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Monitoring cholinergic activity during attentional performance in mice heterozygous for the choline transporter: a model of cholinergic capacity limits

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Abstract

Reductions in the capacity of the human choline transporter (*SLC5A7*, CHT) have been hypothesized to diminish cortical cholinergic neurotransmission, leading to risk for cognitive and mood disorders. To determine the acetylcholine (ACh) release capacity of cortical cholinergic projections in a mouse model of cholinergic hypofunction, the CHT+/− mouse, we assessed extracellular ACh levels while mice performed an operant sustained attention task (SAT). We found that whereas SAT-performance-associated increases in extracellular ACh levels of CHT+/− mice were significantly attenuated relative to wildtype littermates, performance on the SAT was normal. Tetrodotoxin-induced blockade of neuronal excitability reduced both dialysate ACh levels and SAT performance similarly in both genotypes. Likewise, lesions of cholinergic neurons abolished SAT performance in both genotypes. However, cholinergic activation remained more vulnerable to the reverse-dialyzed muscarinic antagonist atropine in CHT+/− mice. Additionally, CHT+/− mice displayed greater SAT-disrupting effects of reverse dialysis of the nAChR antagonist mecamylamine. Receptor binding assays revealed a higher density of α4β2* nAChRs in the cortex of CHT+/− mice compared to controls. These findings reveal compensatory mechanisms that, in the context of moderate cognitive challenges, can overcome the performance deficits expected from the significantly reduced ACh capacity of CHT+/− cholinergic terminals. Further analyses of molecular and functional compensations in the CHT +/− model may provide insights into both risk and resiliency factors involved in cognitive and mood disorders.

Keywords

choline transporter; acetylcholine; attention; microdialysis; nicotinic acetylcholine receptor; mouse

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1. Introduction

The basal forebrain cholinergic projections to the cortex are necessary for attentional performance. Decline in the regulation and integrity of the basal forebrain cholinergic system has been attributed to age- and disease-associated impairments in cognitive capacities (Xie and Guo, 2004; Counts and Mufson, 2005; Mufson et al., 2000). In animals, removal of these cholinergic neurons (McGaughy et al., 1996, 1998; Turchi and Sarter, 1997; Dalley et al., 2004; Botly and De Rosa, 2011) or molecular or pharmacological manipulations of their excitability (e.g., Holley et al., 1995; Turchi and Sarter, 2001a,b; Parikh et al., 2013b) all impair attentional performance. Cholinergic neurotransmission supports attention by amplifying the processing of thalamic input in the cortex, thereby enhancing the detection and processing of selected stimuli, by optimizing the synchronization of task-related neuronal activity across cortical regions (e.g., Disney et al., 2007; Hasselmo and Bower, 1992; Herrero et al., 2008; Oldford and Castro-Alamancos, 2003; Thiele et al., 2012; Howe et al., 2013).

The choline transporter (CHT) imports choline for the synthesis of acetylcholine (ACh) and therefore regulates the capacity of cholinergic neurons to release ACh (Simon et al., 1976; Simon and Kuhar, 1976; Yamada et al., 1989). The capacity of CHTs is regulated by the trafficking of CHTs between intracellular membrane and the cell surface (or plasma membrane), as well as by steps concerning the incorporation and state of CHTs in the plasma membrane (Ferguson et al., 2003; Ferguson and Blakely, 2004; Gates et al., 2004; Ribeiro et al., 2006, 2007; Xie and Guo, 2004; Apparsundaram et al., 2005). Recent studies demonstrated associations between genetic variations of the CHT and psychiatric disorders and related psychological vulnerabilities (Hahn et al., 2008; English et al., 2009; Neumann et al., 2006), thereby indicating the significance of understanding the impact of suboptimal CHT capacity for cholinergic neurotransmission and for the behavioral and cognitive processes that depend on, or are modulated by, cholinergic activity (for review see Hasselmo and Sarter, 2011).

Mice that are heterozygous for the CHT have proven valuable for evaluation of the cholinergic dependence of neuromuscular, cardiovascular and behavioral capacities (Ferguson et al., 2004; Bazalakova et al., 2007; English et al., 2010). We previously employed electrical stimulation of basal forebrain (BF) projections to demonstrate the limited capacity of the cortical cholinergic input system in these animals. Furthermore, we showed that the outward trafficking of CHTs produced by an operant, sustained attention task (SAT), **and in the presence of a distractor**, was attenuated in these mutants (Parikh et al., 2013a). The present experiments addressed the essential question of whether CHT heterozygosity also limits cholinergic output *in vivo* while performing an attention task known to require and increase cortical cholinergic activity (e.g., Kozak et al., 2007; St Peters et al., 2011b; Paolone et al., 2012, 2013).

For the experiments described below, we modified established microdialysis methods for use in mice performing the SAT. The SAT previously has been used in rats and humans to study the regulation and function of cholinergic neurotransmission and brain circuitry for attention in general (Demeter et al., 2008, 2013; Nuechterlein et al., 2009; Luck et al., 2011). We developed a mouse version of this task, in part by introducing a new response device that supports levels of performance in mice comparable to levels seen in rats (St Peters et al., 2011a). The present use of microdialysis in mice performing a cognitive task, to our knowledge, has rarely been achieved before. The collective results from our experiments indicate that SAT performance in CHT+/− mice, as in wildtype animals, depends on cholinergic activity, though the expected deficits appear to be partly compensated by an elevation in the density of cortical nicotinic ACh receptors (nAChRs).

2. Materials and methods

2.1. Subjects

Subjects were male and female mice (N=90), bred in a laboratory colony at the University of Michigan. Mice heterozygous for the choline high-affinity transporter (CHT+/−; Ferguson et al., 2004) were originally obtained from Vanderbilt University (R.D. Blakely's Laboratory), maintained on a C57BL/6 genetic background for over seven generations, and they were continued to be bred on this background at the University of Michigan. The analyses of attentional performance, performance-associated ACh release, and pharmacological manipulations did not reveal an effect of sex or interactions between genotype and sex, and thus this factor was not included in the final analyses.

Mice were genotyped at weaning by a commercial vendor (Transnetyx, Cordova, TN). CHT +/− and wild-type mice (CHT+/+; congenic on a C57 background) were used (n=45 each). Animals were housed individually in a temperature (23°C) and humidity-controlled (45%) environment, and kept on a 12:12 light/dark cycle (lights on at 7:30 a.m.). Training and testing on the SAT took place during the light phase; this method is justified given that in rodents, daily attention practice evokes a diurnal activity pattern (Gritton et al., 2009, 2012, 2013; Paolone et al., 2012). Mice were 12 weeks of age and weighed 20-30 g at the beginning experiments. Mice not performing the SAT had *ad libitum* access to food and water. SAT-performing mice were gradually water-deprived over a five-day period prior to the onset of training. Water was then restricted to a 4-min period following each training session. During SAT sessions, correct responses were rewarded using sweetened water (0.2% saccharin; 6 μL per reward; total average session delivery: 0.45 mL). On days without SAT practice, water access in the animals' home cages was increased to 10 min. Food (Rodent Chow, Harlan Teklad, Madison, WI) was available *ad libitum*. During SAT training, animals' body weights remained stable at 90-100% of their *ad libitum* body weights (weighed twice weekly). SAT performing mice were trained six days per week, and training and testing took place between 12:00 PM and 2:00 PM. All procedures were conducted in adherence with protocols approved by the University Committee on Use and Care of Animals at the University of Michigan and in AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care)-accredited laboratories. The statistical results include degrees of freedom to indicate the number of animals per experiment and condition.

2.2. Behavioral methods

2.2.1. Behavioral apparatus, task acquisition and performance criteria—The SAT was recently adapted for use mice (for a detailed description of the equipment and procedural modifications see St Peters et al., 2011a). Behavioral training and testing took place in twelve modified operant chambers (24.10 cm L × 20.00 cm W × 29.50 cm H; MedAssociates, Inc., St. Albans, VT), situated inside sound-attenuated chambers fabricated at the University of Michigan (Ann Arbor, MI). Each operant chamber contained an intelligence panel equipped with two panel lights (2.8 W), two retractable "Michigan Controlled Access Response Ports" (MICARP; St Peters et al., 2011a), and a liquid dispenser (6 μL of 0.2% saccharin in de-ionized water per delivery). Stimuli and response recordings were implemented using SmartCtrl Package 8-In/16-Out with additional interfacing by MED-PC for Windows (Med Associates, Inc., St. Albans, VT) and custom programming. The MICARPs were located laterally on either side of the liquid dispenser, and the two panel lights were located directly above the liquid dispenser. The MICARPs and the liquid dispenser each contained perpendicular infrared photo-beams to detect nose-poke responses. A house light (2.8 W) was located at the top of the rear wall. Each sound-

attenuating chamber was also equipped with a ventilation fan, a video camera, and 4 red LEDs (JameCo P/N 333489; Jameco Valuepro, Belmont, CA).

The task, training procedures, and validity of the performance measures in terms of indicating sustained attention in humans, rats, and most recently, mice have been described previously (Demeter et al., 2008; St Peters et al., 2011a). Briefly, animals were initially shaped to reach the water dispenser using a modified fixed ratio-1 (FR-1) reward schedule. After obtaining ≥100 water rewards within the 40-min training session for two consecutive days, animals began SAT training. First, mice were familiarized with moving MICARPS. Both MICARPS extended until a nose-poke triggered retraction. Mice were required to generate 90 rewards/session and retrieve at least 80% of these rewards in 1.5 s after the nose poke for two consecutive sessions before moving to the next training stage. During all subsequent training stages, mice were required to distinguish between signal (illumination of the central panel light) and non-signal (no illumination) events. The house lights of the cage were kept off until the last stage of training, to increase the relative saliency of the signal. On signal trials, a left nose-poke was reinforced and termed at “hit.” On non-signal trials, a right nose-poke was reinforced and termed a “correct rejection.” Half of the animals were trained following the reverse rules. Incorrect responses to signal trials (“misses”) and non-signal trials (“false alarms”) triggered an inter-trial interval (12±3 s) but had no further scheduled consequences. An omission was recorded if the animal failed to nose-poke during the lever extension following an event. Signal and non-signal trials were presented in a pseudo-randomized order.

In the first training stage, the signal light remained illuminated for 500 ms on signal trials. MICARPs were extended 1 s after the signal onset and remained extended for 4 s or until a nose poke triggered a retraction. During non-signal trials, the MICARPs were likewise extended for 4 s or until a nose poke was initiated. Incorrect responses were followed with up to three correction trials, during which the previous trial was repeated. If the animal failed to respond correctly to all three correction trials, a forced trial was initiated, during which only the MICARP corresponding to the correct response extended. After reaching stable performance, defined by at least three consecutive days of obtaining >60% hits and correct rejections with <20% omissions, signal length and time delay before nose-poke extension were reduced (1 sec and 0.5 s, respectively). When animals again reached stable performance, multiple signal durations were introduced (500, 50, or 25 ms). The session length was 40 min, allowing for *post hoc* analysis of performance over five 8-min blocks. Pseudo-randomization was designed to ensure equal numbers of signal and non-signal trials as well as trials with 500, 50 or 25 ms signals (approximately 160 trials/session). Forced correction trials were eliminated at this point. After reaching stable performance (>60% hits to 500 ms trials and correct rejections, <20% omissions for three consecutive days), animals moved to the final stage training and the actual SAT task. During this stage, the operant chamber house light was illuminated, requiring animals to sustain orientation towards the signal panel. When performance on the SAT was stable at criterion (identical to the previous stage), animals underwent either implantation of a microdialysis cannula into the right mPFC to allow for determination of ACh levels, or received bilateral BF cholinergic lesions (see below) to determine the necessity of cholinergic signaling for maintaining attentional performance. Once animals recovered from surgery water deprivation and behavioral training resumed.

After recovery from cannulation surgery, SAT training continued using operant chambers modified to accommodate head stages and in-and outlet. To provide additional overhead clearance for the guide cannula and dental cement head cap, the roof of the water reward port (Med Associates ENV-303LPHD) was raised by 1 cm. Reward access was also enhanced by trimming the front of the housing 5 mm to bring the water cup and the photo

cells closer to the port entrance. Openings in the ceilings of the operant and sound attenuating chambers accommodated the microdialysis tubing. To mimic dialysis conditions, animals were tethered with glass capillary tubing (Polymicro Technologies, Phoenix, Arizona; ID: 101.0 μm, OD: 362 μm) encased in protective plastic tubing. For at least two weeks preceding dialysis, animals remained tethered in the operant chambers for two hours prior to task-onset to mimic the two-hour ACh release stabilization period allotted before the start of microdialysis collections. Microdialysis was conducted once mice achieved stable SAT performance under mock-dialysis conditions.

2.2.2. Performance measures—Hits, misses, correct rejections, false alarms, and omissions were recorded for each SAT session. The relative number of hits (hits/hits +misses) was calculated for each signal length, as was the relative number of correct rejections (correct rejections/correct rejections+false alarms). As an overall measure of attentional performance, that takes into account both the relative number of hits (h) and false alarms (f), an overall performance measure (SAT score) was calculated in accordance to: $SAT=(h-f)/[2(h+f)–(h+f)^2]$, where h is the relative number of hits and f is the relative number of false alarms. This measure was derived from the Sensitivity Index (Si; Frey and Colliver, 1973), except that the SAT score is based on the relative number of hits and false alarms, as opposed to probabilities for hits and false alarms, and thus is not confounded by errors of omissions. Scores range from +1.0 to –1.0, with +1.0 indicating all responses were hits and correct rejections, 0 representing an inability to discriminate between signal and non-signal events, and –1.0 indicating all responses were misses and false alarms. SAT scores were calculated separately for each signal duration ($SAT_{500,50,25}$) or averaged over all durations. Errors of omission were recorded separately. Measures of performance were calculated separately for each of the five, 8-min task blocks.

2.3. Surgical procedures

2.3.1. Implantation of guide cannulas for the measurement of atropine-associated and task-associated ACh release—Surgery was performed under aseptic conditions. Animals' heads were shaved using electric clippers and cleaned with an alcohol wipe. Ophthalmic ointment was applied to lubricate animals' eyes. Animals were initially anesthetized with 2-3% isoflurane in an anesthetic chamber (Anesco/SurgiVet). Gas was carried via oxygen at a flow rate of 0.6 L/min. Animals were then mounted to a stereotaxic instrument (David Kopf Instruments, Tujunga, CA). Isoflurane was administered via a facemask and the levels were reduced to 1-2% for the remainder of surgery. Microdialysis guide cannulas (Model CMA 7, Harvard Apparatus, Holliston, MA) were implanted into the right medial prefrontal cortex (mPFC) using the following coordinates, relative to bregma: AP: +1.8 mm, ML: +0.4 mm, DV: −0.1 mm below dura. The focus on the right mPFC is based on evidence indicating that only right cholinergic lesions impair the detection of signals in this SAT (Martinez and Sarter, 2004) and that SAT performance elevates markers of cholinergic activity in the right but not left mPFC of rats as well as mice (Apparsundaram et al., 2005; Parikh et al., 2013a). Likewise, the SAT activates prefrontal regions in the right hemisphere of humans (Demeter et al., 2011). The guide cannula was secured to the skull using stainless steel screws and dental cement. To prevent clogging of the guide cannula, a stainless steel stylet (O.D. 0.38 mm) with no projection past the guide cannula tip was inserted until microdialysis sessions. Animals were given injections of an antibiotic (amikacin; 5.0 mg/0.02 mL) and an analgesic (buprenorphine; 0.01 mg/kg, s.c.). Neosporin was applied to the area around the headstage. Animals recovered in their home cages for 4 days, with *ad libitum* access to food and water.

2.3.2. Cholinergic lesions—Cholinergic neurons projecting to the cortex were removed by infusing the immunotoxin murine-p75NTR-saporin (mu-p75-SAP, Advanced Targeting

Systems, San Diego, CA) bilaterally into the nucleus basalis and substantia innominata of the basal forebrain. Mice were anesthetized and placed on a stereotaxic apparatus as described above. Animals were kept hydrated with 0.2 mL injections (i.p.) of 0.9% NaCl, and body temperature was maintained at 37°C using an isothermal pad. mu-p75-SAP was diluted to a concentration of 0.65 μg/μL in 0.9% NaCl and a bolus-delivered, using a 1 μL Hamilton syringe. A total volume of 0.5 μL/site, with one site per hemisphere, was infused at the following stereotaxic coordinates relative to bregma: AP: −0.7 mm, ML ±1.9 mm, DV −4.0 mm below dura. At each infusion site, the needle was left in place for an additional 5 min to allow for proper absorption of the toxin in the target region (see above for post-surgery procedures). Animals had *ad libitum* access to food and water for 7 days prior to the start of post-surgery retraining.

2.4. Microdialysis methods and determination of ACh concentrations

2.4.1. General methods—To acclimate animals to testing conditions, mice were transferred in their home cages from the housing room to the testing room, and tethered with polyethylene tubing for 4 h each on five separate days. Microdialysis began with the removal of the stylet and insertion of a concentric probe with a 2.0 mm membrane tip (Model CMA 7.0; membrane o.d. 0.24 mm, shaft o.d. 0.38 mm; Harvard Apparatus) into the guide cannula. Mice were perfused at a rate of 2 μL/min with artificial cerebral spinal fluid (aCSF), pH 6.8±0.1, containing the following (in mM): 126.5 NaCl, 27.5 $NaHCO_3$, 2.4 KCL, 0.5 Na_2SO_4, 0.5 KH_2PO_4, 1.2 $CaCl_2$, 0.8 $MgCl_2$, and 5.0 dextrose. Animals were perfused for 120 minutes prior to the start of collections to allow ACh release to stabilize. After the stabilization period, six 8-min baseline dialysates were collected. As in our prior experiments in rats, animals were dialyzed for a maximum of 4 times because additional probe insertions result in alterations in basal release levels (e.g., Kozak et al., 2007; St Peters et al., 2011b).

2.4.2. Analytical methods—Dialysate samples were kept at −80°C until analyzed with high-performance liquid chromatography (HPLC) with electrochemical detection (ESA, Chelmsford, MA; mobile phase: 35 mM sodium phosphate, 0.43 mM ethylenediamine tetracetic acid tetrasodium salt and 5 mL/L ProClin; BASi, West Lafayette, IN). ACh was separated from choline on a 250 mm analytical column and catalyzed on a post-column solid-phase reactor containing acetylcholinesterase and choline oxidase. ACh was then hydrolyzed to acetate and choline, and choline oxidized to hydrogen peroxide and betaine. The amount of hydrogen peroxide corresponding to ACh was then detected using a 'peroxidase-wired' glassy carbon electrode with an applied potential of −200 mV. To calculate the concentration of ACh in each sample, the integral of the area under the peak was taken and fit to a regression line containing known values of ACh in the expected range of the *in vivo* dialysates. *In vitro* recovery of probes varied from 8 to 15%; ACh concentrations were not adjusted for probe recovery. The detection limit of this system averaged 5 fmol/15 μL.

2.4.3. Reverse dialysis of atropine in non-performing mice—The muscarinic acetylcholine receptor (mAChR) blocker atropine is a potent ACh releaser, by blocking presynaptic mAChR (e.g., Moore et al., 1996). Atropine administration served to challenge the capacity of ACh release in CHT+/− mice. After the release stabilization period (above), six 8-min baseline dialysates were collected. Following the sixth baseline collection, the input line was switched from the aCSF syringe to a second syringe containing 50 M atropine dissolved in aCSF. After a wait-period that accounted for the probe and inlet and outlet tubing dead volume, collections resumed. Five 8-min collections were taken as the drug was perfused (D1-5). The input line was then switched back to the aCSF syringe, and four post-drug collections were taken after the dead-volume wait-period (AD1-4). The probe was then

removed, the stylet replaced, and the animals returned to their housing room. In each dialysis session, the last three baseline collections prior to the onset of drug perfusion were averaged to calculate basal ACh release. Atropine-associated release was expressed as a percent change from baseline.

2.4.4. Microdialysis in task-performing mice—Prior to probe insertion, the polyethylene input and output lines attached to the probes were cut off and replaced with glass capillary lines (Polymicro Technologies, Phoenix, Arizona; ID: 101.0 μm, OD: 362 μm) encased in protective plastic tubing (Zeus PTFE, Teflon; ID: 305 μm). The length of these lines was customized for the height of the operant box and sound-attenuating chambers; these lines also proved more resistant to coiling compared to polyethylene tubing. At the start of the test session, the stylet was removed and the probe inserted through the guide cannula. Animals were placed into the operant chambers and the probes were perfused with aCSF at a rate of 2 μL/min for 120 minutes to allow ACh release to stabilize. The operant chamber house light remained on prior to, during, and after the SAT. After the stabilization period, six 8-min baseline dialysates were collected. Following baseline collections, the SAT began. After the onset of the task, the timing before dialysate collections was adjusted to correct for the dead volume of the probe and outlet tubing, and then five 8-min collections taken. Five additional 8-min collections were taken post-task. Mice were then removed from the operant chambers, the microdialysis probe removed and the stylet reinserted. Mice returned to their home cages. The last three baseline collections prior to the task onset were averaged to calculate basal ACh release. Task associated release was expressed as percent increase from baseline.

2.4.5. TTX sensitivity of performance-associated ACh release—In order to demonstrate that performance-associated ACh increases were due to local depolarization of axons, the voltage-gated sodium channel blocker tetrodotoxin (TTX, 1 μM dissolved in aCSF) was reverse dialyzed during task blocks 3-4 of a SAT session. The input line was switched from the aCSF syringe to a second, TTX-containing syringe prior to the third task block, and switched back to aCSF prior to the fifth task block. The timing of the input lines switches between aCSF and TTX-containing syringes was adjusted for the dead-volume of the probe and tubing inlet. As with standard SAT dialysis, five dialysate collections each were taken pre-task, during SAT performance, and post-task. Following task onset, the start of collections was adjusted for the dead volume of the probe and tubing outlet. ACh release is reported as “0” in cases in which ACh was undetectable in collections taken during or following TTX perfusion.

2.4.6. SAT performance challenge by reverse-dialysis of mecamylamine—Extracellular ACh as measured by microdialysis detects the tonic component of cholinergic neurotransmission and this measure can be dissociated from electrochemical measures of second-based cholinergic transients (Sarter et al., 2012). Tonic cholinergic activity modulates cortical circuitry via stimulating nicotinic ACh receptors, thereby optimizing cue detection operations (Sarter et al., 2009; Parikh et al., 2010; Howe et al., 2010; Hasselmo and Sarter, 2011). Based on the finding that SAT performance-associated increases in ACh release were attenuated in CHT+/− mice (below), we reverse-dialyzed the non-specific nAChR antagonist mecamylamine to test the hypothesis that blocking tonic cholinergic modulation of mPFC circuitry reveals a greater attentional vulnerability in CHT+/− mice. Mecamylamine (MEC; 50 μM dissolved in aCSF) was perfused via reverse dialysis during SAT blocks 3-5. The input line was switched from the aCSF syringe to a second syringe containing MEC prior to the third task block, and switched back to the aCSF syringe prior to the first post-task collection. Again, the timing of these syringe switches was adjusted for the probe and input line dead volume. Five dialysate collections each were taken pre-task,

during SAT performance, and post-task. The timing of the start of collections following task onset was adjusted for the dead volume of the probe and output tubing.

2.5. Histological verification of probe placement and cholinergic legion

Following completion of the microdialysis experiments, animals were given an overdose of sodium pentobarbital and perfused with 0.1% phosphate buffer solution (PBS) followed by 4% paraformaldehyde in 0.15 M phosphate buffer and 15% saturated picric acid, pH 7.4. Brains were postfixed for 4 hrs and thereafter cryoprotected in 30% sucrose in PBS. For probe placement verification, coronal brain sections (35 μm) near the probe and cannula sites were sliced using a freezing microtome (CM 2000R; Leica). Slices were mounted on gelatin-coated glass slides and allowed to dry completely before being Nissl-stained and examined for probe placement.

To estimate the extent of cortical cholinergic deafferentation, sections were stained using a polyclonal goat anti-choline acetyltransferase (ChAT) antibody (Millipore, Temecula, CA] and the Vestastain Elite ABC kit (PK-6105; Vector Laboratories; Burlingame, CA). Sections were first rinsed in 0.1 M PBS (pH 7.4) three times for 10 min each in an orbital shaker and were then incubated in 0.3% peroxide for 30 min. Sections were then incubated in blocking buffer (10% goat serum in 0.1 M PBS) for 60 min under constant shaking followed by an overnight incubation in the primary antibody (goat anti-ChAT made in rabbit, 1:300 dilution in 0.1M PBS containing 1% goat serum and 0.1% triton X-100) at 4°C. The following day, sections were rinsed three times 10 min each in 0.1 M PBS with 0.1% triton X-100. They were then incubated in a biotinylated goat anti-rabbit IgG (Vectastain Elite ABC; PK-6105; Vector Laboratories; Burlingame, CA) 1:200 for 2 hours. Sections were then rinsed three times for 5 minutes each in 0.2% Triton-X in 0.1M PBS followed by incubation with the avidin-biotin complex (Vectastain) for 30 minutes. Following rinsing in 0.1M PBS, sections were incubated in a peroxidase substrate solution containing 0.4% DAB and 0.19% nickel (II) chloride in 0.1M PBS. 10μL of 30% hydrogen peroxide was added after the tissue began the incubation. Once sections showed a significant level of background stain (5-10 min), sections were treated with four 5 min rinses with 0.1 M PBS and mounted on gelatin-coated slides to dry overnight. Following dehydration in an ascending series of alcohol rinses and clearing with xylene, slides were coverslipped with DPX.

Semi-quantitative estimates of residual ChAT-positive fiber counts were obtained from frontoparietal cortex (between 0.5 and 1.00 mm anterior to bregma) of both hemispheres (e.g., Burk and Sarter, 2001; Burk et al., 2002). 40X images were taken using a Leica DM4000B digital microscope equipped with a Spot Digital Camera and using a Spot Software (Diagnostic Inc., Sterling Heights, MI, USA). We used a counting grid method to estimate the number of fibers in each quantified region of interest. Briefly, using Adobe Photoshop CS3 software, a grid containing 50 μm squares was superimposed over each image, generating a 250 × 250 μm region for counting. ChAT-positive stained fibers that traversed any line of this grid were counted and the overall counts from the right and left hemispheres of each mouse was averaged for statistical analysis.

For immunohistochemical visualization of the lesions, free floating sections were incubated overnight with 1/3000 diluted rabbit anti-vesicular acetylcholine transporter (VAChT; No. 139103, Synaptic Systems, Goettingen, Germany) antibody (in PBS containing 0.2% Triton X-100 and 2% normal goat serum, 4°C). On the next day sections were washed and incubated for 30 min in a 1/1000 dilution of Alexia 488-conjugated antibody against rabbit IgG (Molecular Probes, Eugene, OR). Immunofluorescence was visualized by confocal microscopy (LSM 710; Zeiss).

2.6. [^{3}H]cytisine saturation binding

As CHT+/− mice were found to perform the SAT at levels similar to those seen in wildtype mice despite exhibiting significantly attenuated levels of ACh (see Results), we conduced [^{3}H]cytisine saturation binding assays to determine the state of α4β2-containing nAChRs (Pabreza et al., 1991; Bitner et al., 2007; Rollema et al., 2010). We analyzed left and right frontal cortical tissue from naive mice and from mice which had performed the SAT for at least 65 sessions, with brain tissues harvested immediately after their final session. Briefly, animals were anesthetized (urethane, 1.25-1.5 g/kg, i.p.) decapitated within 2 min from the injection and frontal cortical tissues (medial frontal, cingulate and dorsal frontal tissues) were collected, immediately frozen at −80°, and shipped to T.R.M. To prepare cell membranes for [^{3}H]cytisine saturation binding assays, individual tissues were homogenized in 20 ml cold BSS-Tris buffer (in mM: 120 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, and 50 Tris-HCl, pH 7.4, 4°C) using an Ultra-Turrax T25 homogenizer (IKA®, Wilmington, NC). The homogenates were diluted with 10 ml additional BSS-Tris buffer and centrifuged at 41,000×*g* for 20 min at 4°C. The resulting pellets were similarly homogenized in 6 ml BSS-Tris buffer and used immediately in the [^{3}H]cytisine binding assays. Cell membrane suspensions and various concentrations of [^{3}H]cytisine (Perkin Elmer, Downers Grove, IL) (specific activity 33 Ci/mmol) were combined in polypropylene deep well microplates and incubated at 4°C for 75 minutes. Saturation binding assays included 6 concentrations of [^{3}H]cytisine, ranging from approximately 0.1 to 8.0 nM; non-specific binding at each [^{3}H]cytisine concentration was defined by co-incubations with 10 μM (-)nicotine (Sigma-Aldrich, St. Louis, MO). Following incubations, assays were terminated by vacuum filtration through polyethylenimine (0.3%) pre-soaked Whatman GF/C filters (Brandel, Gaithersburg, MD), with extensive rinsing with cold 50 mM Tris-HCl, pH 7.4. The amounts of added and bound [^{3}H]cytisine were determined using Ultima Gold™ LSC-cocktail (Perkin Elmer, Shelton, CT) and the Tri-Carb 2900TR Liquid Scintillation Analyzer (Perkin Elmer, Shelton, CT). Subsequently, cell membrane protein concentrations were determined using the Pierce® BCA Protein Assay Kit (Thermo Scientific, Rockford, IL); the average (± SEM) g membrane protein added per assay well was 78.4±1.8. Saturation binding data were analyzed by nonlinear regression with GraphPad Prism (San Diego, CA) software to determine [^{3}H]cytisine B_{MAX} and K_D values, respective measures of [^{3}H]cytisine binding capacity (indicative of α4β2 nAChR density) and affinity.

2.7. Statistical methods

Because of the complexity of the designs employed for the multiple experiments described below, the main factors and statistical methods are described with the results. Generally, mixed-design ANOVAs were employed to determine the effects of sex, genotype, task block (t1-t5), signal duration (500, 50, or 25 ms), lesion, and drug perfusion on performance. *Post hoc* multiple comparisons were conducted using t test and Fisher's least significant difference (LSD) test. Alpha was set at 0.05. Statistical analyses were performed using SPSS for Windows (V. 17.0; SPSS Inc., Chicago, IL). In cases in which the sphericity assumption was not met, Huyhn-Feldt-corrected *F* values are given; uncorrected degrees of freedom are reported to indicate the number of subjects.

3. Results

3.1. Atropine-induced cortical ACh release in CHT +/− and WT mice

The goal of the first experiment was to determine whether CHT heterozygosity affects the capacity of cholinergic neurons to release ACh *in vivo* and in response tolocally applied atropine. Atropine blocks presynaptic autoinhibitory mAChRs (Yan and Surmeier, 1996), thereby potently releasing ACh (Moore et al., 1996). Prior studies have revealed diminished mAChR expression in multiple brain regions of CHT +/− mice, including cortex, as well as

a blunted locomotor response to mAChR antagonist injection (Bazalakova et al., 2007). Pre-drug basal ACh release did not differ between WT and CHT +/− mice ($t(6)=0.46$, n.s.; M, SEM: 3.46±0.41 fmol/15 μL). Atropine-evoked increases in extracellular ACh concentrations were therefore expressed as percent changes from baseline (see Methods for determination of basal ACh levels). Atropine (50 μM)-induced increases in cortical ACh release were significantly lower in CHT +/− mice (main effect of genotype: $F(1,6)=9.63$, $p<0.05$; percent increase from baseline M; SEM: WT: 74.62±10.70%; CHT: 20.43±13.80%). The effects of atropine varied over the collection periods while drug was reverse-dialyzed (main effect of block: $F(4,24)=3.16$, $p<0.05$), but this effect did not interact with genotype ($F(4,24)=0.28$, n.s.; Fig. 1), reflecting that the rate of the decline in ACh levels did not differ between the two groups. These results are consistent with the hypothesis that a reduced density of CHT limits the capacity of cholinergic neurons to respond to presynaptic muscarinic receptor antagonism.

3.2. Pre-surgery SAT acquisition and criterion performance

SAT performance is mediated by, and requires, increases in prefrontal ACh release (Kozak et al., 2006, 2007; St Peters et al., 2011b; McGaughy et al., 1996). Furthermore, SAT-associated increases in cholinergic activity entrain circadian rhythms and are influenced by neuronal clocks, inducing diurnality in rodents and optimizing fixed-time performance, respectively (Paolone et al., 2012; Gritton et al., 2013). Because of the limited capacity for electrically (Parikh et al., 2013a) and pharmacologically-evoked ACh release in CHT+/− mice (above), the performance of CHT+/− mice as well as SAT-associated increases in ACh release were expected to be lower in CHT+/− mice when compared with WT animals. When tested, WT and CHT+/− mice reached performance criterion (>60% hits on 500 ms signals and correct rejections, <20% omissions for three consecutive days) on the final stage of the SAT equally readily (number of sessions to criterion: $t(21)=0.23$, n.s.; 67.83±3.14 days). For each animal, the average of three days on criterion performance, prior to cannulation surgery, was calculated. In terms of overall SAT performance, as indicated by the SAT score that integrates signal and non-signal trial performance (see Methods), longer signals generally yielded more hits ($F(2,44)=191.39$, $p<0.001$; see Fig 2b for multiple comparisons. SAT scores however did not differ by genotype or across blocks of trials. Furthermore, the effects of genotype, block, and signal duration did not interact significantly (genotype: $F(1,22)=0.18$, n.s.; all other effects and interactions involving genotype as a factor: $p>0.05$).

Analysis of the individual measures of performance likewise did not reveal any effects of genotype. As expected, the effect of signal duration on SAT scores reflected a similar effect on hits ($F(2,44)=147.87$, $p<0.001$), but again, this effect did not interact with genotype (effect of genotype and all interactions involving genotype: $p>0.05$). However, CHT+/− mice correctly rejected fewer non-signal events ($F(1,22)=4.84$, $p<0.05$; WT: 78.6±1.8%, CHT+/−: 72.9±1.8%). Correct rejections also varied across blocks of trials ($F(4,88)=2.90$, $p<0.05$) but the two factors did not interact ($F(4,88)=0.072$, n.s.; Fig. 2c). Errors of omission did not differ by genotype ($F(1,22)=0.06$, n.s.) but increased toward later block of trials ($F(4,88)=13.92$, $p<0.001$; Fig. 2). Thus, with the exception of a small reduction in their correct rejection rate, baseline SAT performance rejected the original hypothesis that CHT+/− mice exhibit lower levels of performance than their WT counterparts.

3.3. SAT performance during microdialysis and performance-associated increase in cortical ACh release

After animals recovered from surgery, they were water deprived and resumed SAT training. To aid recovery of task performance, animals were initially placed back on a discrimination task stage (1-s illumination of the signal light) and allowed to progress through the subsequent task stages as before. The time to return to criterion on the final task stage

following surgery did not differ between WT and CHT+/− mice ($t(21)=0.31$, n.s.; 26.35±3.34 days). Additionally, conducting microdialysis in cognitive task-performing animals requires tethering and connecting inlets and outlets, and these challenges may limit the demonstration of systematic relationships between release events or release levels. Mice were extensively habituated to performing the SAT while tethered and under mock-dialysis conditions. As a result, SAT performance during microdialysis sessions did not significantly differ from the animals' pre-surgery baseline (above; genotype × task block × session type on SAT scores, hits, correct rejections, omissions: all $F<1.74$, n.s.).

3.4. SAT performance-associated ACh release

Pre-task (or 'basal'; see Methods) ACh release ($F(1,9)=0.16$, n.s.) and task-associated ACh release ($F(1,11)<0.001$, n.s.) did not differ by sex. Thus, data from male and female mice were collapsed for all further analyses. Pre-task basal ACh release did not differ between SAT-performing WT and CHT+/− mice ($t(11)=1.13$, n.s.; 6.66±0.77 fmol/15 μL). However, pre-task ACh levels were significantly higher than basal levels in non-performing mice and prior to the atropine challenge (above; $F(1,17)=8.20$, $p<0.05$; basal non-performing: 3.52±0.86 fmol/15 μL; performing: 6.59±0.65 fmol/15 μL; genotype and interaction: both n.s.), reflecting cholinergic activation in both strains as a function of fixed daily practice time (Paolone et al., 2012). In WT mice, SAT performance-associated increase in ACh release was comparable to that seen previously in rats (e.g., St Peters et al., 2011b). In contrast, in CHT+/− mice, this increase was greatly attenuated (main effect of genotype: $F(1,11)=13.703$, $p<0.01$; WT: 131.74±18.14%; CHT+/−: 40.36±16.79%; Fig. 3a). On both WT and CHT+/− mice, performance-associated increases in ACh levels remained stable across the five task blocks ($F(4,44)=1.53$, n.s.), and there was no interaction between genotype and block ($F(4,44)=2.15$, n.s.; Fig. 3a). Thus, although CHT+/− mice performed the SAT as well as WT mice, their performance was associated with levels of ACh release that were less than half of those observed in WT mice.

3.5. Task-associated ACh release is TTX-dependent

Because these experiments were the first to measure ACh release during SAT performing in mice, and because the finding described above warrants the demonstration that ACh release in CHT+/− mice depends on neuronal depolarization, the blocker of voltage-gated sodium channels, tetrodotoxin (TTX; 1 μM), was reverse-dialyzed during SAT trial blocks 3 and 4. Pre-task basal ACh release again did not differ between WT and CHT+/− mice ($t(5)=1.30$, n.s.; 9.61±2.91 fmol/15 μL). Prior to TTX administration (trial blocks 1 and 2), SAT-associated increase in ACh release was again attenuated in CHT+/− mice when compared with WT mice ($t(5)=2.82$, $p<0.05$). TTX suppressed ACh release in 6/14 collections to below the level of detection (5 fmol/15 μL). Detectable residual ACh concentrations (T3-T5) were 58.48±14.57% and 39.72±13.55% below basal ACh levels in WT and CHT+/− mice, respectively (Fig. 3b). ACh levels during and after TTX administration (T3-AT5) did not differ between the strains (genotype and genotype × block, both n.s.; Fig. 3b). Thus, performance-associated increases in ACh release were highly and comparably dependent on neuronal depolarization in both strains.

As a result of TTX perfusion, SAT performance was characterized by near-random selection of the response ports (chance performance: SAT scores <0.17) during the second 8-min period of TTX administration and the final collection period thereafter (blocks 4 and 5; WT: 0.29±0.07; CHT+/−: 0.20±0.19; n.s.). Thus, in both strains, blockade of neuronal transmission in the right mPFC was sufficient to disrupt SAT performance.

3.6. SAT performance in CHT+/− mice depends on the integrity of the cortical cholinergic input system

We previously demonstrated that cortical cholinergic deafferentation permanently impairs SAT performance in rats, specifically by increasing the number of misses in signal trials (McGaughy et al., 1996). Thus cortical cholinergic activity is necessary for this task. Because CHT+/− mice performed the SAT at levels comparable to WT mice but with only ~40% increase in ACh release, contrasting with ~140% in WT mice, we tested the hypothesis that SAT performance in CHT+/− mice does not depend on the presence of the cholinergic system. Therefore, we removed corticopetal cholinergic neurons by bilaterally infusing murine-p75NTR-saporin into the basal forebrain of WT and CHT+/− mice after they reached the SAT performance criterion.

Following recovery from surgery, animals were placed back on a discrimination task stage (1-s illumination of the signal light), from which they progressed until again reaching the final task stage and stable asymptotic performance. The final analyses were based on performance during post-surgery sessions 28-30. This lesion significantly and comparably impaired the performance of both strains of mice (SAT score; lesion: $F(1,9)=49.82$, $p<0.001$; genotype: $F(1,9)=1.06$, n.s.; genotype × lesion: $F(4,36)=0.003$, n.s.; Fig. 4a). As in rats, the cholinergic lesion-induced impairment in SAT performance was due exclusively to a decrease in hits ($F(1,9)=59.54$, $p<0.001$; Fig. 4b). To further describe the effects of the lesions in mice, *post hoc* analyses on the effects of lesion and signal duration on hits indicated a significant interaction between these two factors ($F(2,20)=14.65$, $p<0.01$). As hit rates in lesioned mice remained signal duration-dependent ($F(2,20)=8.13$, $p<0.05$), this interaction reflected the greater lesion-induced decrease in hits to longer when compared to shorter signals (pre-lesion, post-lesion; $hits_{500}$: 78.7±1.6%, 27.0±6.3%; $hits_{50}$: 52.2±2.8%, 18.7±4.1%; $hits_{25}$: 43.2±2.6%, 14.6±3.3%).

In lesioned mice, we also found a small but significant increase in correct rejections ($F(1,9)=5.39$, $p<0.05$; Fig. 4c). This seemingly paradoxical effect likely reflected an increased bias toward the side where misses and correct rejections were reported, resulting from the high increases in misses caused by the lesion. The lesions did not affect errors of omission ($F(1,9)=1.61$, n.s.) and a trend for an increase in omissions across blocks of trials did not reach significance ($F(4,36)=4.08$, n.s.; block 1: 12.4±1.8%; block 5: 20.4±3.3%). The effects of lesion and block interacted significantly, reflecting a lesion-induced increase in omission during the first 2 blocks of trials ($F(4,36)=8.17$, $p<0.01$; block 1: pre-lesion: 5.36±1.21% omission; lesion: 18.92±4.23%; $t(10)=2.84$, $p<0.05$; block 2: pre-lesion: 5.82±1.17%; lesion: 19.24±4.71%; $t(10)=2.63$, $p<0.05$). However, there were no main effect of genotype on omissions, and no significant interactions involving genotype as a factor.

The degree of the loss of cortical cholinergic inputs was estimated using a grid-counting method (see Methods). In both genotypes, counts indicated a loss of about 50% of cholinergic inputs to the cortex. A two-way ANOVA indicated a main effect of lesion ($F(1.13)=85.50$, $p<0.001$) but no effect of genotype and no interaction between the two factors (both $F<0.03$, n.s.; counts (M±SEM, controls: 183.75±8.24; lesioned: 88.94±6.11). The findings from this experiment indicate that although CHT+/− mice are able to perform the SAT with attenuated levels of ACh release, cholinergic signaling remains necessary for sustaining attentional performance.

3.7. Cholinergic vulnerability of SAT performance in CHT +/−, but not WT mice

The evidence described above indicates that, to perform the SAT, cholinergic activity is as necessary in CHT+/− mice as in WT mice. However, performance-associated levels of ACh release in CHT+/− mice were significantly lower. One explanation for these findings is that

an upregulation of postsynaptic cholinergic signaling mechanisms in CHT+/− mice could compensate for lower presynaptic release levels (see below). Because of evidence indicating that the tonic component of cholinergic activity modulates cortical circuitry via nAChRs (Parikh et al., 2008, 2010; Hasselmo and Sarter, 2011), we hypothesized that blocking these receptors could reveal a greater dependency of SAT performance in CHT+/− mice on nAChR-mediated cholinergic neurotransmission. To this end, we reverse-dialyzed the nAChR antagonist mecamylamine (MEC; 50 μM) during trial blocks 3-5 of the SAT.

First, we analyzed performance during the pre-drug blocks 1-2 to verify that both strains performed comparably, and at regular levels. Data from these trial blocks were compared with analogous data from sessions without MEC infusions. As expected, there were no effects of genotype, session type, and no significant interactions involving these factors. We next compared performance in blocks 3-5, during which MEC was perfused, with data from a dialysis session without MEC administration. Overall performance, as indicated by the SAT score, indicated a significant interaction between MEC, genotype, and signal duration ($F(2,40)=3.80$, $p<0.05$). *Post hoc* multiple comparisons indicated that administration of MEC reduced SAT_{500} in CHT+/− mice but not wildtype mice ($p<0.01$). SAT scores calculated on the basis of hits to shorter durations remained unaffected by MEC (both n.s.; Fig. 4). This significantly greater impairment of MEC on SAT scores in CHT+/− mice reflected a trend for a decrease in hits to longest signals ($F(2,40)=3.155$, $p<0.06$; $hits_{500}$: MEC vs control: $p<0.05$; shorter signal durations: n.s.; Fig. 5b). MEC did not affect correct rejections ($F(1,20)=0.21$, n.s.) and did not increase the errors of omission ($F(1,20)=0.33$, n.s.). The relatively lower hit rates to 50 and 25 ms signals may have limited the efficacy of MEC to further lower these scores, yielding a selective effect on the performance of CHT+/−mice in trials with longest signals. Together, these findings reveal SAT performance by CHT+/− mice has a greater dependency on nAChR signaling than seen in wildtype mice.

We also determined ACh levels during reverse-dialysis of MEC in SAT-performing WT and CHT+/− mice and compared these with ACh levels measured during regular SAT sessions. As before, performance-associated increases in ACh levels were attenuated in CHT+/− mice ($F(1,18)=15.72$, $p<0.01$; increases during task blocks T1-T5: WT: 85.59±16.39%; CHT+/−: 37.80±11.05%). However, MEC did not affect levels of ACh release in either genotype (main effect of MEC and genotype × MEC: both n.s.).

3.8. [^{3}H]cytisine saturation binding assays

Our evidence and the derived circuitry model suggest that cholinergic activity modulates cortical target circuitry primarily via α4β2* nAChRs (Parikh et al., 2010; Howe et al., 2010; Hasselmo and Sarter, 2011). We therefore measured the density of α4β2 nAChRs (B_{MAX}) and [^{3}H]cytisine binding affinity (K_D) by saturation binding assays employing cell membranes prepared from frontal cortex tissues obtained from both naïve and SAT-performing mice (see Methods). For the analysis of B_{MAX} values, and in both naive and SAT-performing mice, two-way mixed model ANOVAs analyzing the effects of genotype and hemisphere indicated a significant effect of genotype, reflecting higher B_{MAX} values in CHT+/− mice (naive: genotype: $F(1,8)=8.77$, $p<0.05$; SAT-performing: $F(1,8)=7.78$, $p<0.05$; M±SEM; naive: WT: 47.71±1.23 fmol/mg; CHT+/−: 51.86±0.93 fmol/mg; SAT-performing: WT: 38.73±0.99 fmol/mg; CHT+/−: 41.80±0.64 fmol/mg). The main effects of hemisphere and interactions for between hemisphere and naive vs. SAT-performing did not reach significance (all $F<4.14$). Because our radioligand binding data revealed overall lower density of α4β2 nAChR levels in mice that had performed the SAT, we conducted a follow-up analysis on the effects of genotype and performance (naive vs. SAT; values from the two hemispheres were averaged for each animal). This analysis again revealed higher B_{MAX} values in CHT+/− mice as compared to wildtype littermates ($F(1,4)=12.18$, $p<0.05$), with

values generally lower in mice that had performed the SAT ($F(1,4)=123.23$, $p<0.001$; interaction: $F(1,4)=0.39$, n.s.; M±SEM [fmol/mg]; naive: 49.79±0.63; SAT-performing: 40.27±0.71).

Corresponding analyses of K_D values revealed no effects of genotype and no interaction between the effects of genotype and hemisphere. Likewise, the follow-up analysis on the effects of SAT performance failed to reveal significant effects (all $F<7.26$; n.s.; M, SEM [nM]; naive: WT: 0.17±0.007; CHT+/−: 0.15±0.006; SAT-performing: WT: 0.16±0.01; CHT+/−: 0.15±0.01).

4. Discussion

The present experiments tested the hypothesis that CHT heterozygosity limits attentional performance and the capacity of cholinergic projections to cortex to support such performance. To do so, we pursued microdialysis assessments of extracellular ACh levels in mice performing the SAT. Results from these experiments are summarized as follows. 1. CHT heterozygosity limits the capacity of cortical cholinergic inputs to release ACh in response to presynaptic autoreceptor blockade. 2. Attentional performance did not differ between wild-type and CHT+/− mice. 3. However, attentional performance-associated increases in prefrontal extracellular ACh levels were greatly attenuated in CHT+/− mice. 4. The attentional performance of CHT+/− remained as dependent on cholinergic neurotransmission as in wildtype mice, as indicated by disruption of performance and associated lowering of ACh levels following reverse-dialysis of TTX into PFC, and by the performance effects of lesions of BF cholinergic neurons. 5. SAT performance of CHT+/− mice was significantly more vulnerable to blockade of nAChRs in prefrontal cortex. 6. The frontal cortex of CHT+/− mice contained a higher density of α4β2* nAChRs.

SAT performance did not differ between CHT+/− and wildtype mice but SAT-associated levels of extracellular ACh were greatly attenuated in CHT+− mice when compared to wildtype animals. We previously demonstrated that SAT-associated ACh release, as measured by microdialysis, varies as a function of the demands on performance and increases specifically in response to performance challenges, such as distractors, and while performance suffer from such challenges. Thus, ACh levels are correlated with demands on attention but not with levels of performance (Kozak et al., 2006; St. Peters et al., 2011b; Sarter et al., 2006). In the present experiment, the demands on SAT performance were not explicitly varied and thus ACh levels remained stable across blocks of trials/collection intervals. This finding may be speculated to reflect that CHT+/− mice, when compared with wildtype mice, performed this task based on a lower degree of attentional control than their wildtype littermates. Subjects may reduce the degree of attentional control by deploying, for example, a greater degree of automaticity in processing the response rules governing the two trial types (signal and non-signal trials), and/or being less sensitive to performance variations and to the demand of performing the SAT over a relatively long period of time (for a discussion of attentional control mechanisms see Sarter and Paolone, 2011).

Alternatively, the degree of attentional control may be similar in CHT+/− mice and wildtype mice but, in CHT+/− mice, the lower level of SAT-associated ACh, in combination with a higher density of cortical α4β2* nAChRs, serves to produce a comparable cholinergic modulation of cortical circuitry and similar levels of performance as in wildtype mice. As pointed out above, the focus on α4β2* nAChRs was based on the current circuitry model suggesting that α4β2* nAChRs play a key role in the modulatory effects of tonic cholinergic activity on cortical circuitry (Parikh et al., 2008, 2010; Hasselmo and Sarter, 2011). We can of course not exclude the possibility that similar upregulation of other nAChR subtypes or mAChR contributed to the ability of CHT+/− mice to perform the SAT. Consistent with this

view, cortical mAChR have been demonstrated to mediate key aspects of stimulus processing and attention (e.g., Disney and Aoki, 2008; Herrero et al., 2008; McKenna et al., 1989; Robinson et al., 2012) and they also are capable of undergoing plastic, compensatory changes (Tian et al., 2011).

Although we cannot rule out at this point that an upregulation of postsynaptic mAChR may have contributed to stable SAT performance in CHT+/− mice, the density primarily presynaptic M2 mAChR is known to be reduced in the cortex of these mice (Bazalakova et al., 2007), consistent with the attenuated effects of atropine (Fig. 1). Viewed another way, a lower density of presynaptic M2 mAChR in CHT+/− mice could also limit the autoinhibitory effects of endogenous ACh. Thus, the SAT-associated increases in extracellular ACh in CHT+/− mice might have been even lower in the absence of such an additional compensatory mechanism. Collectively, our findings indicate that moderately demanding attentional performance in CHT+/− mice may be supported by these compensatory pre- and postsynaptic mechanisms.

However, more severe demands on cholinergic activity cannot be fully supported as a result of CHT heterozygosity (Parikh et al., 2013a). We previously observed that electrical stimulation, or challenged SAT performance, by presenting a distractor, revealed, *ex vivo*, the attenuated capacity of cholinergic neurons of heterozygous CHT mice to increase the density of CHTs in synaptosomal plasma membranes extracted from cortex (Parikh et al., 2013a). These findings revealed that a limited constitutive capacity of CHT significantly limits behavioral performance under conditions requiring acute increases in cholinergic activity. In the present experiment, SAT performance was not challenged by a distractor and thus SAT performance did not differ between the strains. At the same time, constitutive CHT heterozygosity, which produces a reduction of ~50% of tissue ACh levels (Bazalakova et al., 2007), will also limit capacities for “tonic” cholinergic activation, as indicated by reductions in SAT-associated elevations in extracellular ACh. Here though, behavioral effects are lacking, possibly because the level of performance does not rely on high levels of ACh release or, as noted here, there may be compensation by elevated nAChR levels. In this context, it is important to note that such compensatory mechanisms are often considered a weakness of genetic models that manipulate neural signaling pathways. However, as humans express disease-associated, CHT coding variation that limits CHT function (English et al., 2009; Hahn et al., 2008; Neumann et al., 2005, 2006), knowledge of these compensations provides critical *in vivo* data that may be of great value in therapeutic studies.

This conceptualization of our collective findings predicts that the attentional limitations of humans expressing diminished cholinergic signaling capacity, either via a subcapacity variant of the CHT or through other mechanisms (e.g., aging), may not be best revealed by tasks that assess steady-state levels of attentional performance, but rather will benefit from conditions requiring performance “spikes”, such as the filtering of intrusive distractors. Our research on humans heterozygous for the low functioning Ile89Val variant (Okuda et al., 2002) of the CHT (I89V) indicates that they are not impaired when performing the SAT or even when performing this task in the presence of a visual distractor (Berry et al., 2013). However, their ability to suppress more meaningful distractors, such as dialogue from popular television shows, is significantly attenuated. The present results allow the speculation that relatively high degree of attentional control required to filter effective cognitive or emotional distractors is mediated in part by the ability to quickly mobilize high levels of cholinergic activity (St Peters et al., 2011b; Sarter et al., 2006) and that deficits in the ability to generate such “spikes” in cholinergic neurotransmission may underlie the association of ADHD and depression seen in the Ile89Val variant.

The present effects of prefrontal blockade of nAChR suggest that the cognitive/behavioral impact of CHT heterozygosity ("first hit") is robustly revealed in interaction with an additional, genetic, environmental or behavioral manipulation of cholinergic neurotransmission ("second cholinergic hit"; see also Bazalakova et al., 2007). Given the upregulation of nAChR in CHT+/− mice (above), their greater vulnerability to nAChR blockade is an expected result. Likewise, the detrimental interactions between CHT heterozygosity and other neuronal manipulations, such as dopaminergic lesions (Zurkovsky et al., 2013) seems likely and should be further investigated, in part to test the hypothesis that the presence of CHT subcapacity enhances the vulnerability of humans to age-related cognitive disorders (Sarter and Parikh, 2005; Bohnen et al., 2003, 2009; Mesulam, 2004).

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- The choline transporter (CHT) influences cholinergic activity and attention.
- CHT +/− mice exhibit impaired cholinergic activity but unchanged attention.
- Cholinergic manipulations revealed vulnerability in CHT+/− mice.
- CHT+/− mice exhibited an increased density of cortical α4β2* nAChRs.

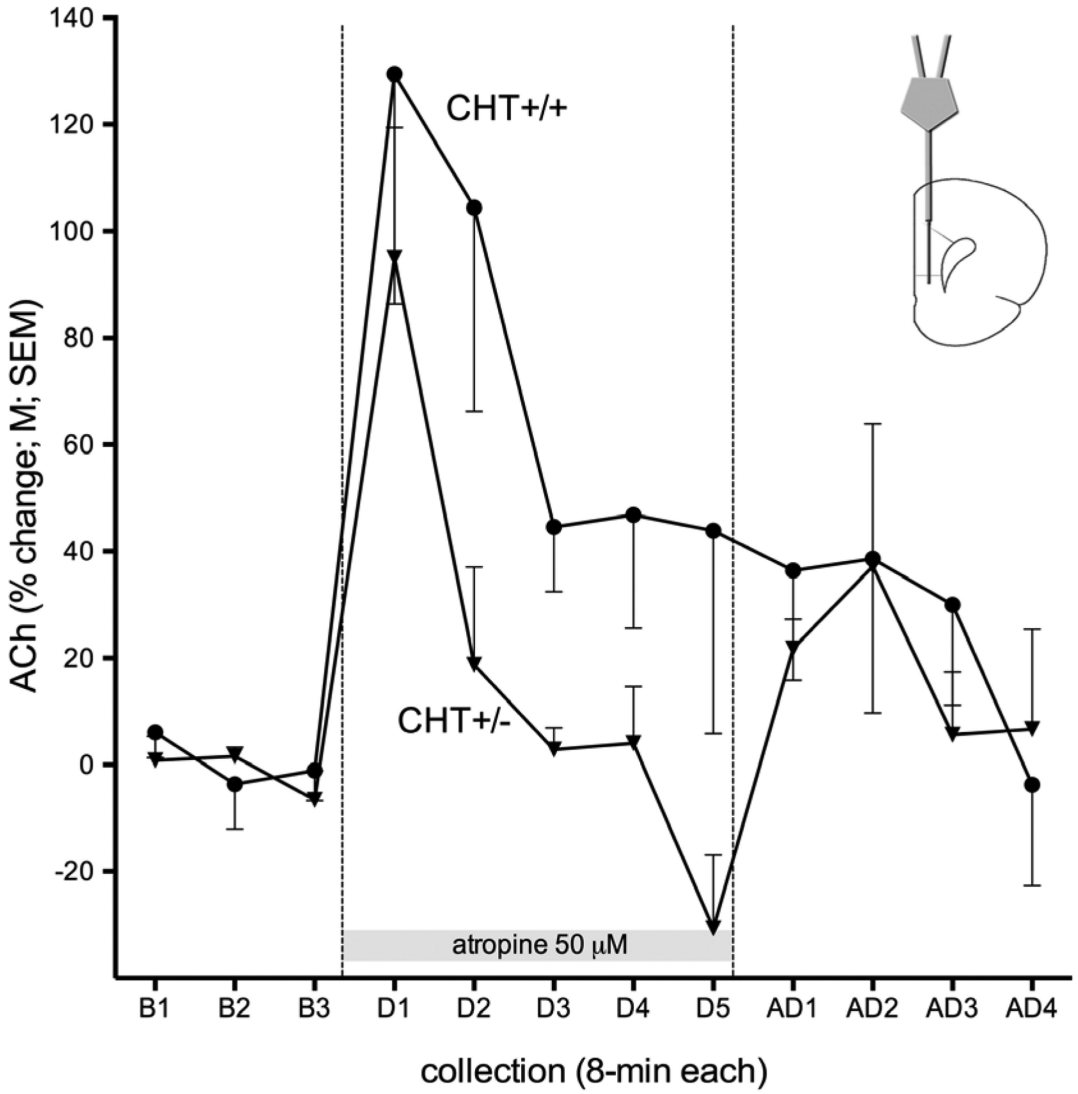

Figure 1.

ACh release in CHT+/− and WT mice in response to reverse-dialyzing the mAChR antagonist atropine. Atropine-induced ACh release results from blocking presynaptic autoinhibitory mAChRs. Basal ACh release did not differ between the strains (see results). Atropine (50 μM) was administered over 5 collection periods (see vertical lines). ACh release in CHT+/− mice was attenuated when compared with WT mice (see Results for main effect and means). ACh release returned to baseline across collections and in both strains (no interaction between genotype and collection).

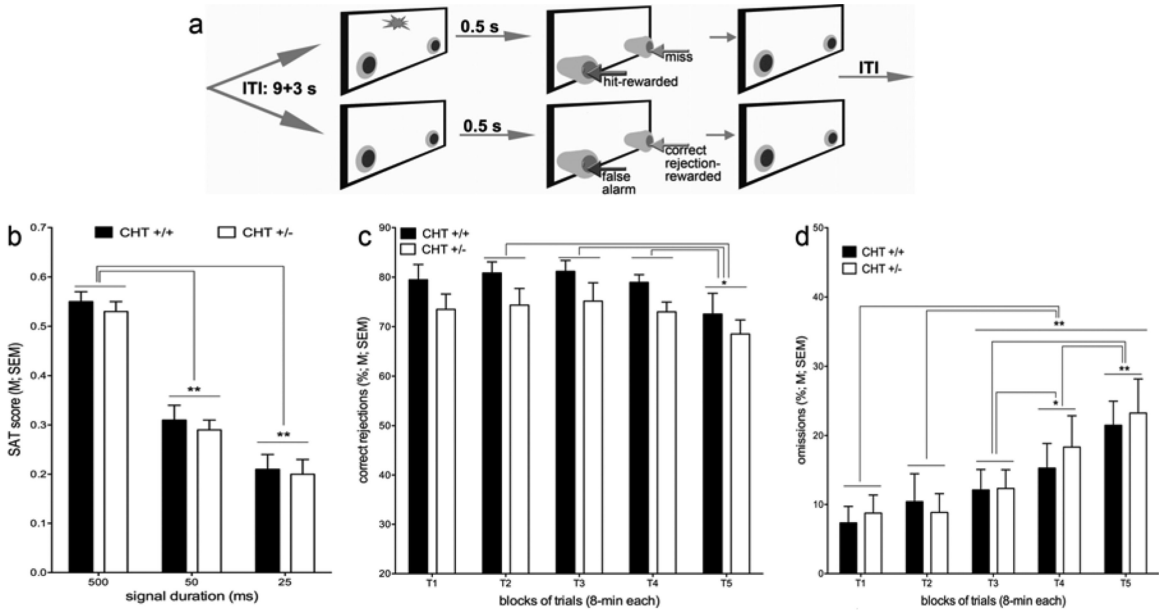

Figure 2.

SAT performance of CHT+/− and WT mice at baseline and prior to cannulation surgery. a: Illustration of trial types (signal and non-signal, respectively; pseudo-randomized sequence) and outcomes (note that half of the animals were trained with reversed outcome assignments to response ports). b: The SAT score indicates overall performance by combining signal and non-signal trial performance (see Methods). SAT scores vary by signal duration, reflecting higher hit rates to longer signals. SAT scores did not indicate an effect of genotype. The number of correct rejections was slightly but significantly lower in CHT+/− mice (see Results for ANOVA and means) and decreased for all animals toward the end of the session (see c). This effect of block did not interact with genotype. d: The number of errors of omission increased towards later blocks of trials, with blocks 3-5 containing more omissions than blocks one and two and additional significant differences between blocks 3, 4 and 5 (multiple comparisons, for this and subsequent figures: *, $p<0.05$; **, $p<0.001$).

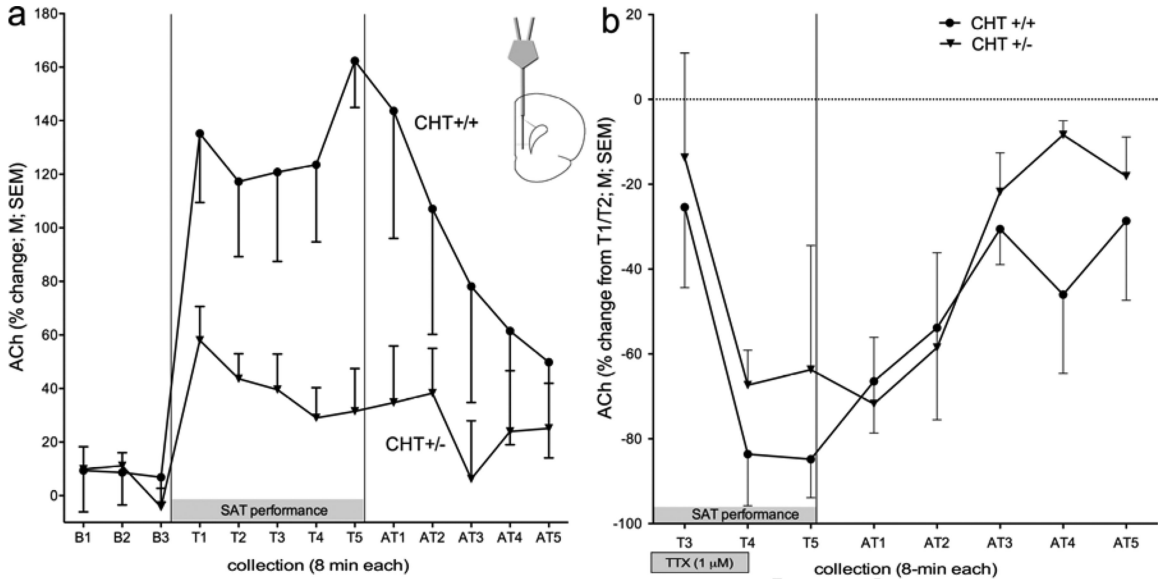

Figure 3.

a: SAT-associated increases in ACh release were significantly lower in CHT+/− than in WT mice (see Results for main effect and group means; no interaction between genotype and collection). Mice were extensively habituated to performing while tethered and dialysis lines connected and therefore their performance did not differ from pre-surgery baseline (Fig. 2) nor among strains. SAT-associated increases in PFC ACh release of WT mice were comparable to levels seen in rats (St Peters et al., 2011b). b: Reverse dialysis of tetrodotoxin (TTX; 1 μM) during task blocks 3 and 4 suppressed ACh release in 43% of all collections below the level of detectability. Detectable ACh concentrations (b) were >60% below performance-associated levels seen prior to TTX administration during the last task blocks and did not differ between the strains.

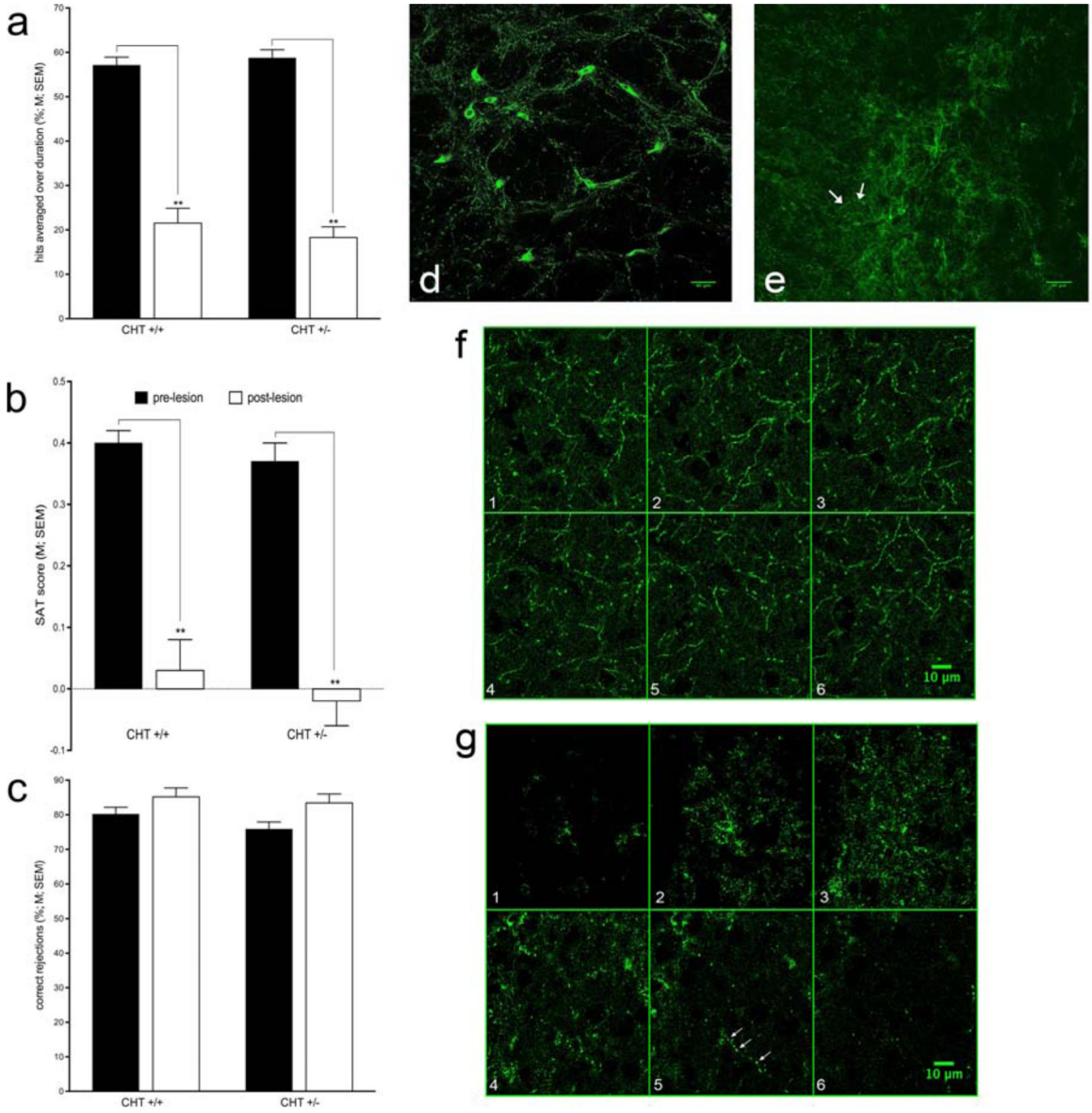

Figure 4.

Impaired SAT performance after BF cholinergic lesions in WT and CHT+/− mice. The lesion-induced decrease in SAT scores (a) reflected decreases in hits (b); the lesion produced a small but significant increase in corrected rejections (c) that likely was due to a bias toward the side where misses or correct rejections were reported. d Nucleus basalis of Meynert of an intact WT mouse, showing neurons and local cholinergic varicosities (VAChT immunoreactivity). e: Near complete loss of cholinergic neurons following the infusion of mu-p75-SAP into the basal forebrain of a CHT+/− mouse. Arrows depict the location of residual cholinergic varicosities. f and g: Consecutive slices (1-6), obtained by confocal microscopy, depicting VAChT immunoreactivity in the frontoparietal cortex of an intact WT (f; 7.27 um/slice) and a lesioned CHT+/− mouse (g; 4.87 um/slice). In g, residual varicosities are visible in the 5th slice. The lesions nearly completely removed cholinergic projections to frontoparietal regions in both WT and CHT+/− mice.

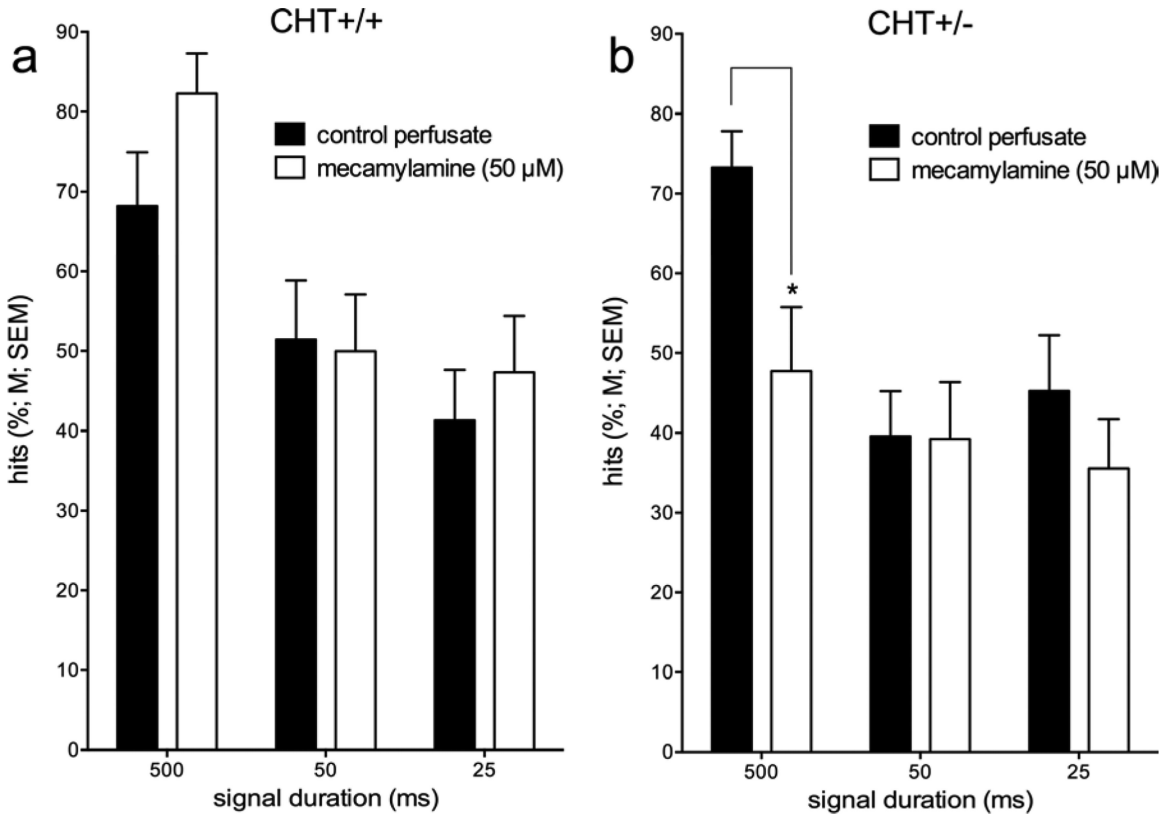

Figure 5.

Reverse-dialysis of the nAChR antagonist mecamylamine impaired the performance of CHT +/− but not WT mice (see Results for the significant interaction between the effects of genotype, drug and signal duration on SAT scores). This interaction was mirrored in the analysis of hits, indicating that mecamylamine impaired hits to the longest signals in CHT+/ − mice (b) but not WT animals (a). “Floor effects” may have limited the efficacy of mecamylamine to further lower hits to shorter signals. Corrected rejections and omissions were unaffected by mecamylamine.