Mice Deficient for the Vesicular Acetylcholine Transporter Are Myasthenic and Have Deficits in Object and Social Recognition

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Summary

An important step for cholinergic transmission involves the vesicular storage of acetylcholine (ACh), a process mediated by the vesicular acetylcholine transporter (VAChT). In order to understand the physiological roles of the VAChT, we developed a genetically altered strain of mice with reduced expression of this transporter. Heterozygous and homozygous VAChT knockdown mice have a 45% and 65% decrease in VAChT protein expression, respectively. VAChT deficiency alters synaptic vesicle filling and affects ACh release. Whereas VAChT homozygous mutant mice demonstrate major neuromuscular deficits, VAChT heterozygous mice appear normal in that respect and could be used for analysis of central cholinergic function. Behavioral analyses revealed that aversive learning and memory are not altered in mutant mice; however, performance in cognitive tasks involving object

and social recognition is severely impaired. These observations suggest a critical role of VAChT in the regulation of ACh release and physiological functions in the peripheral and central nervous system.

Introduction

Acetylcholine (ACh) plays a crucial role in controlling a number of physiological processes in both the peripheral and central nervous system. Synthesis of ACh requires efficient uptake of choline by the high-affinity choline transporter and choline acetylation by the enzyme choline acetyltransferase (ChAT) (Ribeiro et al., 2006). Efficient release of ACh from nerve endings depends on its storage in synaptic vesicles, a step reliant on the activity of a vesicular acetylcholine transporter (VAChT) (Parsons, 2000). VAChT is a twelve-transmembrane domain protein that uses the electrochemical gradient generated by a V-type proton ATPase to accumulate ACh in synaptic vesicles. VAChT and the vesicular monoamine transporters (VMATs) share a high degree of homology in their transmembrane domains and belong to the SLC18 (or solute carrier) family of proton/neurotransmitter antiporters (Erickson et al., 1994; Reimer et al., 1998; Roghani et al., 1994).

The ACh transporter is likely to provide stringent control of the amount of neurotransmitter stored and released by cholinergic nerve endings (Prado et al., 2002). VAChT trafficking to secretory vesicles appears to be the target of cellular regulation, and phosphorylation by protein kinase C (PKC) influences delivery of VAChT to synaptic-like microvesicles in PC12 cells (Cho et al., 2000; Krantz et al., 2000). However, the consequences of reduced targeting of VAChT to synaptic vesicles for ACh output in vivo are unknown.

Deficits in central or peripheral ACh neurotransmission have been described in several human disorders, including Alzheimer's disease (AD), in which certain behavioral and cognitive abnormalities have been related to brain cholinergic dysfunction (Bartus et al., 1982; Mesulam, 2004). However, the relationship between cholinergic decline and specific behavioral deficits is still not completely appreciated. Basal forebrain lesions in rats, with immunotoxins targeting the p75 neurotrophin receptor, indicate that ACh plays an essential role in attention (Sarter and Parikh, 2005), whereas it seems to participate, but it is not essential, in hippocampal-dependent spatial learning and memory (Parent and Baxter, 2004).

To investigate the consequences of reduced expression of VAChT on ACh neurotransmission and function, we genetically modified mice to produce a knockdown (KD) of VAChT gene expression. We observed a strong relationship between the levels of VAChT expression and ACh release in both the peripheral and central nervous systems. A marked reduction of VAChT expression was necessary to affect neurotransmission at the neuromuscular junction, while even modest deficiency was sufficient to interfere with brain ACh release and affect

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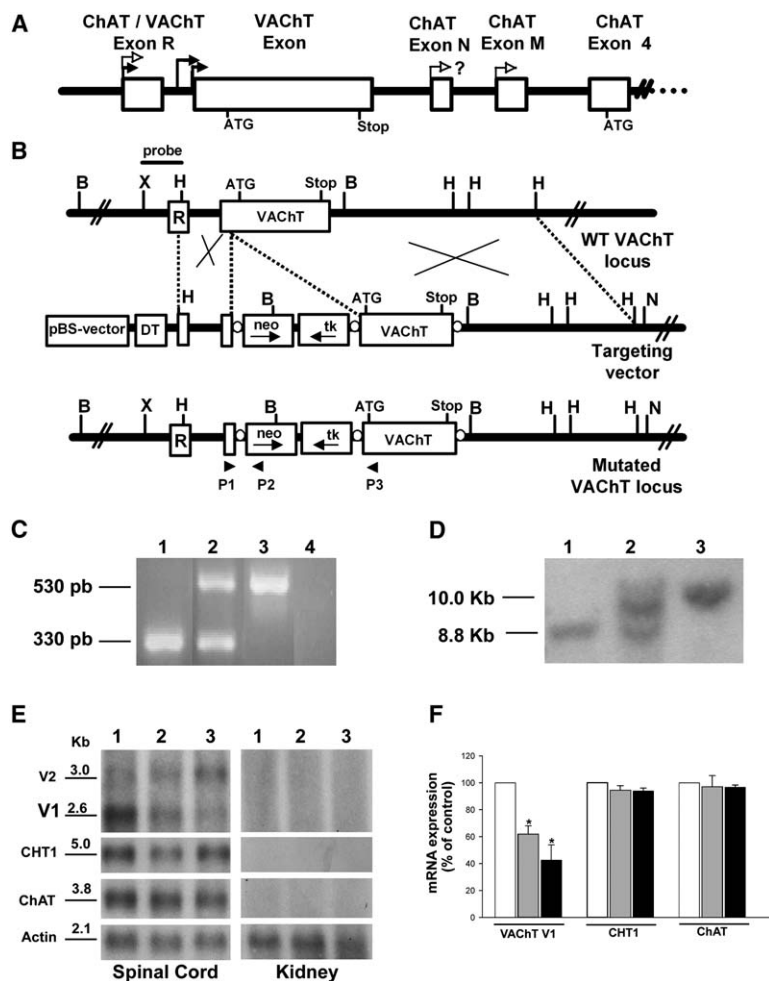
Figure 1. Schematic Drawing of the Cholinergic Gene Locus and Generation of VAChT-Deficient Mice

(A) Boxes represent the different exons of ChAT or VAChT. The position of the initiation codon (ATG) for VAChT and ChAT and the stop codon (stop) of VAChT are indicated. Potential transcription initiation sites are indicated for VAChT (filled arrowheads) and ChAT (open arrowheads). Note that the VAChT gene is within the first intron of ChAT.

(B) Schematic representation of the VAChT gene locus, the targeting construct, and the recombinant DNA. P1, P2, and P3 indicate position of PCR primers used for genotyping. Open white circles indicate loxP sites.

(C) PCR analysis of wild-type (lane 1), heterozygous VAChT KD mice (lane 2), and homozygous VAChT KD mice (lane 3). Lane 4 is a negative control without DNA.

(D) Southern analysis of wild-type (lanes 1), VAChT KD^{HET} (lane 2), and VAChT KD^{HOM} mice (lane 3).

(E) Northern blot analysis of VAChT, ChAT, and CHT1 in spinal cord for wild-type (lane 1), VAChT KD^{HET} (lane 2), and VAChT KD^{HOM} mice (lane 3). Kidney mRNA was isolated, and Northern analysis detected no signal for VAChT, ChAT, and CHT1 transcripts.

(F) Quantification of cholinergic transcripts. Blots were scanned and densitometric analysis was performed using the actin signal to normalize mRNA levels. Data are presented as a percentage of wild-type levels. (*) indicates statistical significant differences as described in the text.

behavior. Moreover, these investigations revealed a role for cholinergic tone in processing complex cues, which manifested as cognitive deficits in mutant mice for object and social memory.

Results

Molecular Analysis

To investigate the physiological consequences of altered expression of VAChT as related to vesicle filling and ACh release, we generated a mouse line with decreased expression rather than complete deletion, of this transporter, so we could investigate the consequences of reduced cholinergic tone in vivo (Figures 1A and 1B for wild-type and mutant alleles, respectively). PCR and Southern analyses confirmed homologous recombination and targeting of the 5′ untranslated region of the VAChT gene in genetically altered mice (Figures 1C and 1D). Mutant mice were born at the expected Mendelian frequency, survived, and exhibited no gross abnormalities.

Northern analysis of spinal cord indicated that the major mRNA species for VAChT (V1, 2.6 kb) was significantly reduced by 40% and 62% in VAChT KD^{HET} and KD^{HOM} mice, respectively [$F(2,11) = 11.09$, $p < 0.005$, one-way ANOVA, Figures 1E and 1F]. Surprisingly, a second VAChT species of 3.0 kb, which was especially

apparent in spinal cord, was significantly increased in VAChT KD mice, suggesting that compensatory transcriptional mechanisms operate in response to changes in VAChT expression. The changes in mRNA were specific for VAChT transcripts, as we detected no significant changes in mRNA levels for ChAT [$F(2,3) = 0.0311$, $p = 0.970$] and CHT1 [$F(2,12) = 0.0921$, $p = 0.9127$] in mutant mice (Figures 1E and 1F). These results agree with the lack of significant alterations found in ChAT activity and high-affinity choline transport in mutant mice (see Figure S1 in the Supplemental Data). Control experiments using kidney mRNA demonstrated the specificity of the probes (Figure 1E).

We investigated the consequences of altered expression of VAChT mRNA in VAChT KD mice by probing protein expression by immunoblot analysis. These experiments show a reduction of close to 50% in immunoreactivity for VAChT in the hippocampus [two-way ANOVA followed by Bonferroni post hoc, $F(2,23) = 70.95$, $p < 0.001$, Figures 2D and 2E] and in other brain regions (Figures 2A–2E) of VAChT KD^{HET} mice compared to wild-type control mice. In contrast, levels of other presynaptic proteins were not altered (Figures 2A–2E). Results were similar in all brain regions and in spinal cord (Figure 2E, overall decrease in all tissues was 56% ± 4% of the wild-type levels, n = 20). These results indicate a significant reduction in VAChT protein in VAChT KD^{HET}
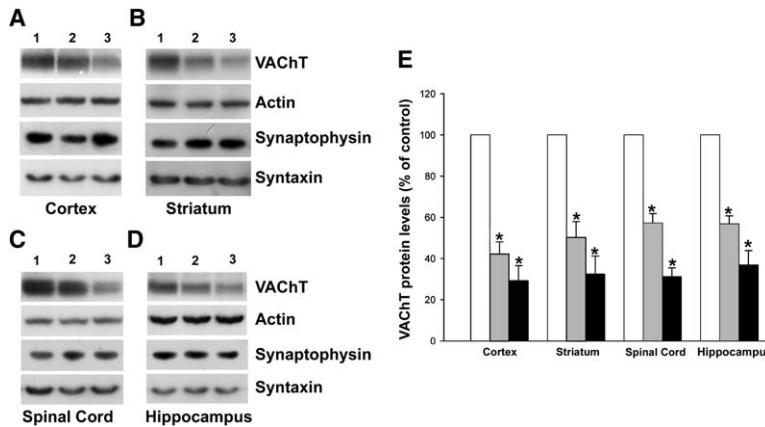
Figure 2. Gene Targeting Alters VAChT Protein Levels

Western blot analysis of VAChT, synaptophysin, and syntaxin in the cortex (A), striatum (B), spinal cord (C), and hippocampus (D) of wild-type (lane 1), VAChT KD[HET] (lane 2), and VAChT KD[HOM] mice (lane 3). (E) Quantification of protein levels. Actin immunoreactivity was used to correct for protein loading between experiments. Data are presented as a percentage of wild-type levels. (*) indicates statistical significant difference (one-way Anova with Bonferroni post hoc [cortex, $F_{(2,9)}$ = 49.11, p < 0.001; striatum, $F_{(2,6)}$ = 27.24, p < 0.001; spinal cord, $F_{(2,9)}$ = 95.75, p < 0.001; Hippocampus, $F_{(2,23)}$ = 70.95, p < 0.001]).

mice. VAChT KD[HOM] mice showed further decrease in VAChT protein levels (65% to 70%, Figures 2A–2E). Thus, VAChT KD[HOM] mice present an even larger decrease in levels of transporter than VAChT KD[HET] mice, but VAChT expression in homozygous mutant mice is sufficient for survival.

Electrophysiological Analysis and Neuromuscular Function

In order to evaluate the impact of reduced VAChT expression on quantal ACh release, we examined neuromuscular transmission. Miniature end-plate potentials (MEPPs) were readily recorded at neuromuscular junctions from either wild-type, VAChT KD[HET], or VAChT KD[HOM] mice. To compare quantal size we recorded at least 100 MEPPs from each of five fibers from five to seven animals of each genotype. MEPPs from mutant mice were smaller than those from wild-type, as can be seen in histograms of MEPP amplitudes (Figure 3A). To avoid possible histogram binning artifacts, we also analyzed the cumulative distribution of MEPP amplitudes, which showed a similar shift to smaller MEPPs in mutant animals (Figure 3B, p< 0.001 for VAChT KD[HOM], p < 0.05 for VAChT KD[HET], Kolmogorov-Smirnoff test). Further statistical analysis using ANOVA on averages of either the peak amplitude or the area of MEPPs confirmed the statistical significance of the differences in quantal sizes between wild-type and VAChT KD[HOM] animals [$F_{(1,71)}$ = 8.7, p < 0.005]. Therefore, mutant mice appear to pack less ACh in each synaptic vesicle.

In addition to quantal size, MEPP frequency was also strongly reduced in VAChT KD[HOM] animals, as shown in Figure 3C. The frequency of MEPPs was 0.69 ± 0.08 s^{-1} in wild-type animals (40 synapses from seven animals), 0.79 ± 0.18 s^{-1} in VAChT KD[HET] animals (30 synapses from five animals), and 0.37 ± 0.05 s^{-1} in VAChT KD[HOM] mice (41 synapses from seven animals). The difference in MEPP frequency between wild-type and VAChT KD[HOM] mice was statistically significant [two-way ANOVA followed by Bonferroni post hoc, $F_{(1,18)}$ = 10.3, p < 0.005].

The observed decrease in MEPP frequency at junctions from KD[HOM] mice could be due to a reduction in the number of synaptic vesicles available for release, a reduction in vesicle release probability, or a population of synaptic vesicles whose ACh load is below our detection limit. To investigate these possibilities, we measured evoked end-plate potentials (EPPs) during 100 Hz trains after cutting the muscle fibers to avoid contraction. Under these conditions, EPP amplitudes during a train rapidly fell from their initial level to a depressed steady state over the course of the first ten stimuli (Figure 3D). Overall, initial depression of normalized EPPs was similar in recordings from wild-type and KD[HOM] animals, suggesting similar release probabilities. Quantal content of each EPP during a train was calculated based on measured MEPP amplitudes, thus permitting an estimate of the size of the readily releasable pool of vesicles as described (Elmqvist and Quastel, 1965). This analysis considered only the first eight responses during a train for which the relationship between EPP versus cumulative EPP was linear. With this method, the readily releasable pool was similar for both genotypes and estimated at 439 ± 73 vesicles in synapses from wild-type animals and 550 ± 59 vesicles in VAChT KD[HOM] synapses (p = 0.52, two-tailed Student's t test). In contrast, the extent of steady-state depression of EPPs was significantly greater in VAChT KD[HOM] animals compared with wild-type [one-way ANOVA, $F_{(1,70)}$ = 197, p < 0.001].

Assuming constant quantal size, the increase in depression uncovered in the above experiments would suggest a defect in mobilizing or recycling of ACh-filled vesicles; however, the assumption of constant quantal size during the stimulus train may not be valid for mutant animals. Therefore, we attempted to directly test whether synaptic vesicle exo- and endocytosis would be altered in mutant mice. For this we performed experiments with the vital dye FM1-43 (Richards et al., 2000), which provides the opportunity for optical detection of both exocytosis and endocytosis of synaptic vesicles.

Labeling of nerve terminals in junctions from both wild-type and KD[HOM] animals in response to 60 mM KCl (10 min) was indistinguishable, and no differences were detected upon quantification of fluorescent spots (Figure 3E), suggesting that endocytosis occurs to the same extent in both genotypes. Destaining of fluorescent spots in response to 60 mM KCl was calcium dependent (not shown), and was not different between wild-type and KD[HOM] animals (Figure 3F), indicating that synaptic vesicle exocytosis is not changed in VAChT KD[HOM] mice. Thus, taken together, our observations would suggest that the alterations in MEPP frequency and EPP
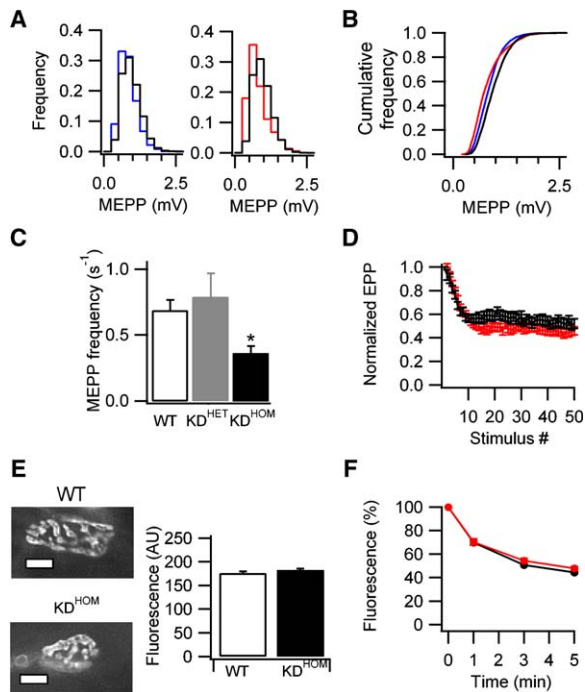
Figure 3. Neuromuscular Transmission in VAChT KD[HET] and VAChT KD[HOM] Mice

(A) Normalized histogram of MEPP amplitudes for wild-type (black line, 3302 MEPPs), VAChT KD[HET] (blue line, 4319 MEPPs), and VAChT KD[HOM] (red line, 3690 MEPPs) mice. Data are from five synapses from five to seven animals for each genotype.

(B) Quantal size of the three genotypes quantified by plotting the cumulative frequency of MEPP amplitudes. Black line, wild-type; blue line, VAChT KD[HET]; red line, VAChT KD[HOM].

(C) Frequency of MEPPs at synapses from the three genotypes. (*) indicates statistically significant difference from control wild-type mice (two-way ANOVA followed by Bonferroni post hoc; $F_{(1,18)}$ = 10.3, p < 0.005).

(D) Normalized EPP amplitude (to the first stimulus) for wild-type (black line) and VAChT KD[HOM] (red line) mice in response to a train of 100 Hz (0.5 s). Data are from ten synapses from three wild-type animals and 16 synapses from three KD[HOM] animals.

(E) Nerve terminals from wild-type and VAChT KD[HOM] mice were labeled with FM1-43 and show similar patterns of staining. Data are mean ± SEM of 109 fluorescent spots from 21 nerve terminals of wild-type mice and 111 fluorescent spots from 26 nerve terminals from VAChT KD[HOM]. Scale bar, 10 μm.

(F) Destaining of FM1-43-labeled nerve endings from wild-type (black line) and VAChT KD[HOM] (red line). Data are mean ± SEM of 26 fluorescent spots (wild-type mice) and 21 fluorescent spots (VAChT KD[HOM]) from four mice per genotype.

depression in VAChT KD[HOM] are more than likely a consequence of decreased transport of ACh by synaptic vesicles.

To evaluate whether the alterations detected in neuromuscular transmission may affect neuromuscular function, we tested the performance of wild-type and mutant mice in motor tasks (Figure 4). In the wire-hang test (Figure 4A), wild-type and VAChT KD[HET] mice show no differences in performance; however, VAChT KD[HOM] mice were significantly impaired [$F_{(2,37)}$ = 28.77, p < 0.001]. This altered performance of VAChT KD[HOM] animals is likely the result of altered neuromuscular force, since these mutants were also severely impaired in a

grip strength test when compared with wild-type mice [Figure 4B, $F_{(3,48)}$ = 9.52, p < 0.001]. By comparison, VAChT KD[HET] mice present no deficit in neuromuscular function as assessed in this test. Importantly, reduced grip strength in VAChT KD[HOM] mice was improved by prior injection of one of three cholinesterase inhibitors: pyridostigmine (i.p., 1 mg/kg), galantamine (s.c., 1 mg/kg) or physostigmine (i.p., 0.3 mg/kg) [Figure 4B, $F_{(3,47)}$ = 8.323, p < 0.05]. No change in grip force was observed in wild-type mice treated similarly with any of the above cholinesterase inhibitors at the doses used (not shown). Since pyridostigmine is charged and should not cross the blood-brain barrier, its efficacy in improving grip force observed in homozygous mutant mice directly implicates peripheral cholinergic transmission in this effect.

To further study neuromuscular output, we examined performance of VAChT mice on the rotarod. This test depends not only on the ability of mice to learn motor skills, but also on their ability to maintain prolonged motor function. Wild-type mice were able to learn this motor task, and after five trials their performance was significantly better than their performance during the first trial [Figure 4C, repeated measures ANOVA, $F_{(13,195)}$ = 16.9, p < 0.05]. The performance of VAChT KD[HET] mice improved significantly only after 12 trials on the rotarod [repeated measures ANOVA, $F_{(13,117)}$ = 4.63, p < 0.05]. In contrast, VAChT KD[HOM] mice never learned this motor task [Figure 4C, $F_{(13,91)}$ = 0.653] and their performance was significantly worse than those of wild-type and VAChT KD[HET] mice [$F_{(2,434)}$ = 60.16, p < 0.05 on trials 12, 13, and 14, two-way ANOVA followed by Bonferroni post hoc tests].

The performance of VAChT KD[HOM] mice may indicate either motor learning deficits on the rotarod or that mutant mice are incapable of sustained physical activity. To evaluate the latter possibility, we used a treadmill to evaluate the performance of wild-type, VAChT KD[HET], and VAChT KD[HOM] mice in exhaustive physical activity. Figure 4D shows that VAChT KD[HOM] mice were not able to maintain long periods of physical activity and performed poorly compared with wild-type or VAChT KD[HET] mice [one-way ANOVA followed by Bonferroni post hoc, $F_{(2,28)}$ = 22.09, p < 0.001]. Indeed, VAChT KD[HOM] mice could run for no more than 5 min on the treadmill, whereas wild-type or VAChT KD[HET] mice could usually run for longer than 60 min. These results indicate that VAChT KD[HOM] mice are unable to perform on the rotarod due to their decreased capacity to maintain physical activity. They also indicate that VAChT KD[HET] mice appear as physically fit as wild-type control mice under the conditions tested.

Neurochemical Analysis

VAChT KD[HOM] mice display significant neuromuscular deficiency, which may confound the outcomes of complex behavioral tests aimed at assessing consequences of central ACh deficiency. In contrast, VAChT KD[HET] mice have essentially normal neuromuscular transmission, thereby indicating their potential usefulness as test subjects for investigating the behavioral consequences of mild reductions of central cholinergic function.

To investigate the functional consequences of reduced VAChT expression, we first used brain microdialysis to
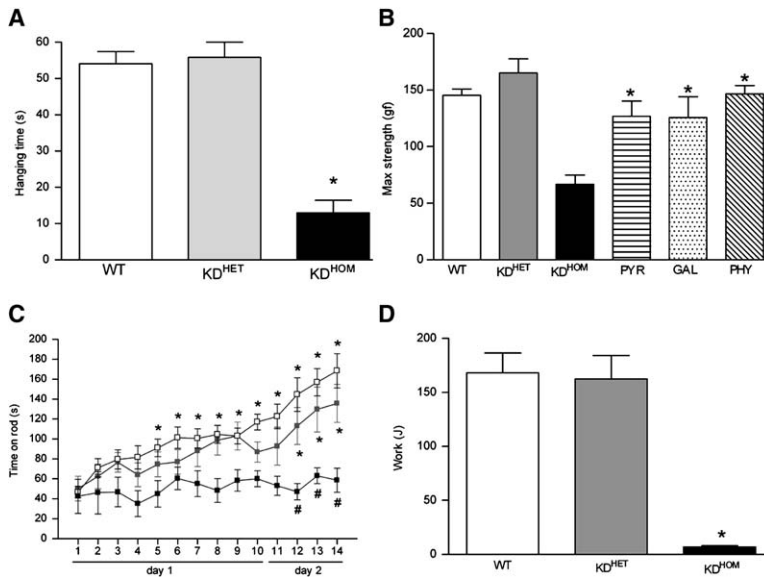
Figure 4. Neuromuscular Function of VAChT KD[HET] and VAChT KD[HOM] Mice

(A) Time spent hanging upside-down from a cage by wild-type, VAChT KD[HET], and VAChT KD[HOM] mice. *p < 0.05 from wild-type controls (one-way ANOVA followed by Bonferroni post hoc; $F[2,37] = 28.77$, p < 0.05, n = 20 wild-type, n = 12 VAChT KD[HET], and n = 8 VAChT KD[HOM]).

(B) Grip force measured for wild-type, VAChT KD[HET], VAChT KD[HOM], and VAChT KD[HOM] mice treated with pyridostigmine (i.p., 1 mg/kg), galantamine (s.c., 1 mg/kg) and physostigmine (i.p., 0.3 mg/kg) 30 min prior to the test. (*) indicates statistical difference when compared with VAChT KD[HOM] mice without cholinesterase treatment.

(C) Performance of wild-type (clear squares), VAChT KD[HET] (gray squares), and VAChT KD[HOM] mice (black squares) on the rotarod task. (*) indicates statistically significant differences compared with the first trial for each genotype (repeated measures ANOVA, p < 0.05). (#) indicates statistically different performance when compared with wild-type mice [two-way ANOVA shows an effect of genotype; $F(2,434) = 60.16$, p < 0.05].

(D) Exercise capacity of wild-type, VAChT KD[HET], and VAChT KD[HOM] mice. Mice were trained on the treadmill with a protocol that evaluated physical capacity (see Experimental Procedures). After training mice, were tested for performance, and the work (in J) done was calculated.

establish extracellular levels of ACh in freely moving VAChT KD[HET] mice. Because all brain regions examined appeared to show similar reductions in VAChT expression, we chose to determine extracellular ACh levels in frontal cortex and striatum. Frontal cortex was selected because this brain region receives innervation from nucleus basalis and substantia innominata, areas known to be affected in Alzheimer's disease. Striatum was chosen because it contains the largest concentration of cholinergic nerve endings and is therefore particularly suitable to evaluate possible decreases in extracellular ACh. The quantitative "low perfusion rate" microdialysis approach, which allows precise determination of a given extracellular neurotransmitter (Gainetdinov et al., 2003), revealed that levels of extracellular ACh were depressed by more than 35% in frontal cortex [$t(1,19) = 2.642$, p < 0.016] and by approximately 31% in striatum [$t(1,18) = 2.560$, p < 0.020] of VAChT KD[HET] mice (Figure 5A). Next, by using the conventional microdialysis approach, we examined the dynamic responses to KCl-stimulated ACh release in the striatum. After establishing basal extracellular ACh levels, artificial cerebrospinal fluid (CSF) containing 60 mM [K$^+$] was perfused through the microdialysis probe over the next 40 min, and the probe was returned to normal artificial CSF for the remaining 40 min of the experiment (Figure 5B). A repeated measures ANOVA revealed a significant main effect of time [$F(5,60) = 31.541$, p < 0.001] and a significant time by genotype interaction [$F(5,60) = 7.502$, p < 0.001]. Bonferroni-corrected pairwise comparisons showed genotype effects at 40 (p < 0.044), 60 (p < 0.023), and 80 min (p < 0.026). Hence, both genotypes responded to KCl depolarization; however, stimulated release in KD[HET] striatum was reduced relative to that of the wild-type controls.

Since VAChT is responsible for sequestering ACh into secretory vesicles, we evaluated the effects of decreased VAChT expression on total ACh levels in brain tissue. When tissue concentrations of ACh were measured by high-performance liquid chromatography (HPLC) with eletrochemical detection (HPLC-EC), levels in frontal cortex and striatum of VAChT KD[HET] mice were significantly increased by approximately 49% [$t(1,25) = 4.082$, p < 0.001] and 30% [$t(1,10) = 3.408$, p < 0.007], respectively, over that of the wild-type controls (Figure 5C). These data were replicated in a complementary chemiluminescence assay in a separate group of mice using both striatum and hippocampus (Figure 5D; p < 0.05). Moreover, VAChT KD[HOM] mice show an even larger increase in ACh content in the brain, and this increase was statistically different from that of VAChT KD[HET] mice or wild-type mice (Figure S1C; p < 0.05). This increase in ACh content for mutant mice cannot be attributed to an increase in ChAT activity (Figure S1A), high-affinity choline transporter activation (Figure S1D), or increased levels of expression of ChAT (Figures S1B and S1E) or CHT1 (Figure 1E). Whereas the mechanism of such an increase in total tissue ACh content it is not immediately apparent, it is important to emphasize that the functional "releasable" ACh pool seems to be decreased, as evidenced by in vivo microdialysis experiments and quantal analysis at the neuromuscular junction. All together, these results demonstrate that a reduction of approximately 50% in the levels of VAChT expression in the brain results in a significant decrease in the release of ACh in vivo, despite enhanced intracellular content of neurotransmitter. These observations suggest a complex relationship between the control of storage and release of ACh in CNS neurons.
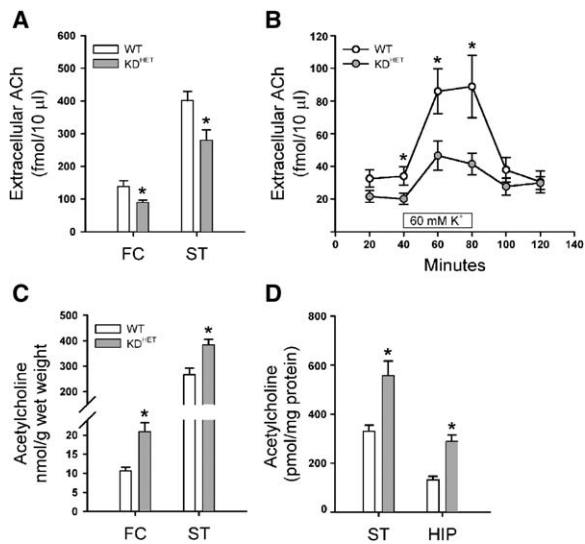
Figure 5. Neurochemical Alterations in VAChT KD[HET] Mice

(A) Extracellular ACh levels as determined by quantitative low perfusion rate microdialysis in frontal cortex and striatum. n = 10 mice per genotype per brain region.

(B) KCI-stimulated release of ACh in striatum of freely moving mice. Following 40 min of baseline collection of ACh, 60 mM [K+] was infused through the microdialysis probe for 40 min, and artificial CSF was infused over the last 40 min of the experiment. n = 7 mice per genotype. *p < 0.05 for wild-type controls.

(C) Tissue ACh contents in frontal cortex (FC) and striatum (ST) of wild-type and KD[HET] mice measured by HPLC with electrochemical detection. FC: n = 14 (wild-type), n = 13 (KD[HET]); ST: n = 6 mice per genotype.

(D) Striatal (ST) and hippocampal (HIP) tissue ACh levels assayed by chemiluminescent detection in wild-type (open bars) and VAChT KD[HET] (gray bars) mice (n = 5). In all panes, data are mean ± SEM. *p < 0.05 for wild-type controls.

Behavioral Evaluation

After documenting normal performance of VAChT KD[HET] mice in tests of neuromuscular strength, despite reduced cholinergic tone in the brain, we proceeded to evaluate performance of mutants in behavioral tasks reflecting CNS cholinergic function. VAChT KD[HET] mice were tested for performance in the step-down inhibitory avoidance task, a task that depends upon hippocampal and amygdala networks and may be sensitive to manipulations in central cholinergic function (Izquierdo and Medina, 1997). Both genotypes exhibited learning, as latency to step-down from the platform increased from 10 to 15 s to approximately 80 to 100 s after training. In parallel experiments, we determined in another cohort of mice that the unconditioned stimulus was essential for both genotypes to learn the task (data not shown). VAChT KD[HET] performed as well as wild-type littermates on this task on a short-term (1.5 hr after learning) and long-term (24 hr after learning) memory test, suggesting that this specific aspect of learning and memory is preserved in animals with a mild decrease in cholinergic tone (Figure 6A).

A second test for memory, based on the ability to discriminate novel objects, was used to evaluate the performance of mutant mice. In the object recognition task, mice explore two objects, and after a latency of 1.5 or 24 hr they are presented with one of the familiar objects and a nonfamiliar object. Initial exploration time of two objects was identical for both genotypes, indicating that they both show preference for novelty (data not shown). However, whereas wild-type mice exhibited a significant increase in the exploration of the unfamiliar object, mutant mice performed poorly compared to wild-type mice in their ability to remember the familiar object both 1.5 and 24 hr after learning (Figure 6B, p < 0.05, Kruskal-Wallis analysis of variance and Mann-Whitney U tests, n = 12–18). Thus, VAChT KD[HET] mice appear to have a cognitive deficit that impacts behavior in this test.

Recognition of a familiar conspecific is the basis of several social interactions, including hierarchical social relationship and mate choice (Winslow and Insel, 2004). There is evidence for the participation of nicotinic and muscarinic central systems in social recognition in rodents (Prediger et al., 2006; van Kampen et al., 2004; Winslow and Camacho, 1995), and social recognition deficits may relate to cholinergic decline in a mouse model of AD (Ohno et al., 2004). We evaluated social interactions of VAChT KD[HET] mice in a habituation-dishabituation paradigm using a mouse intruder (Choleris et al., 2003). Wild-type control mice showed extensive exploration of the intruder (e.g., sniffing) during first contact. This response decreased with subsequent exposure to the same juvenile [$F_{(4,11)}$ = 60.93, p < 0.01], indicating that wild-type control mice readily habituated to the conspecific (Figure 6C). Hence, after four exposures to the same juvenile, wild-type mice explored the intruder for only one-third of the length of time of the initial exploration. Upon changing to an unfamiliar mouse, wild-type animals showed a renewed interest in investigation, and explored the new mice as much as they explored the original intruder during the first contact (Figure 6C). These results indicate that lack of interest in exploring the first intruder upon recurring exposure was not attributable to lack of motivation, but appears to be due to habituation, i.e., learning. Exploration of the intruder mice by VAChT KD[HET] mice on the first contact was slightly less than that observed for wild-type animals (p < 0.05, two-way ANOVA with Bonferroni post hoc). Upon subsequent exposures, VAChT KD[HET] mice show statistically significant differences in exploration of the intruder mice as compared with wild-type mice (p < 0.001, two-way ANOVA with Bonferroni post hoc). In sharp contrast to wild-type littermates, VAChT KD[HET] mice failed to habituate to the juvenile intruder in the subsequent exposures after the initial contact, and only after the fourth contact was there a significant difference in exploratory behavior compared with the first encounter [$F_{(4,10)}$ = 5.293, Figure 6C)]. Introduction of an unfamiliar mouse led VAChT KD[HET] mice to increase their exploration, indicating that the decrease in exploration during the fourth exposure for the first intruder was not due to nonspecific effects such as physical exhaustion or motivation.

One possible explanation for the inability of VAChT KD[HET] mice to habituate to a conspecific is that mutant mice have olfactory deficits. In a control experiment, we evaluated olfactory responses in these mice. However, both wild-type and VAChT KD[HET] mice showed similar abilities in finding a hidden food reward (data not shown), suggesting that the differences observed
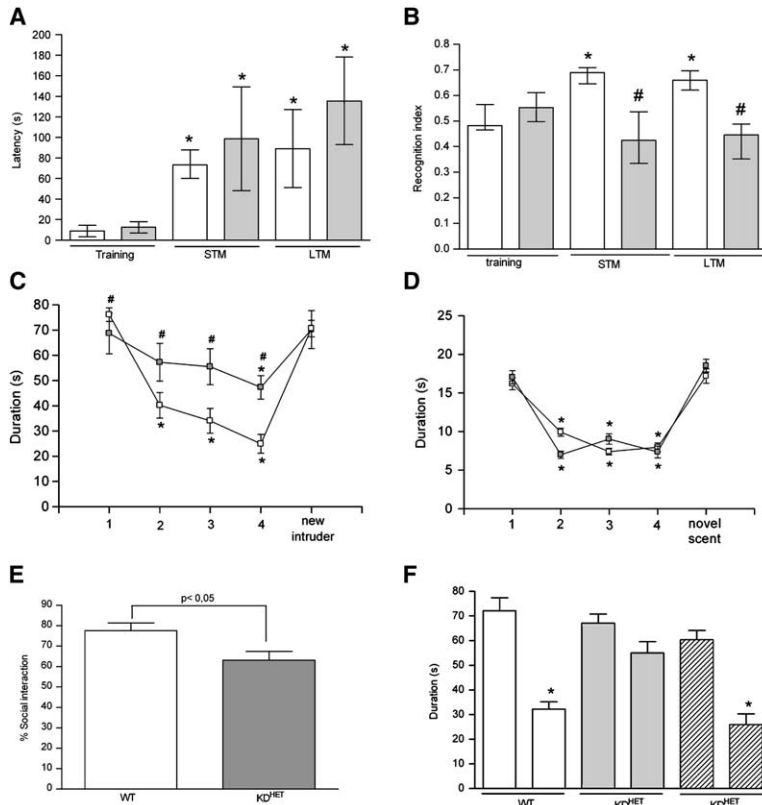
Figure 6. Behavioral Alterations of VAChT KD[HET] Mice

(A) Step-down inhibitory avoidance task. Retention test latency measured 90 min after training (STM) and again at 24 hr (LTM). Ordinates express median (interquartile range) test session latency, in seconds. Open bars represent the performance of wild-type mice and shadowed bars represent that of VAChT KD[HET] mice (n = 13–18 per group). *p < 0.05 compared with performance of mice during training.

(B) Object recognition test. Results are shown as median (interquartile ranges) recognition indexes of short-term (STM) and long-term (LTM) retention test trials. Clear bars represent data from wild-type mice and shadow bars are the data from VAChT KD[HET] mice. (#) indicates a significant difference from wild-type; p < 0.05, n = 12–18. *p < 0.05 compared with performance of mice during training.

(C) Social memory of wild-type (open squares) and KD[HET] (gray squares) mice was measured as olfactory investigation during each of four successive 5 min trials with an intertrial interval of 15 min. A fifth dishabituation trial depicts the response of mice to the presentation of a new intruder in a 5 min pairing, 15 min after the fourth trial. *p < 0.05 compared with performance on the first trial within the genotype, and #p < 0.05 when compared with wild-type control mice; n = 10–12.

(D) Olfactory function of wild-type and VAChT KD[HET] mice. Mice were presented a strawberry essence for 1 min in 4 sequential trials with an intertrial interval of 10 min. On the 5th trial, vanilla essence was presented. *p < 0.05 from the first trial within genotype. No between group differences were observed.

(E) Social preference of wild-type (open bar) and VAChT KD[HET] mice. Only the percentage of exploration for the social stimulus is shown.

(F) Social memory of wild-type (open bars, n = 14), VAChT KD[HET] (gray bars, n = 14), and VAChT KD[HET] mice treated with galantamine (s.c., 1 mg/kg) 30 min prior to the first exposure to an intruder (hatched bars, n = 8). The intruder is presented in each of two 5 min trials with an intertrial interval of 30 min. *p < 0.05 for the first trial within the genotype. Unless otherwise stated, data are mean ± SEM.

in social recognition do not relate to deficits in olfactory function. In addition, wild-type and VAChT KD[HET] mice habituated to a test odor similarly [wild-type, $F(4,6) = 11.35$, and VAChT KD[HET] mice, $F(4,6) = 18.11$, p < 0.05 by repeated measures ANOVA]. There were no differences between the two genotypes in olfactory habituation or in their ability to discriminate between two test odors (Figure 6D).

A second possibility to explain the deficit in social habituation is that VAChT KD[HET] mice are more social than wild-type mice, i.e., they prefer the company of intruder mice more than wild-type mice do. This would be the converse of the autistic-like behavior found in PTEN mutant mice (Kwon et al., 2006). To specifically test this possibility, we evaluated the choice of wild-type and VAChT KD[HET] mice for a social stimulus (an adult mouse in an acrylic cage that allowed minimum tactile exploration but allowed olfactory exploration) against a nonsocial stimulus (an identical acrylic cage which was never previously presented to the mice). This experiment was done in specially designed boxes containing two separate rooms, each of which the mice had to enter to explore the social or nonsocial target, respectively (Kwon et al., 2006). As expected from the previous experiment, both genotypes had a stronger preference for the social against the nonsocial stimuli (Figure 6F);

however, VAChT KD[HET] mice expended significantly less time with the social stimulus and consequently more time with the nonsocial stimulus than wild-type control mice did (p < 0.05, Student's t test). Therefore, increased social preference of VAChT KD[HET] mice cannot explain the lack of habituation observed in the social recognition test. If anything, the data indicate that VAChT KD[HET] mice are less social than control mice.

The results above suggest that VAChT KD[HET] mice have a deficit in social memory. This deficit could be a consequence of decreased ACh release, or it could result from adaptative changes in brain neurochemistry during development in response to the decreased expression levels of VAChT. If the deficits in social recognition are related to decreased acetylcholine output, acute inhibition of cholinesterase, which preserves ACh in the synapse, might rescue the phenotype. Therefore, we retested mice in the social memory task using a paradigm that allowed us to treat mice with a cholinesterase inhibitor prior to the experiment. The social recognition memory lasted at least 30 min, as wild-type mice exposed to an intruder for 5 min twice, with an intertrial interval of 30 min, explored the intruder significantly less in the second exposure [$F(5,70) = 17.21$, p < 0.001] (Figure 6F). In contrast, there was no difference for VAChT KD[HET] mice between the first and second

exposure to intruder mice with this protocol (Figure 6E). We repeated these experiments after injecting mice with either saline or galantamine (Figure 6F). The dose of galantamine used (s.c., 1 mg/kg) has been shown to be effective in improving cholinergic function in mice (Csernansky et al., 2005), and it was sufficient to improve the performance of VAChT KD^{HET} in this social recognition task (Figure 6F). Injection of saline had no effect on the performance of wild-type or VAChT KD^{HET} mice, ruling out that prior manipulation of mice affected the outcome of these experiments (data not shown). In addition, galantamine did not alter the response of wild-type mice (data not shown). It should be noted that the deficit in social recognition memory was also observed in a small number of VAChT KD^{HOM} mice studied with an identical protocol (Figure S2), thus confirming this phenotype for the two mutant genotypes. Hence, it appears that VAChT KD^{HET} mice have a deficit in social memory due to decreased cholinergic tone.

Discussion

To define the role of VAChT and ACh in physiological functions and behavior, we generated a mouse line with reduced expression of this transporter. The partial decrease in VAChT expression is essential in these investigations as complete lack of the vesicular transporter is likely to be incompatible with life, as shown for other presynaptic cholinergic genes (Brandon et al., 2004; Ferguson et al., 2004; Misgeld et al., 2002). Thus, this reduced expression mouse line allowed us to examine the consequences of reduced cholinergic tone in function, behavior, and cholinergic neurochemistry.

It has been demonstrated that several putative mRNA species exist for VAChT, although V1 is predominant in cholinergic tissues (Bejanin et al., 1994). In the present experiments, we show that VAChT KD^{HET} and KD^{HOM} mice have reduced levels of this major VAChT mRNA, whereas an increase in a less common mRNA for VAChT was detected, suggesting the existence of a compensatory mechanism in mutant mice. The open reading frame of VAChT is within the first intron of the ChAT gene. Interestingly, we detected no changes in ChAT mRNA levels in all CNS regions investigated, even though ChAT and VAChT transcripts might be, under certain conditions, coregulated (Eiden, 1998). In vertebrates, regulation of the cholinergic gene locus expression is complex; ChAT- and VAChT-specific mRNAs can be produced either from different promoters or by alternative RNA splicing (Oda, 1999).

In addition to the decrease in VAChT transcript, we detected a 45% reduction in VAChT protein levels in several CNS regions in VAChT KD^{HET} mice, whereas the reduction in VAChT protein levels in homozygous mutant mice was 65%–70% of that found in wild-type littermates. Therefore, the data indicate that protein levels of VAChT closely follow the reduction of the major VAChT mRNA species.

To evaluate how a decrease in VAChT levels affects transmitter release, we examined quantal secretion of ACh at the neuromuscular junction. Surprisingly, we observed relatively mild alterations in the distribution of quantal sizes in VAChT KD^{HET} mice. A robust change in quantal size distribution for VAChT KD^{HOM} mice was

detected; however, a very pronounced decrease in the frequency of MEPPs was also observed. This decrease in MEPP frequency is not the result of alterations in the readily releasable pool of vesicles. It also seems unlikely that the alteration in MEPP frequency is the result of decreased exocytosis, endocytosis, and total pool of vesicles, as FM1-43 experiments have shown no difference in these parameters between wild-type and VAChT KD^{HOM} mice. We hypothesized that, if in synapses, the number of copies of VAChT per synaptic vesicles is low (Parsons et al., 1993; Van der Kloot, 2003), a reduction in VAChT abundance could result in electrophysiologically "silent" vesicles, and thus a decrease in MEPP frequency. Prior experiments have demonstrated that overexpression of VAChT in immature *Xenopus* spinal neurons increases not only the amplitude but also the frequency of miniature excitatory postsynaptic currents (Song et al., 1997), indicating that, at least under certain conditions, VAChT expression levels can affect electrophysiological detection of exocytosis. Similarly, in *Drosophila* mutants with decreased neuromuscular expression of the vesicular glutamate transporter, there are major deficits in frequency of miniature end-plate currents, but no alterations in quantal size (Daniels et al., 2006). Remarkably, VAChT phosphorylation by PKC affects its trafficking to secretory vesicles, suggesting that alterations in VAChT expression in synaptic vesicles could occur physiologically (Krantz et al., 2000).

The results herein with VAChT mutant mice also indicate that synaptic vesicle exocytosis is not altered by decreased levels of the transporter; in this regard, these results agree with similar observations in VMAT2-deficient mice (Croft et al., 2005), which also present no deficits in monoaminergic vesicle exocytosis. Nonetheless, the data show that VAChT KD^{HET} mice have mild changes in ACh release at the neuromuscular junction, whereas VAChT KD^{HOM} mice have a more profound deficit in transmitter release.

Analysis of neuromuscular function in the three genotypes corroborated these electrophysiological data. VAChT KD^{HET} mice performed as well as wild-type mice in tests of motor function, whereas VAChT KD^{HOM} mice were significantly impaired in grip strength and ability to hold their weight. Importantly, the deficit in grip strength could be ameliorated by prior treatment of mutant mice with cholinesterase inhibitors. The effect of pyridostigmine, which is used to treat myasthenia, is of particular importance, as it indicates that a peripheral cholinergic deficit due to alteration in neuromuscular transmission is the cause of neuromuscular dysfunction.

Investigation of VAChT KD mice on the rotarod, a task that depends upon motor learning and physical endurance, reveals that VAChT KD^{HET} are slower to learn this motor task than wild-type control mice, but the former are able to reach the same level of performance in time. In contrast, VAChT KD^{HOM} mice are significantly impaired and never improve their performance. That VAChT KD^{HOM} mice have limited capacity for exercise is clearly observed on the treadmill, indicating that performance of the homozygous mutants on the rotarod also reflects their inability to maintain prolonged physical activity. These results suggest that VAChT KD^{HOM} mice may provide a model for study of the effects of markedly reduced ACh release on neuromuscular

function, such as the ones observed in certain types of congenital presynaptic myasthenia (Ohno et al., 2001).

In contrast, we were unable to detect any alteration in neuromuscular function in VAChT KD[HET] mice. Release of ACh accompanied the reduction of protein expression in the brain for VAChT KD[HET] mice, and both basal and stimulated extracellular levels were affected. This decrease in ACh release appears to be related to the reduction of VAChT expression, as ChAT activity was not decreased in these mutants. Overall, the approximately 45% reduction in VAChT expression appears to decrease ACh secretion to a similar extent in the brain. Unexpectedly, tissue ACh was significantly increased in several brain regions from VAChT KD[HET] and VAChT KD[HOM] mice, indicating a previously unrecognized connection between ACh storage, nonvesicular ACh pools, and tissue content. Molecular mechanisms responsible for this increased tissue ACh content have not been uncovered yet, but it does not seem to be due to altered ChAT activity or high-affinity choline uptake. It is interesting that pharmacological experiments with vesamicol, an inhibitor of VAChT, have revealed a similar relationship, whereby decreased secretion of ACh leads to accumulation of intracellular transmitter during nerve stimulation (Collier et al., 1986).

Whereas VAChT KD[HET] mice present only mild defects in neuromuscular neurotransmission, there is a relatively larger deficiency in central ACh release in vivo. Neuromuscular transmission has a high safety margin, and neuromuscular weakness is not observed until a significant proportion of neuromotor units are compromised (Paton and Waud, 1967; Waud and Waud, 1975). Therefore, it is reasonable to envision that a partial decrease in VAChT expression will cause more profound consequences on cholinergic transmission in the brain, where a relatively small number of synaptic vesicles (100–200 vesicles) need to be constantly recycled and refilled with neurotransmitter. In contrast, at neuromuscular synapses, there is a very large population of vesicles; fast refilling of vesicles may not be as crucial for neurotransmission at the neuromuscular junction as it is for brain synapses, at least when under low neuromuscular demand.

The VAChT KD[HET] mice present a unique opportunity to investigate the consequences of homogeneous decrease of ACh tone in cognitive tasks, as the results show that these mice represent a model of moderate, predominantly central cholinergic dysfunction. We observed no deficits in performance of VAChT KD[HET] mice in the step-down inhibitory avoidance test. A number of experiments have demonstrated that inhibition of nicotinic and muscarinic central receptor activity can affect performance of rats in this paradigm (Barros et al., 2002), indicating an important cholinergic contribution toward performance in this test. It is likely that the reduction of cholinergic function in VAChT KD[HET] mice was below the threshold for detecting a learning or memory impairment for this task. This result supports the notion that ACh participates in, but is not essential for, some hippocampal-dependent paradigms of learning and memory (Parent and Baxter, 2004).

Interestingly, VAChT KD[HET] mice performed worse than wild-type mice in an object recognition test, suggesting that even mild decline of cholinergic function can affect cognitive processes required for this task. Indeed, rats treated with 192 IgG-saporin, which leads to cholinergic degeneration in the basal forebrain, also present object recognition deficits (Paban et al., 2005), and object recognition alterations are observed in certain mouse models of AD (Dewachter et al., 2002). It is likely that VAChT KD[HET] present such deficits because they have impairments in their ability to learn or remember the intricate cues necessary for discriminating the novel object.

Our data also revealed an important role of cholinergic tone in recognition of mouse conspecifics. In these experiments, the KD[HET] mice explore unfamiliar mice; however, their preference for a social stimuli is somewhat decreased compared with wild-type mice. Nonetheless, the mutant mice are not socially deficient, but they are clearly impaired in remembering intruder mice when compared with wild-type mice. Absence of deficits in olfactory discrimination in VAChT KD[HET] mice supports the notion that the decreased social memory is due to cognitive impairments rather than a simple incapacity to process olfactory cues. An important role of cholinergic tone in social recognition is supported by reversal of this phenotype in mice treated with a cholinesterase inhibitor and by the fact that VAChT KD[HOM] mice also present a significant deficit in social recognition.

Social memory in rodents depends upon the activity of vasopressin on V1A receptors in the lateral septum (Bielsky et al., 2005) and upon the activity of oxytocin (Bielsky and Young, 2004; Ferguson et al., 2000; Winslow and Insel, 2004). However, central muscarinic and $\alpha7$ nicotinic receptors have also been suggested to play a role in social memory (Prediger et al., 2006; van Kampen et al., 2004; Winslow and Camacho, 1995), indicating a potential mechanism for ameliorating social memory deficits in response to cholinergic decline. Our observations support the notion that reduced cholinergic tone in AD mouse models can indeed cause deficits in social memory. However, based on somewhat similar impairments found in the object and social recognition tasks, it is possible that mild cholinergic deficits may cause a more general memory deficit for recognizing previously learned complex cues, whether social or not. Future detailed investigations will be necessary to further define the specific type of cognitive processing affected by cholinergic deficits in these mutants. Such studies in mouse models of reduced cholinergic tone may be particularly informative for understanding the contribution of cholinergic decline to specific behavioral alterations observed in certain pathologies of the CNS, and may even be helpful in understanding physiological aging (Cummings, 2004).

In conclusion, we have generated an animal model to study the impact of decreased VAChT expression on peripheral and central ACh neurotransmission and function. The present results illuminate the role of VAChT in vesicular ACh release and reveal that deficits in VAChT-mediated filling of synaptic vesicles may have important behavioral consequences. Furthermore, these observations indicate an important role for ACh in cognitive processes involved in object and social recognition and memory. In this respect, a decrease in VAChT expression is much less tolerated than a decrease in

ChAT activity, a parameter that is used extensively to evaluate cholinergic deficits in AD.

Experimental Procedures

Animal Care

Heterozygous mutant VAChT mice were backcrossed with C57BL/6J animals for three generations; the N3 mice were used in most experiments. Homozygous mutant VAChT mice were obtained by intercrossing N3 heterozygous animals. Control animals were wild-type age- and sex-matched littermates, and all behavioral and most of the biochemical studies were conducted with researchers "blind" to the genotypes of the mice. For all behavioral experiments, male mice were used.

Animals were housed in groups of three to five animals per cage in a temperature-controlled room with a 12:12 light-dark cycles, and food and water were provided ad libitum. All studies were conducted in accordance with NIH guidelines for the care and use of animals and with approved animal protocols from the Institutional Animal Care and Use Committees at the Federal University of Minas Gerais in Brazil, Pontificia Universidade Catolica de Rio Grande do Sul in Brazil, and Duke University in the United States.

Wire-Hang, Grip Force, Rotarod, and Treadmill Tests

The wire-hang experiments were conducted as described (Sango et al., 1996) and time spent hanging upside down was determined with a cutoff time of 60 s.

To measure grip force, we used a custom-built force transducer connected to a small support that could be grasped by the mouse as described (Fowler et al., 2002). Five tests were performed per mouse with a maximum period of 50 s for each animal over 2 different days. The force transducer was coupled to a computer and a routine was developed to record the maximal grip force exerted.

For the rotarod task, we followed the protocol described by Brandon et al. (1998). Mice were placed on the rotarod apparatus (Insight Equipments, Ribeirão Preto, Brazil) and rotation was increased from 5 to 35 rpm. Latency to fall was recorded automatically. The test was run within the last 4 hr of the light phase of the 12:12 cycle. Ten trials were given on the first day and four trials on the second day, with a 10 min intertrial interval. In the time between trials, mice were placed in their home cage.

For the treadmill test (Insight Equipments, Ribeirão Preto, Brazil), mice were trained for 4 days (3 min a day). On the first day, inclination was set to 5°. The inclination was increased by 5° for each training day until reaching 20°. The initial training speed was 8 m/min, and the treadmill was accelerated by 1 m/min. In the second training session, the initial speed was 10 m/min increased to 11 and 12 m/min in the third and fourth training days, respectively. During testing, the initial speed was set to 12 m/min, which was increased by 1 m/min at 2, 5, 10, 20, 30, 40, 50, and 60 min after starting the exercise, essentially as described by Pederson et al. (2005). The work performed (in J) was calculated with the following formula: $W(J) =$ body weight (kg) $\times \cos 20° \times 9.8$ (J/kg $\times$ m) $\times$ distance (m).

Step-Down Inhibitory Avoidance

The step-down inhibitory avoidance apparatus was a $50 \times 25 \times 25$ cm acrylic box which had a floor consisting of a grid of parallel stainless steel bars 1 mm in diameter spaced 1 cm apart. A 10 cm^2 wide, 2 cm high acrylic platform was placed in the center of the floor. Animals were placed on the platform and their latency to step down on the grid with all four paws was measured with an automatic device. In the training session, immediately after stepping down on the grid, the animals received a 2.0 s, 0.3 mA scrambled foot-shock. Retention test sessions were procedurally identical, except that no foot-shock was given. The latency to step down during testing was taken as a measure of retention. A ceiling of 180 s was imposed in this measure, i.e., animals whose test latency was over than 180 s were counted as 180 s. Each animal was tested twice, once at 1.5 hr after training, to measure short-term retention, and once at 24 hr after training, to measure long-term retention (Izquierdo et al., 2002; Lorenzini et al., 1996). Since the variable being analyzed (step-down latency) does not follow a normal distribution, the data were analyzed by Mann-Whitney U or Kruskal-Wallis non-

parametric tests, followed by Dunn's post hoc comparisons where appropriated.

Object Recognition

All animals were given a single 5 min habituation session with no objects in the open-field arena (as described above). Twenty-four hours after habituation, training was conducted by placing individual mice for 5 min into the field, in which two identical objects (objects A1 and A2; Duplo Lego toys) were positioned in two adjacent corners, 10 cm from the walls. A minimum of 30 s exploration time for objects was allowed in this first exposure. In a short-term memory (STM) test given 1.5 hr after training, the mice explored the open field for 5 min in the presence of one familiar (A) and one novel (B) object. All objects presented similar textures, colors, and sizes, but distinctive shapes. A recognition index calculated for each animal was expressed by the ratio $T_B/(T_A + T_B)$ [T_A = time spent exploring the familiar object A; T_B = time spent exploring the novel object B]. Between trials the objects were washed with 10% ethanol solution and air-dried. In a long-term memory (LTM) test given 24 hr after training, the same mice explored the field for 5 min in the presence of familiar object A and a novel object C. Recognition memory was evaluated as for the short-term memory test. Exploration was defined as sniffing or touching the object with nose and/or forepaws (de Lima et al., 2005). Data for recognition indexes are expressed as median (interquartile ranges). Comparisons among groups were performed using a Kruskal-Wallis analysis of variance and Mann-Whitney U tests. Recognition indexes within individual groups were analyzed with Wilcoxon tests.

Social Recognition

Mice were housed in individual cages in a quiet room for 4 days to establish territory dominance. Swiss juvenile male mice were used as the intruder. To test for social interaction, the intruder was placed inside a transparent acrylic chamber containing several holes and introduced into the test cage exactly as described (Choleris et al., 2003). Time spent sniffing was measured as the amount of time that VAChT KD[HET] mice or wild-type littermates spent poking their noses into the holes of the chamber. Initially, the subject tested (wild-type or VAChT KD[HET] mouse) was exposed to an empty acrylic chamber for 10 min; subsequently, this chamber was exchanged by one containing the intruder and left for 5 min. The entire procedure was repeated four times. After the fourth exposure to the same intruder, a novel intruder was added to the acrylic chamber. The experiment was videotaped and a trained researcher, blind to genotype, evaluated time spent sniffing in each condition.

A second experiment consisted of exposing the subject to the same intruder twice with an intertrial interval of 30 min. The standard measure for the statistical analysis in social recognition tests was the time spent exploring the juvenile intruder. To evaluate the contribution of acute cholinergic deficits, saline or 1 mg/kg galantamine (s.c.) was injected 30 min before beginning of the tests.

For evaluation of sociability, we followed the protocol described by Kwon et al. (2006). Testing was done in a three-chambered apparatus ($15 \times 90 \times 18.5$ cm divided into three chambers of 15×29 cm and separated by dividers with a central 3.8×3.8 cm door) that offers the subject a choice between a social stimulus and an inanimate target. In the habituation session, mice were allowed to explore the entire box for 10 min. Subsequently, mice stayed 5 min in the center and were then allowed to interact for 10 min with an empty cage in one chamber versus a caged social target in the opposite chamber. Social and nonsocial stimuli were varied among the chambers and the box was cleaned between tests. Results are presented as percentage of total exploration time.

Two tests to evaluate the olfactory responses of the mice were conducted (Bielsky et al., 2005; Ferguson et al., 2000). The first consisted of measuring the time that both genotypes took to find a candy located on the surface of bedding or hidden within the bedding (Ferguson et al., 2000). The second test investigated whether VAChT KD[HET] mice presented olfactory habituation and discrimination. Experiments were performed 7 days after completing the social recognition tests in the same groups of mice. For this test, a microtube, with a piece of cotton containing 10 μl of strawberry essence, was presented to mice four times for 1 min with a 10 min intertrial interval. On the fifth trial, the microtube was exchanged with one containing

vanilla essence. The significance of differences between the groups was determined by Student's t test or two-way ANOVA, and a post hoc Bonferroni test was performed when appropriate. Changes across trials were assessed with repeated measures ANOVA with Bonferroni's post hoc analysis.

For all other methods, see the Supplemental Data.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/51/5/601/DC1/.

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