³					
Average number of cells counted (ΣQ)	24-hr	PF	PTD	2-wk	PF	PTD
	VEx	471.5	318.1	VEx	483.3	486.8
	Stat	423.6	312.3	Stat	407.2	351.5
Thickness of mounted section (t)	measured in software at each site = average ~35 μ m					
Height of disector (h)	26 μ m					
Observed coefficient of variation of estimator (OCE)	0.10					
Smoothness factor (m)	1.0					
Number of sections used (i)	6					

Table 2

Percent co-localization and soma size of ChAT+/nestin- and ChAT+/nestin+ neurons within in the MS/DB

A. Nestin/ChAT Co-Localization

24-hrs	PF	PTD	2-wks	PF	PTD
Stationary Control	36.60 ± 3.82	31.75 ± 3.05	Stationary Control	35.80 ± 1.49	30.80 ± 2.33
Voluntary Exercise	37.26 ± 2.14	37.52 ± 2.49	Voluntary Exercise	41.87 ± 3.20 [^]	40.80 ± 2.67 [^]

B. ChAT Soma Size

24-hrs	PF	PTD	2-wks	PF	PTD
Stationary Control	190.33 ± 5.51	172.98 ± 6.32 [*]	Stationary Control	180.58 ± 9.66	165.69 ± 9.11
Voluntary Exercise	186.70 ± 7.91	173.43 ± 8.39 [*]	Voluntary Exercise	188.12 ± 14.70	200.37 ± 8.18 [^]

C. Nestin Soma Size

24-hrs	PF	PTD	2-wks	PF	PTD
Stationary Control	165.16 ± 10.17	153.37 ± 11.42	Stationary Control	156.97 ± 10.24	160.31 ± 6.82
Voluntary Exercise	174.48 ± 14.42	182.30 ± 11.10	Voluntary Exercise	180.12 ± 8.55 [^]	176.52 ± 12.00 [^]

Exercise increased the co-localization rate at the 2-wk time point (**A**). PTD treatment decreased ChAT soma size, but this was recused by exercise after a 2-wk adaption period (**B**). Exercise increased Nestin soma size at the 2-wk period in both PF and PTD rats (**C**).

* indicates a significant effect of Treatment,

[^] indicates a significant effect of Exercise.