Sustained Attention and Associated Acetylcholine Release in Choline High-Affinity Transporter Hemizygous Mice

by

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Abstract

Transport of choline from the synaptic cleft into cholinergic neurons is mediated by the choline high-affinity transporter (CHT). This process represents the rate-limiting step in the synthesis of acetylcholine (ACh), a neurotransmitter critically involved with processes requiring sustained attention. Compromised CHT capacity limits the ability to release ACh and may be implicated in disorders affecting attention. Mice hemizygous for the CHT gene were trained alongside wildtype (WT) littermates on a sustained attention task (SAT), and performance-associated ACh release in the medial prefrontal cortex (mPFC) was determined through microdialysis. CHT +/- mice express roughly half the amount of the CHT protein, and were predicted to show impaired performance on the SAT in addition to a reduced capacity to sustain elevated levels of ACh release. We found no differences between WT and CHT +/- mice on measures of SAT performance, however task-associated ACh increases appeared to be greatly reduced in hemizygotes. In a complimentary experiment, we taxed cholinergic signaling pharmacologically by perfusing, via reverse dialysis, the muscarnic antagonist atropine, which causes increased ACh release in rats (Moore, Stuckman, Sarter, & Bruno, 1995). We found CHT +/- mice are initially able to release levels of ACh equivalent to WT, but are unable to sustain elevated release for as long a period. Together our data suggest that CHT +/- mice are limited in their capacity to sustain elevated ACh release, and further research is needed to explore both

compensatory mechanisms enabling good performance on attentionally demanding tasks, as well as limitations of CHT hemizygosity.

Sustained Attention and Associated Acetylcholine Release in Choline High-Affinity Transporter Hemizygous Mice

Cholinergic neurons originating in the basal forebrain and projecting widely throughout the cortex are critical in mediating attentional processes (Sarter, Givens, & Bruno, 2001). Sustained attention, the focus of our study, is characterized by the subjects' ability to detect unpredictably occurring signals over a prolonged period. The necessity of cortical cholinergic neurotransmission on maintaining sustained attentional performance has been demonstrated in rats through lesion studies, measurements of acetylcholine (ACh) during task performance, and pharmacological manipulations (Sarter & Parikh, 2005). In particular, ACh signaling within the right medial prefrontal cortex (mPFC) appears to be critical for performance on the sustained attention task (SAT). Microdialysis studies have consistently found elevated levels of ACh in the right mPFC of rats performing the SAT and lesions to the right hemisphere cholinergic inputs impair signal detection (Martinez & Sarter, 2004). Dysfunction of cholinergic neurotransmission compromises attentional abilities and is implicated in a number of cognitive impairments including aging and dementia (Sarter & Parikh, 2005). It is therefore important to study the molecular mechanisms that contribute to compromised cholinergic signaling.

Of particular interest is the choline high-affinity transporter (CHT), which mediates high-affinity uptake of choline into presynaptic neurons and limits the rate of ACh synthesis from

choline and Acetyl coenzyme A (Apparsundaram, Martinez, Parikh, Kozak, & Sarter, 2005). In the brain, CHTs are found within cholinergic neurons, both at the membrane and within vesicles in the membrane terminal. CHT capacity for choline-uptake depends on the density of CHTs localized at the synaptosomal membrane, which is regulated by intracellular trafficking of CHTs from reserve stores to the terminal membrane (Sarter & Parikh, 2005). While 15% of the reserve pool is constitutively trafficked to the synaptosomal membrane to maintain basal CHT capacity, increased trafficking is necessary to maintain sustained, elevated levels of ACh release, and is thought to be regulated by intracellular mechanisms independent of neuronal depolarization. Evidence in support of this model comes from findings that rats performing the SAT have a higher proportion of CHTs localized at the neuronal membrane in the right mPFC than rats performing a control task lacking explicit attentional demand (Apparsundaram et al., 2005).

Determining the mechanisms behind CHT regulation will help to understand diseases and abnormalities associated with alterations in CHT functioning. It has been suggested that disruptions in CHT regulation may contribute to the declines in cholinergic transmission implicated in Alzheimer's disease (Sarter & Parikh, 2005). Additionally, a short nucleotide polymorphism of the human CHT gene has been identified (Ile89Val) that is associated with a 40-50% decrease in choline-uptake capacity (Okuda, Okamura, Kaitsuka, Haga, & Gurwitz, 2002). Present in 11% of the general population, this allele is overrepresented in the ADHD

population. Compared to control subjects, humans heterozygous for the variant self-report higher distractibility and poorer concentration (unpublished findings, Sarter, 2009).

While CHT knockout mice die shortly after birth, CHT hemizygotes (CHT +/-) are fertile and viable, providing a model to study phenotypes associated with altered CHT functioning. Hemizygotes express 50% less CHT protein and display lower overall brain ACh levels, elevated choline levels, and decreased expression of muscarinic ACh receptors in certain brain regions (Bazalakova et al., 2007). Under basal conditions, CHT +/- mice express equal amounts of CHT at the synaptosomal membrane, but have a disproportionately small intracellular reserve pool compared to WT (unpublished findings, Sarter, 2009). This may explain why under basal conditions hemizygotes display normal ACh release and are largely indistinguishable from WT mice (Sarter & Parikh, 2005). While CHT +/- mice perform equivalently to WT on measures of coordination, general activity, anxiety, and spatial learning and memory, they more quickly reach exhaustion on a treadmill test, and are less sensitive to the hyperlocomotive effects of the muscarinic ACh receptor antagonist scopolamine (Bazalakova et al., 2007). It is hypothesized that processes that significantly tax cholinergic transmission may reveal impaired phenotypes in CHT +/- mice, due to their limited CHT capacity.

In two experiments, we challenged cholinergic signaling behaviorally and pharmacologically to investigate differences in ACh release between WT and CHT +/- mice.

We first studied the impact of CHT hemizygosity on attentional performance and task-associated ACh release in the mPFC. To assess differences in attentional capacity, WT and CHT +/- mice were trained on the SAT, which has been used extensively as a measure of sustained attention (McGaughly & Sarter, 1995). SAT training begins with a discrimination task, in which animals must distinguish between the presence or absence of a signal. In subsequent levels, the task becomes more attentionally challenging. For example, the introduction of varying signal durations and the presence of a house light require the animal to maintain its focus towards the intelligence panel.

As research with rats has consistently demonstrated increases in ACh release in the right mPFC during the SAT, a major goal of this study was to investigate the presence of a similar pattern in mice and determine if CHT +/- mice exhibit attenuated task-associated ACh release due to their reduced CHT capacity. As predicted, WT ACh release looked similar to patterns observed in rats, while CHT +/- mice showed considerably less task-associated ACh release. Despite releasing less ACh during task, CHT +/- mice performed equivalently to WT on the SAT.

To explore the possibility that the SAT does not sufficiently tax cholinergic transmission to reveal phenotype differences, we exposed mice to the distractor condition of the SAT (*d*SAT). In the *d*SAT, a visual distractor (house light flashes on/off at 0.5 Hz) is presented during a period

of the task. This manipulation is associated with further increases in cholinergic activity in the mPFC, and is thought to demand increased attentional effort (Sarter, Gehring, & Kozak, 2006). Experiments in rats have found the greatest ACh release during presentation of the distractor (St. Peters, Demeter, Lustig, Bruno, & Sarter, 2011). We were interested to see how the distractor condition affects ACh release in CHT +/- mice, and how release patterns differ from WT. Preliminary data suggest that CHT +/- mice are more impaired on signal trials during the block following the distractor (unpublished findings, Sarter, 2009), suggesting that CHT trafficking is exhausted during distractor presentation. According to this model, we expected to find increased ACh release during the distractor period, followed by low level release and poor performance in the post-distractor period. Results from a preliminary dialysis session in a CHT +/- mouse performing the *d*SAT are discussed below. Due to the limited time frame of this study, we were unable to obtain comprehensive *d*SAT data.

As we found that CHT +/- mice are able to perform the SAT with surprisingly low levels of task-associated ACh release, we next explored whether this small increase was necessary for attentional performance. We attempted to block ACh signaling in the mPFC by perfusing, via reverse dialysis, the muscarinic receptor antagonist atropine during a period of the task. While atropine evokes ACh release, by inhibiting presynaptic autoreceptors located on cholinergic neurons, it also blocks cholinergic signaling through its actions on postsynaptic receptors. We

expected that if a certain degree of ACh signaling were indeed necessary for SAT performance, the animals' ability to discriminate between signal and nonsignal events would be impaired during drug perfusion. Additionally, we confirmed that task-associated ACh-release relies on impulse-dependent neurotransmission by adding 1 μM of the voltage-gated sodium channel blocker tetrodotoxin (TTX) to the perfusion medium for a brief period during a single SAT dialysis session.

In a second experiment, we taxed the mPFC cholinergic system pharmacologically by perfusing, via reverse dialysis, atropine, which evokes increased ACh release in rats (Moore, Stuckman, Sarter, & Bruno, 1995). We expected that while WT mice would similarly show increased ACh release over the course of the drug, CHT +/- mice would be unable to sustain heightened levels of choline-uptake and ACh release due to an insufficient intracellular CHT reserve pool. In addition, CHT +/- mice express fewer M2 autoreceptors in the cortex, suggesting that atropine may be less effective at inhibiting ACh negative feedback mechanisms (Bazalakova & Blakely, 2006). Consistent with our hypothesis, we found that CHT +/- mice were unable to release elevated levels of ACh for as long a time period as WT mice.

Together, the two experiments aimed to tax cholinergic signaling in order to elucidate differences in ACh release patterns between WT and CHT +/- mice. Understanding such differences is a critical step in understanding the normal regulation of CHT trafficking and

capacity as well as the limitations and compensatory mechanisms associated with CHT hemizygosity. A major undertaking of this study was to perform microdialysis, which enables detection of ACh, on freely moving mice. While microdialysis has been previously conducted on anesthetized mice, this is, to our knowledge, the first study to employ the method in awake and performing mice. By overcoming the many challenges that arose due to the novelty of the procedure (see discussion), we were able to refine a technique that will benefit future studies of genetically modified mice.

Method

Subjects

WT and CHT +/- C57Bl/6J mice were bred at the University of Michigan. Male and female mice weighing 20-25 g prior to the onset of training were housed individually in a humidity (45%) and temperature (23 °C) controlled environment. Animals were kept on a 12hrlight/dark cycle (lights on at 7:30 A.M.) and trained and tested during the light phase. Performing mice were gradually water deprived over a one-week period prior to the onset of training, with *ad libitum* access to food. Animals received water as a reward during task performance and for 3 min following completion of the task. On days not tested, animals received 10 min water. A separate, nonperforming, group of mice was used in the second experiment, and provided with *ad libitum* access to food and water.

Behavioral Training Procedure

The training and testing took place in operant chambers (20 × 24 × 29.8 cm; width × length × height), situated inside sound-attenuated chambers. Each operant chamber contained an intelligence panel equipped with two lights (2.8 W), two retractable nose-pokes, and a water dispenser (6 μL of 0.2% Saccharin in de-ionized water per delivery). A house light (2.8 W) was located on the rear wall.

Mice were trained on a SAT modified from the version used with rats (McGaughly & Sarter, 1995). Animals were initially shaped to reach the water dispenser using a modified fixed ratio-1 (FR-1) reward schedule. After obtaining ≥100 water rewards for three days, animals began training on the SAT. SAT training began with a series of levels in which animals learned to distinguish between signal (illumination of the central panel light) and non-signal (no illumination) events. Importantly, the house light was kept off, making the signal more salient. On signal trials, a left nose-poke was termed at "hit," while a right nose-poke was termed a "miss." On non-signal trials, a right nose-poke was termed a "correct rejection," while a left nose-poke was termed a "false alarm." Half of the animals were trained following the reverse rules. Only hits and correct rejections were rewarded. An omission was recorded if the animal failed to nose-poke during the nose-poke extension following an event. Signal and non-signal trials were presented in a pseudorandom order.

In the first task level, a longer cue (5 sec) illuminated during signal trials. 1 sec after signal onset, both nose-pokes extended for 4 sec. In non-signal trials, nose-pokes extended for 4 sec. 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 nose-poke corresponding to the correct response extended. After reaching criterion (at least three consecutive days of obtaining >60% hits and correct rejections) animals moved to the subsequent level, in which signal length and time delay before nose-poke extension were reduced (1 sec and 0.5 sec, respectively). After reaching criterion (same as previous level), animals moved to next level, during which the length of the signal varied (either 500, 50, or 25 ms) throughout the session. The session consisted of five, 8-min blocks, designed to ensure equal numbers of signal and non signal trials, as well as 500, 50 or 25 ms signals. Trial type and signal duration were presented in a pseudorandom order, with approximately 160 trials per session and an inter-trial interval of 12 ± 3 sec. Forced and correction trials were eliminated at this point.

After reaching criterion at this stage (three days of obtaining a SAT score >0.6, see below for an explanation of SAT score), animals moved to the final level of the SAT (the real sustained attention task per se) during which the house light was illuminated. After stable performance for at least three days (hits and correct rejections >0.60, omissions <0.20) some animals were

exposed to the distractor (house light flashes on/off at 0.5 Hz) during task blocks 2 and 3. Following a distraction session, animals were trained on the SAT for three days or until returning to criterion. When performance on the SAT was stable at criterion, animals were surgically implanted with guide cannulas to allow for microdialysis.

Surgical Method

Animals were placed in an anesthetic chamber and administered 2-3% isoflurane. Prior to operation, animals were injected with an antibiotic (Amoxicillin, 100 mg/kg) and atropine (0.05 mg/kg) subcutaneously. The animals' heads were shaved with electric clippers and cleaned with ethanol and a topical antiseptic, and animals' eyes were lubricated with opthalmic ointment. Throughout the surgery, body temperature was maintained using an isothermal heat pad. Animals were set in a stereotaxic instrument, and 1-3% isoflurane was administered continuously during the surgery via a face-mask. A microdialysis guide cannula (CMA 7) was implanted into the mPFC of the right hemisphere according to the following coordinates, relative to Bregma: AP: +1.8 mm, ML: -0.4 mm, DV: -0.1 mm. These coordinates placed the tip of the probe 2.1 mm beneath the dura surface. 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 was inserted until microdialysis sessions, at which point it was replaced by a 2 mm

probe (CMA 7MD). Post-surgery, animals were returned to their cages and allowed to recover for at least 7 days before dialysis.

Microdialysis Procedure

Up to four microdialysis sessions, separated by at least five days, were conducted on each animal while performing the SAT. After surgery animals resumed SAT training in modified operant chambers that contained a taller water delivery area and openings in the ceilings of the operant and sound attenuating chambers to accommodate the microdialysis tubing. Furthermore, animals were tethered with polyethylene tubing to mimic dialysis conditions. Microdialysis was conducted once SAT performance returned to stable performance on criterion. On one animal, microdialysis was conducted during *d*SAT performance.

At the beginning of the test session, the stylet was removed and a probe was inserted through the guide cannula into the mPFC. 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. After a 2 hr stabilization period, six 8-min baseline dialysates were collected and the last three used to calculate basal ACh release. The SAT then began, and five 8-min dialysates were collected after a 3:40 min wait period that accounted for the dead volume of the probe and outlet tubing. Five 8-min collections were taken after completion of the task.

During one SAT session, TTX (1 μM) was dissolved in aCSF and perfused, via reverse dialysis, for the duration of the third and fourth blocks of the task. The input line was switched to the drug-containing syringe prior to the third SAT block, with adequate time allotted for the dead-volume of the probe and tubing inlet, and switched back to the aCSF containing syringe prior to the fifth block. In a different session, atropine (50 μM) was dissolved in aCSF and perfused, via reverse dialysis, during task (blocks t2-t5). At the conclusion of the session, probes were removed and replaced with stylets, and animals returned to their cages.

In the second experiment, to test differences in ACh release in response to atropine, microdialysis was conducted on a group of nonperforming animals (WT: $n = 4$, CHT: $n = 2$). The basic dialysis procedure was the same as that described above, with the following modifications. Dialysis took place in the animals' home cage, and acclimation to testing conditions required staying in the testing room, tethered for 4 hr each on five separate days. During the test session, a 2 hr stabilization period was allowed after probe insertion before six 8-min baseline dialysates were collected. The microdialysis tubing was then switched to a second syringe containing 50 μM atropine dissolved in aCSF. Collections resumed after a 6-min wait-period to allow for the dead-volume of the probe and inlet and outlet tubing. Four 8-min collections were taken as the drug was perfused. The perfusion medium was then switched back to aCSF, and 5 post-drug collections were taken after the dead-volume wait-period.

Determination of ACh Concentrations

Dialysate samples were kept at -80 ºC until analyzed with high-performance liquid chromatography (HPLC) with electrochemical detection.

Histological Verification of Probe and Cannula Placements

Animals were perfused within one week after the last microdialysis session. The brains were preserved in formalin overnight and then transferred to a 30% sucrose phosphate buffer solution. Sections (35 μm) near the probe and cannula sites were mounted on glass slides, Nissl-stained, and examined to confirm the cannula and probe placements.

Behavioral measures

Hits, misses, correct rejections, false alarms, and omissions were recorded for each SAT session. Performance was assessed according to the SAT score, defined as $(h-f)/[2(h + f) – (h + f)^2]$, where h is the relative number of hits and f is the relative number of false alarms. Scores range from +1.0 to –1.0, with +1.0 indicating all responses were hits and correct rejections, –1.0 indicating all responses were misses and false alarms, and 0 representing chance performance (the animal is unable to discriminate between signal and nonsignal events). SAT scores were calculated separately for each signal duration ($SAT_{500,50,25}$), the average of which was taken as the SAT score. Measures of performance were calculated separately for each of the five, 8-min task blocks.

Statistical Analyses

Performance data analyses. Complete sets of SAT performance data were available from five WT and six CHT +/- mice. Upon reaching criterion on the SAT, data from three consecutive days of stable performance was analyzed to establish baseline performance. Mixed analysis of variance (ANOVA) was used to determine the effects of sex (male vs. female), genotype (WT vs. CHT +/-), task block (t1–t5) and signal duration (500, 50, or 25 ms) on performance.

ACh release. In each dialysis session, the last three baseline collections were averaged to calculate basal ACh release. Task and drug-associated release values were expressed as a percent change from baseline. Basal release was constant across dialysis sessions and also comparable between performing and nonperforming mice. Therefore, all sessions were collapsed to determine the average basal release for WT and CHT +/- mice. Performance-associated ACh release was evaluated using a paired *t*-test comparing basal release to release over the task blocks. Interactions between genotype and perfusion of atropine were assessed using a genotype × drug mixed ANOVA with drug as a repeated measure. *Post hoc* analyses are described further with the results.

General statistical methods. In cases in which the sphericity assumption was not met, Huyhn-Feldt-corrected *F* values are given; degrees of freedom were not adjusted.

Results

Measures of performance

There was no difference in SAT score between male and female mice, as demonstrated by a lack of effect of sex on SAT score in a block (5) × duration (3) × sex (2) mixed ANOVA, $F(1,9) = 0.07$, $p = 0.795$. Thus, data from male and female mice were collapsed for further comparisons. WT and CHT +/- mice scored comparably on all measures of task performance, as illustrated in Figure 1. There was no difference in overall performance (SAT score ± SEM: WT: 0.30 ± 0.03, CHT +/-: 0.27 + 0.02), as indicated by a lack of effect of genotype on SAT score (main effect of genotype: $F(1,9) = 0.19$, $p = 0.674$). There was no main effect of genotype on hits [$F(1,9) = 0.09$; $p = 0.784$], correct rejections [$F(1,9) = 0.00$; $p = 0.980$], or omissions [$F(1,9) = 0.19$, $p = 0.677$]. As expected, there was a significant effect of signal duration on signal trial responses (main effect of duration, $F(2,18) = 91.68$, $p = 0.000$). In a dialysis session conducted on a CHT +/- mouse performing the *d*SAT, we observed only a marginal impairment in performance. The *d*SAT score was 0.11 bellow the average SAT score of the previous three days. Compared to the first block, hits were 7% lower in the distractor blocks and 7% higher in the block following distractor.

Basal ACh release

Basal release did not vary between nonperforming and performing animals (WT: $t(3) = 0.35$, $p = 0.748$, CHT +/-: $t(6) = 1.60$, $p = 0.161$). There was also no difference in basal release between WT and CHT +/- mice (basal release ± SEM: WT: 6.34 ± 0.34 pmol/15 µL; CHT: 6.56 ± 0.52 pmol/15 µL; $t(11) = 0.82$, $p = 0.430$). Repeated measures ANOVA revealed that basal release remained steady across four dialysis sessions, $F(3,12) = 2.47$, $p = 0.112$.

SAT/*d*SAT performance-associated ACh release

SAT performance-associated increase in ACh release, measured in the mPFC is illustrated in Figure 2. Due to the small WT subject pool ($n = 1$), statistical comparisons between groups were not conducted. Among CHT +/- subjects, ACh release over the task blocks (t1-t5) was averaged and compared to average baseline release, revealing a trend towards SAT-associated ACh release, $t(3) = 2.55$, $p = 0.084$. Repeated measures ANOVA comparing the task blocks showed that release remained steady across the task (main effect of task block: $F(4,12) = 1.42$, $p = 0.297$). ACh release during the task blocks was higher in WT than CHT +/- mice [WT ($n = 1$): 107.46%; CHT ($n = 4$): 36.93 ± 6%]. A preliminary dialysis session was conducted on a CHT +/- mouse performing the *d*SAT (see Figure 3). ACh release was greatest in the block immediately following the distractor (49.4%).

SAT with perfusion of atropine

To determine the effect of ACh signaling blockade on task performance in CHT +/- mice, the muscarinic ACh receptor antagonist atropine was perfused during blocks t2-t5 of one SAT session. ACh levels increased 111% above baseline during reverse dialysis of atropine and remained elevated after the task/drug period (see Figure 4). During drug perfusion, omissions increased by 162.8%, hits increased by 21.9% and correct rejections decreased by 5.9%.

Impaired ACh release with TTX

To demonstrate that task-associated ACh increases were dependent on local depolarization of axons, we administered 1 μM of the voltage-gated Na^+ channel blocker TTX through blocks 3 and 4 during one SAT session (see Figure 5). By the second collection following drug administration, TTX completely abolished the task-associated ACh increase observed during blocks one and two, with ACh levels falling on average 43% below baseline over four collection periods. Performance during blocks 4 and 5 was also affected, with the SAT score dropping by 0.38 points, and hits dropping by 43%.

Atropine-induced ACh release in nonperforming mice

Atropine (50 μM) was perfused in nonperforming WT and CHT +/- mice to investigate differences in the ability to sustain elevated ACh release (see Figure 6). To analyze for possible effects of atropine as a function of group, the mean of the 4 atropine collections was calculated, and this value and the average baseline release were subjected to a genotype (2) × drug (2) mixed

ANOVA. Reverse dialysis of atropine was associated with increases in cortical ACh release, as indicated by a main effect of drug, $F(1,4) = 54.24$, $p = 0.002$. *Post hoc* analysis confirmed a significant difference between the average baseline collections and the first drug collection, $t(5) = 3.05$, $p = 0.028$. A main effect of genotype did not reach statistical significance, and ACh increases during the first collection were comparable (WT: 88.49 ± 28%; CHT: 117.96 ± 57%). However, while WT release levels remained elevated over the drug period, CHT +/- release levels showed a tendency to drop back to baseline after the first drug collection period.

Discussion

The present study is the first to conduct microdialysis in the mPFC in freely moving and performing mice and establishes a research method that will benefit future studies. The ability to associate ACh release levels with behaviors in mice genetically modified to model diseases will add to our understanding of normal functioning and disease processes. The procedure described above was refined as complications arose over the course of the study. For example, several pilot studies were required to adjust the coordinates, relative to bregma, for guide cannula placement. Following surgery, we had to determine if mice could break the photo-beams of the nose-pokes, and then modify the water-delivery ports to allow access to water.

We discovered that compared to rats, mice require a longer recovery period between consecutive dialysis sessions. This was especially true amongst CHT +/- mice, possibly because

of vulnerability to cardiovascular complications due to impaired CHT capacity in the autonomic nervous system (English et al., 2010). To help speed recovery, we provided CHT +/- mice with a heating pad and *ad libitum* access to water for two days following each dialysis session. Mice also required more time (approximately two weeks) to return to criterion performance levels on the SAT following surgery. A number of mice had to be excluded from our microdialysis data because their performance on the SAT never returned to criterion following surgery. The atropine study also required extensive piloting, as three doses (5 μM, 50 μM, and 100 μM) of drug were tested before determining which to use in our study. We selected the intermediate dose, at which results were most consistent and drug-related ACh release increases were observed for both WT and CHT +/- mice.

Another accomplishment of this study was obtaining detectable levels of ACh release without the addition of an acetylcholinesterase inhibitor. Most dialysis studies of mice have relied on the local perfusion of neostigmine to facilitate the detection of low levels of ACh. However, because acetylcholinesterase inhibitors cause ACh levels to remain elevated by reversibly inhibiting ACh breakdown in the synapse, the use of such drugs is not ideal in studies that aim to distinguish potentially important patterns of ACh release across time.

We found that basal ACh release was comparable between WT and CHT +/- mice, consistent with the current understanding that CHT +/- mice have equivalent basal levels of CHT

localized at the synaptosomal membrane (Sarter & Parikh, 2005). In nonperforming mice, we observed that CHT +/- mice released ACh levels equal to WT during the first 8-min block of drug perfusion, but were unable to sustain this elevated release for the remainder of the drug period. This release pattern is congruent with the prevailing model of the CHT +/- deficiency, in which sustained, heightened ACh synthesis and release are limited by a reduced intracellular CHT reserve pool. In both WT and CHT +/- mice, atropine may initially induce trafficking of CHT from intracellular stores to the membrane to enable elevated ACh synthesis and release. However, as these intracellular stores are depleted, CHT hemizygotes are unable sustain heightened ACh turnover.

The release pattern observed during CHT +/- task performance looked quite different from that observed in nonperforming animals. During the task blocks we observed a ≈30% increase in ACh release, substantially lower than the ≈100% increase seen in the WT. Intriguingly, despite lower levels of performance-associated ACh release, CHT +/- mice performed equally well to WT on all measures of the SAT. While an interpretation of these surprising results will follow, a clear limitation of this study was the restricted number of WT microdialysis data available for analysis. Further experiments will include a larger sample size to confirm the findings here. Encouragingly, the ACh release pattern exhibited by the WT task-performing mouse in this study was congruent with previous findings in rats. For example the

≈100% increased release observed during the first task block was comparable to the 138.2 ± 14.24% reported in SAT-performing rats (Kozak, Bruno, & Sarter, 2006). Likewise, we found an average task-associated increase of 107.46%, consistent with the 88.70 ± 19.28% increase reported in rats (St. Peters et al., 2011). The finding that CHT +/- mice performed equally well despite lower levels of ACh release demonstrates an impressive ability of CHT +/- mice to compensate for reduced CHT capacity and provokes many questions.

First, it appears that the SAT alone does not sufficiently tax the cholinergic system to reveal differences in attentional performance that may be inherent of CHT hemizygosity. To investigate this possibility, we began training mice on the *d*SAT and conducted a preliminary dialysis session on a CHT +/- mouse. We observed the greatest ACh release not during the distractor blocks, as we had originally predicted, but in the block immediately following. This finding was especially intriguing in light of recent findings that suggest CHT +/- are more impaired than WT on signal trials in the block following the distractor (unpublished findings, Sarter, 2009). As increased cholinergic neurotransmission has been linked to attentional effort, the increased ACh release observed after the distractor may reflect the animal's attempt to recover performance. Further dialysis sessions during *d*SAT performance are needed to explore differences in ACh release patterns between WT and CHT +/- mice. If performance on the *d*SAT turns out to be equivalent in future studies, we expect to find that fluctuations in ACh

release during *d*SAT are mirrored in WT and CHT +/- animals, but on different scales. However, if CHT +/- mice continue to show an impaired ability to recover from the distractor, we predict that CHT +/- mice will not be able to reach proportionally high post-distractor release levels.

After finding that CHT +/- mice perform the SAT and *d*SAT with only a small amount of ACh release, we were interested to determine if the small increase in cholinergic signaling is in fact required for attentional performance. In an initial attempt to address this question, we blocked local ACh signaling by perfusing the muscarnic ACh receptor antagonist atropine during task. Beginning in the second block after drug onset, we observed a robust increase in omissions. While this may be an initial indication that ACh is required for attentional performance, the fact that signal and nonsignal responses were unimpaired makes it difficult to interpret the specific effects of the drug. Likely the animal's behavior was altered as it became aware of its "drug-state." Subsequent experiments will include a lower dose of atropine, which will hopefully allow for determination of the specific effects of ACh signaling blockade.

More illuminating will be lesions of the basal forebrain cholinergic neurons, effectively blocking cholinergic transmission to the mPFC. Similar lesions, conducted in rats after reaching criterion on the SAT, showed cholinergic inputs to the mPFC to be critical for performance (Martinez, & Sarter, 2004). If CHT +/- mice are able to recover performance after these lesions,

we must investigate the alternative strategies and signaling pathways employed by hemizygotes to accomplish the SAT. We can at this point only speculate as to the compensatory mechanisms CHT +/- animals develop throughout life (for example, down regulation of postsynaptic receptors, re-regulation of autoreceptors, or recruitment of different neurotransmitter systems to perform attention tasks).

Our data support the model that CHT +/- mice are unable to sustain elevated levels of ACh release, further validating the use of these mice to model diseases associated with impaired CHT capacity. The fact that we were unable to detect phenotype differences suggests that these animals are able to impressively compensate for their reduced CHT capacity to maintain sustained attention. These findings, as well as future research regarding the limitations of the CHT +/- genotype and the compensatory mechanisms enabling sustained attentional performance will help in understanding diseases associated with altered CHT functioning.

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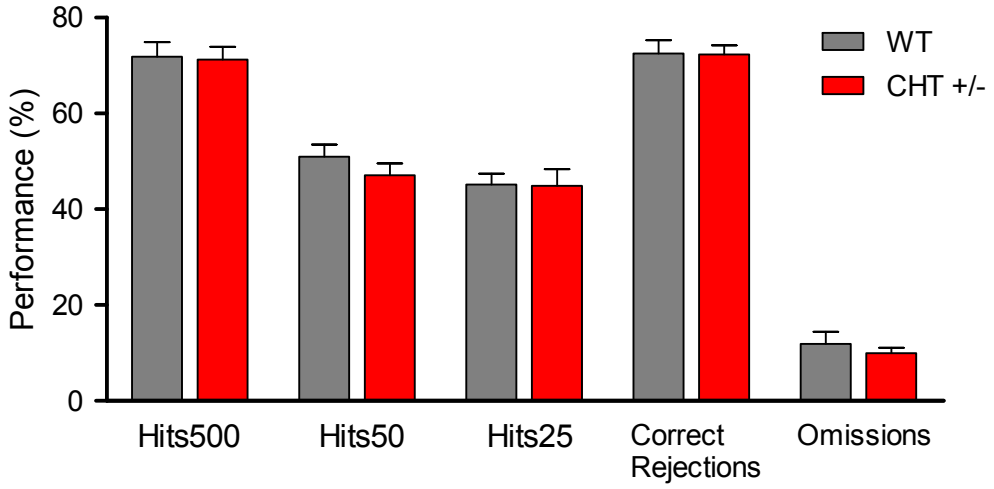

Figure 1. Sustained Attention Task performance of WT (n = 5) and CHT +/- (n = 6) mice. Measures of performance were averaged over three consecutive days at criterion. Hits, correct rejections, and omissions were comparable between WT and CHT +/- groups.

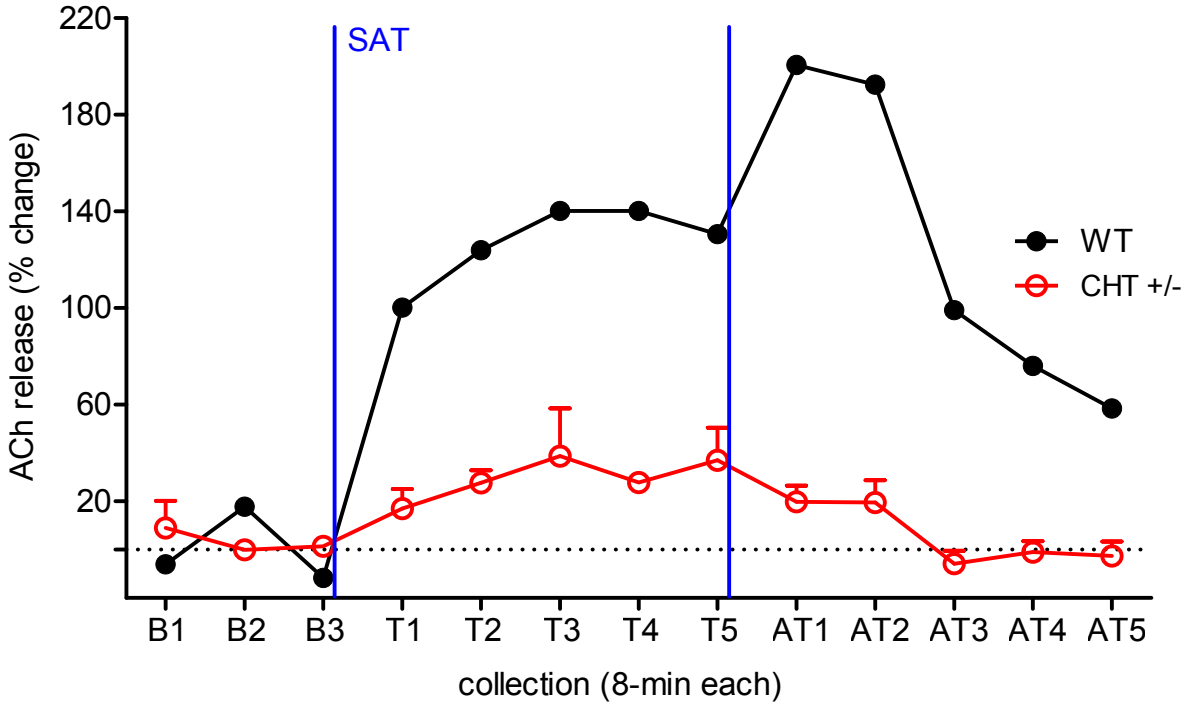

Figure 2. Acetylcholine (ACh) release in the medial prefrontal cortex of CHT +/- (n = 4) and WT (n = 1) mice performing the Sustained Attention Task (SAT). Vertical lines indicate the task period. Among CHT +/- animals, ACh release during SAT performance was not significantly different from baseline release.

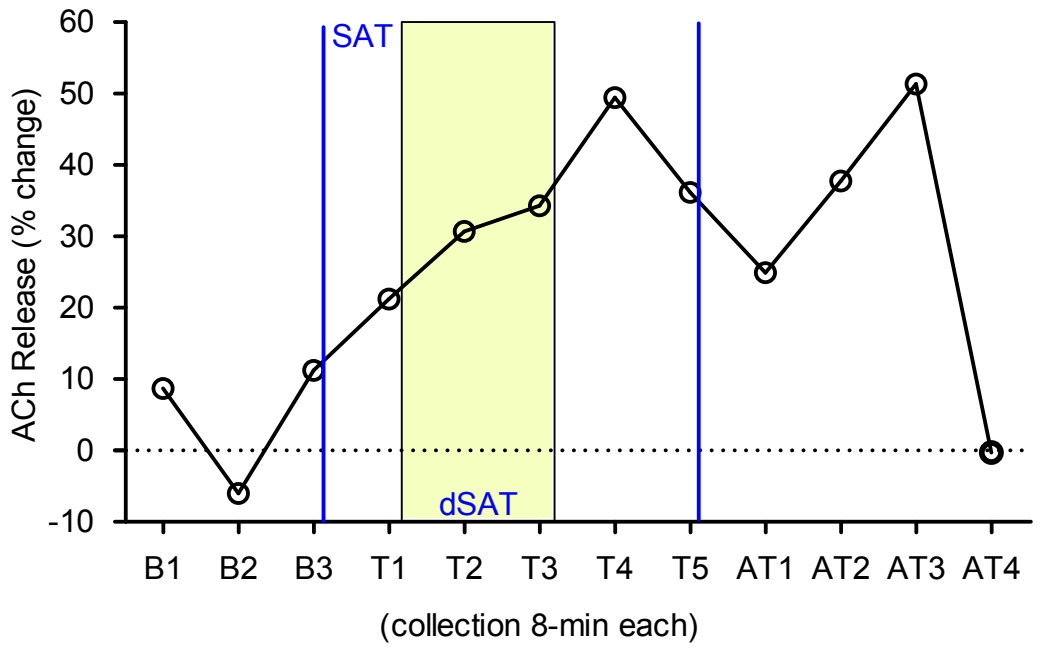

Figure 3. ACh release during performance on the *d*SAT. The visual distractor was presented during task blocks 2 and 3, as indicated by the shaded box. This graph depicts the performance-associated ACh release of a CHT +/- mouse (n = 1).

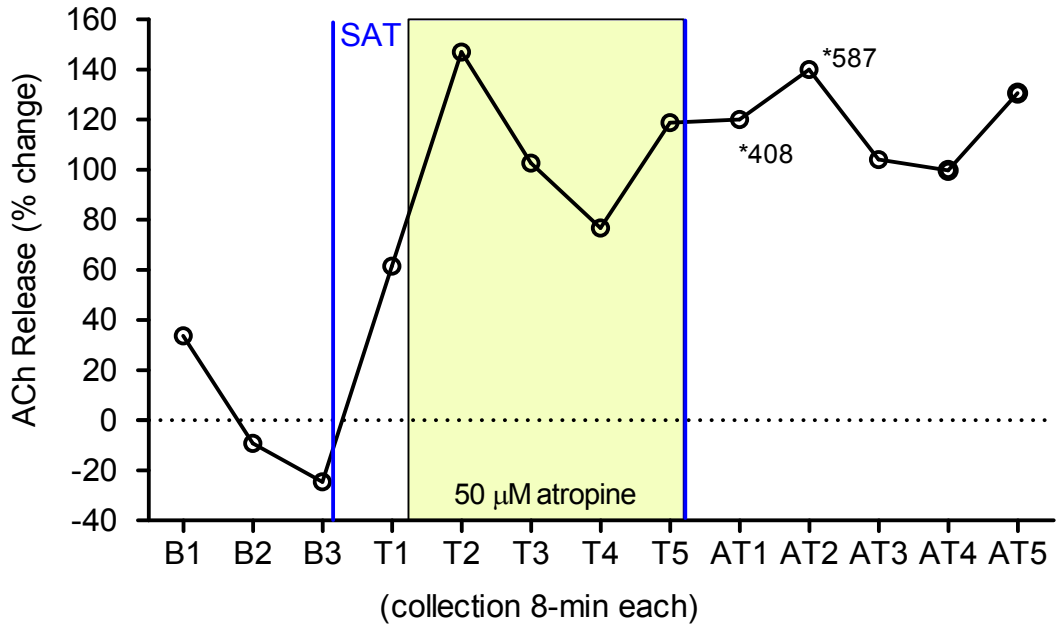

Figure 4. Effects of atropine on mPFC ACh release in a SAT-performing CHT +/- mouse. The shaded box indicates the time during which atropine (50 μM, $n = 1$) was perfused via reverse dialysis. This manipulation was designed primarily to block cholinergic signaling in the mPFC. The increases in ACh release observed are due to the drug's inhibitory effect on presynaptic autoreceptors. Note that the AT1 and AT2 values (indicated by asterisks) are misrepresented on the graph in order to allow better visualization of the task-associated release.

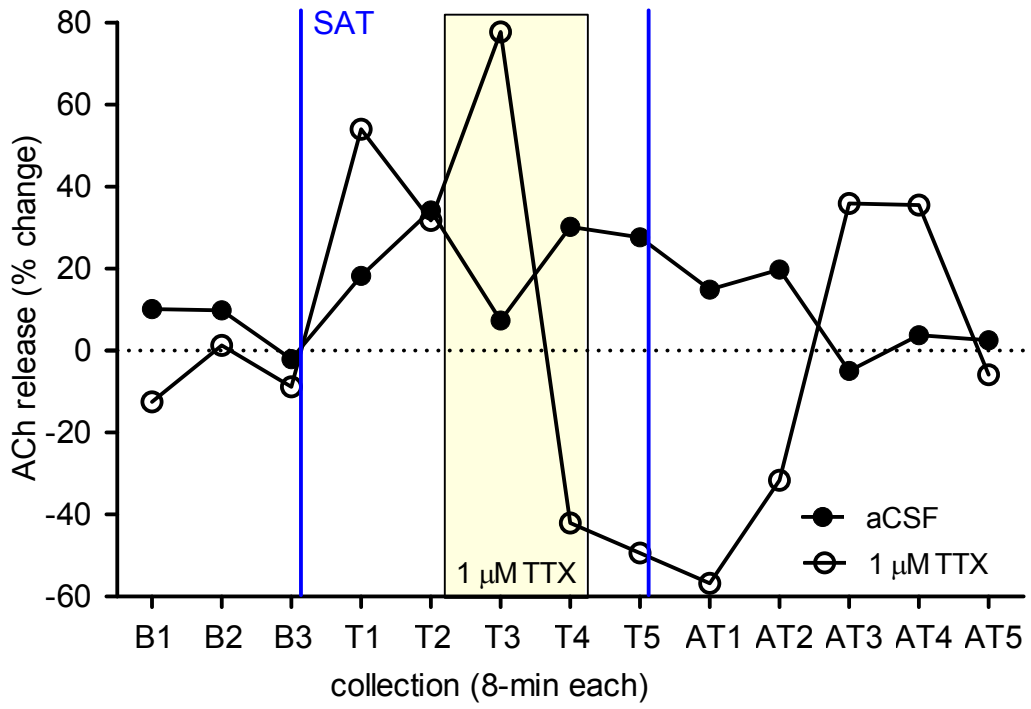

Figure 5. Effects of reverse dialysis of tetrodotoxin (TTX) on performance-associated ACh release in the mPFC. This graph depicts two different dialysis sessions performed on the same, CHT +/- mouse (n = 1). In one session TTX (1 μM) was perfused via reverse dialysis through blocks t3 and t4; in a control session, aCSF alone was perfused. This manipulation was designed to show that task-associated increases in ACh release are dependent on impulse-dependent transmission within the mPFC.

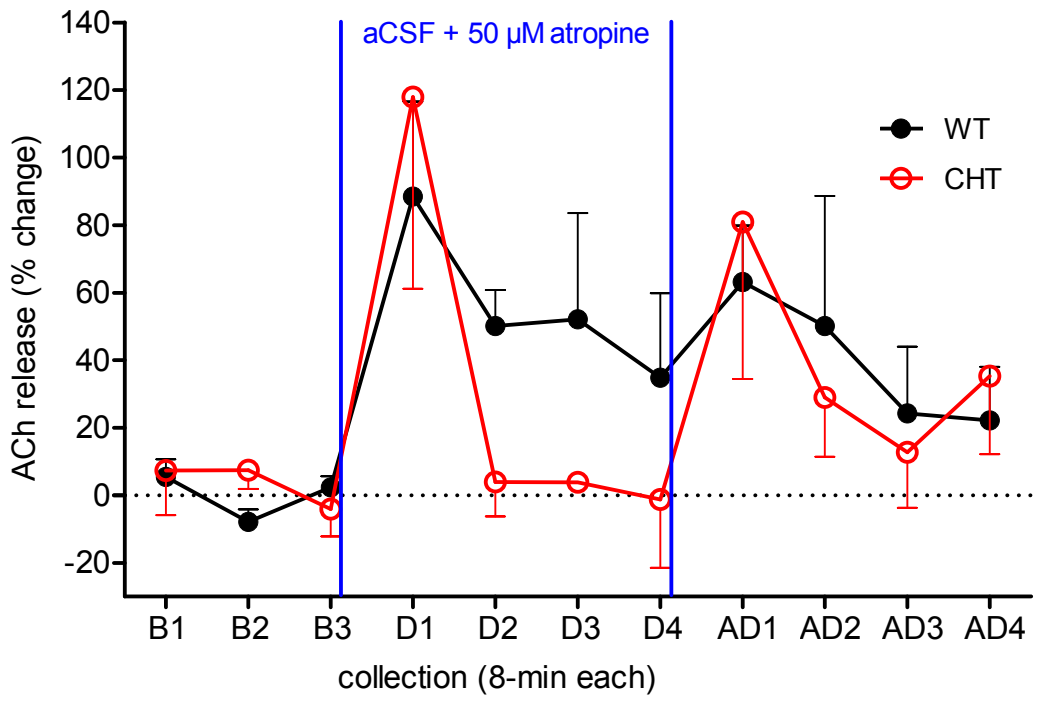

Figure 6. Temporal pattern of ACh in the mPFC following reverse dialysis of atropine (50 µM) in nonperforming CHT +/- (n = 2) and WT (n = 4) mice. ACh release during the first drug collection (D1) was significantly higher than average baseline release ($p<0.05$). An effect of genotype did not reach statistical significance, likely due to a lack of statistical power. CHT +/- mice released levels of ACh comparable to WT during the first drug collection period, but appeared unable to sustain this elevated release over the remaining collections.