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Elimination of the vesicular acetylcholine transporter in the forebrain causes hyperactivity and deficits in spatial memory and long-term potentiation

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Basal forebrain cholinergic neurons, which innervate the hippocampus and cortex, have been implicated in many forms of cognitive function. Immunolesion-based methods in animal models have been widely used to study the role of acetylcholine (ACh) neurotransmission in these processes, with variable results. Cholinergic neurons have been shown to release both glutamate and ACh, making it difficult to deduce the specific contribution of each neurotransmitter on cognition when neurons are eliminated. Understanding the precise roles of ACh in learning and memory is critical because drugs that preserve ACh are used as treatment for cognitive deficits. It is therefore important to define which cholinergic-dependent behaviors could be improved pharmacologically. Here we investigate the contributions of forebrain ACh on hippocampal synaptic plasticity and cognitive behavior by selective elimination of the vesicular ACh transporter, which interferes with synaptic storage and release of ACh. We show that elimination of vesicular ACh transporter in the hippocampus results in deficits in long-term potentiation and causes selective deficits in spatial memory. Moreover, decreased cholinergic tone in the forebrain is linked to hyperactivity, without changes in anxiety or depression-related behavior. These data uncover the specific contribution of forebrain cholinergic tone for synaptic plasticity and behavior. Moreover, these experiments define specific cognitive functions that could be targeted by cholinergic replacement therapy.

Alzheimer's disease | Morris water maze | synaptic vesicle | Barnes maze

The mechanisms that underlie the formation of hippocampal-dependent spatial memory have been broadly explored (1). However, the neurochemical basis underlying changes in the strength of synaptic connections necessary for memories to persist is still not precisely understood. In the case of the hippocampus, mechanisms for memory processing include mRNA-dependent (2) and mammalian target of rapamycin (mTOR)-mediated protein synthesis (3) as well as a sequence of biochemical events shared with or closely similar to that of long-term potentiation (LTP) (2). Indeed, the consolidation of two different aversive tasks (4) and of spatial recognition memory (5) is accompanied by LTP of the CA3-CA1 synapse and can be occluded by a preceding LTP.

The basal forebrain cholinergic system, which innervates the hippocampus and cortex, has been suggested to modulate LTP in the hippocampus (6–9) and has been implicated in many forms of behavior (10). In addition, spatial memory has also been suggested to depend on cholinergic activity (10), although there are numerous controversies surrounding which behaviors acetylcholine (ACh) regulates (11, 12). Moreover, in few studies cholinergic denervation did not affect expression of LTP (13). Understanding the precise roles of ACh in learning and memory is of importance because in different types of dementia cholinergic function is decreased (14), and manipulations that boost ACh levels at synapses are used as treatment for cognitive deficits.

Animal models of cholinergic dysfunction have been generated by elimination of basal forebrain cholinergic neurons using electrolytic or excitotoxic methods, as well by the more selective strategy of cholinergic immunolesion (14). These studies have given inconsistent results concerning the cognitive and behavioral processes that are affected by altering cholinergic transmission (12, 15, 16). This is likely related to the fact that in many cases both noncholinergic and cholinergic projection neurons are destroyed, or that the lesions produced do not fully deplete cholinergic neurons. Moreover, most immunolesion experiments were performed initially in rats (15, 16), and only recently immunotoxins have been developed for mice (11), a species in which genetic tools are available. In mice these toxins show poor selectivity depending on the dose used (11). In addition to these technical problems, cholinergic neurons usually release glutamate as a neurotransmitter with ACh (17, 18), which complicates the interpretation of dysfunction using toxin-based methodologies that eliminate secretion of both neurotransmitters simultaneously. This is relevant in the striatum, where there are remarkable differences in behavior between ablation of cholinergic neurons and elimination of the vesicular acetylcholine transporter (VAChT) used to selectively impair the release of ACh (19).

In the present study, we used genetic manipulation of VAChT (20) to investigate the specific contribution of forebrain cholinergic neurotransmission to behavioral manifestations and cellular mechanisms thought to be associated with learning and memory. We report that hippocampal cholinergic deficits caused LTP impairment in the CA1-CA3 pathway. Moreover, VAChT mutant mice were hyperactive and had deficits in spatial memory acquisition assessed using the Morris water maze (MWM). Finally, mutants showed impaired ability to relearn a spatial memory task, suggesting that they lack behavioral flexibility. Our data demonstrate an important role for forebrain ACh in controlling locomotion as well as in modulating hippocampal memory processing.

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Results and Discussion

Synaptic Plasticity Is Impaired in VAChT KD^{HOM} Mice. Although cholinergic denervation has been shown in some studies to affect LTP (6–9), in others decreased cholinergic tone did not affect synaptic plasticity (13). We attribute these differences to variability of lesion-based strategies. Therefore, to investigate the specific roles of ACh in synaptic plasticity, we initially tested whether LTP was affected in VAChT KD^{HOM} mice. For these experiments, we recorded fEPSP in the CA1 region of WT and VAChT KD^{HOM} mice, which have 70% decrease in VAChT expression and similar deficit in ACh release (20, 21). These mice present social memory deficits and also object recognition memory impairment (20, 21). We induced LTP in hippocampal slices using high frequency stimulation (100 Hz, 1 s) delivered to the Schaffer-collaterals. Recordings from WT mice showed a robust potentiation of fEPSPs indicative of synaptic plasticity (Fig. 1*A*; 134.7 ± 3.8, $n = 7$). In contrast, slices obtained from VAChT KD^{HOM} mice did not show any potentiation (Fig. 1*A*; 105.6 ± 3.1, $n = 5$, $P < 0.001$ in a Student t test).

Interestingly, not all forms of synaptic plasticity were blocked in VAChT-deficient mice. Long-term depression (LTD) elicited by low-frequency stimulation (1 Hz, 600 s) was similar between WT and VAChT KD^{HOM} mice (Fig. 1*B*; WT ($n = 7$), 83.9 ± 3.1; VAChT KD^{HOM} ($n = 6$), 77.3 ± 5.8; $P = 0.32$ in a Student t test). Analysis of synaptic transmission using input-output relationship (I/O) indicated a small, but significant, decrease in I/O slope for VAChT KD^{HOM} mice compared with WT mice (Fig. S1).

VAChT KD^{HOM} Mice Display Hyperactivity and Impaired Spatial Acquisition. Genetic and pharmacological manipulation of ACh receptors change locomotor activity (22). Additionally, using other VAChT-deficient strains of mice with global reduction of the transporter in the brain, we have reported that ACh regulates locomotion (22). In agreement with these previous observations (22), we found that VAChT KD^{HOM} mice were hyperactive compared with WT controls (Fig. 2*A*, $P < 0.001$ in a two-tailed t test).

Cholinergic tone has been associated with several memory-related functions (20, 21), but depending on the approach and investigator, lesion of the medial septum cholinergic system caused varying degrees of spatial navigation impairment (11, 12, 16). Indeed, for some investigators cholinergic lesions did not cause impairment in spatial memory (15, 23). Spatial memory, determined by the MWM, has been associated with LTP in mice in vivo (5). However, because VAChT KD^{HOM} mice cannot swim (24), we tested spatial memory in these mice using the Barnes maze (25). Latency to find the target hole was significantly longer for VAChT KD^{HOM} mice compared with WT controls over the 4-d training phase of the task (Fig. 2*B*, $F_{(18,90)} = 5.280$, $P < 0.001$), although no differences were observed in the number of primary errors made or path length taken to find the target hole (Fig. 2*B*). Furthermore, latency to find the target hole in the day-5 probe trial was no different between genotypes and time spent investigating the target quadrant was significantly longer compared with the other quadrants for both VAChT KD^{HOM} and control mice [Fig. 2*B*, $F_{(3,72)} = 12.903$, $P < 0.01$]. Together, these data suggest that spatial acquisition in the Barnes maze may be slightly impaired in this VAChT-deficient mouse line, but spatial memory retrieval is normal.

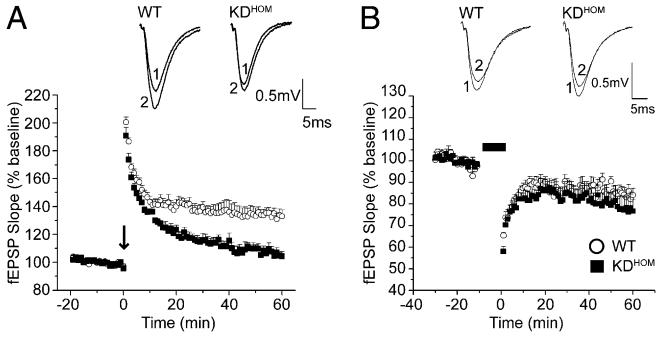

Fig. 1. Plasticity of glutamatergic synaptic transmission in the hippocampus of VAChT KD^{HOM} mice. Field EPSPs, recorded in slices derived from VAChT KD^{HOM} (black square) and WT (white circle) mice. (*A*) 100 stimuli delivered at 100 Hz (timing indicated by arrow) or (*B*) 600 stimuli delivered at 1 Hz (timing indicated by bar). (*Insets*) Representative traces.

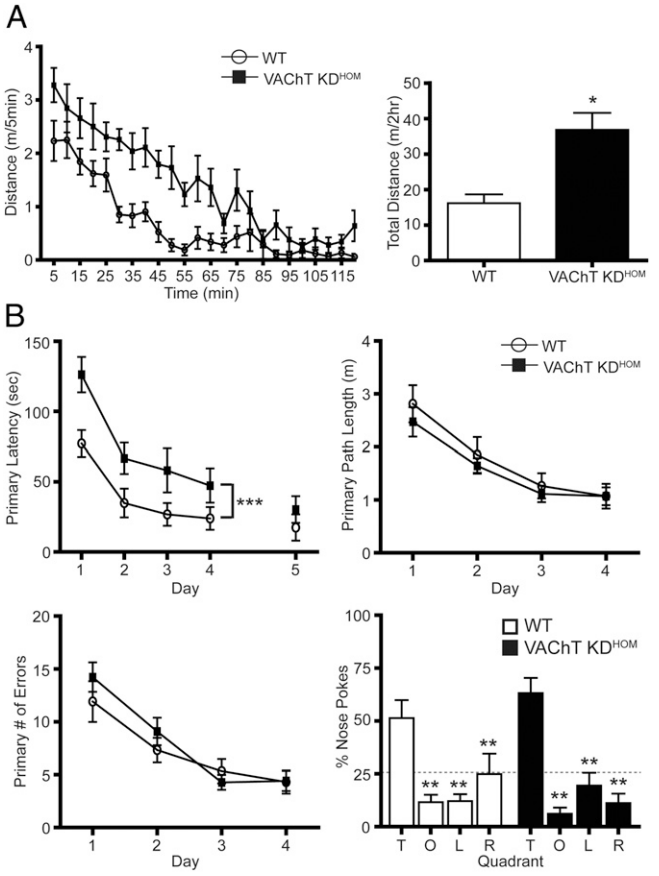

Fig. 2. VAChT KD^{HOM} mice show hyperactivity and deficit in spatial memory acquisition. (*A*) Locomotor activity of VAChT KD^{HOM} mice. Horizontal activity in an open-field for WT (white, $n = 12$) and VAChT KD^{HOM} (black, $n = 11$) mice was measured over time (*Left*) and cumulatively over 2 h (bar graph). (*B*) WT (white, $n = 9$) and VAChT KD^{HOM} (black, $n = 11$) mice were subject to the Barnes maze. Latency to find the target hole (primary latency), distance traveled to find the target hole (primary distance), and number of errors made before finding the target hole (primary errors) are presented. The average of four 3-min trials per day is plotted. Primary latency and the percentage of nose pokes per quadrant were measured on day 5 in a 90-s probe trial. All data are plotted with SEM. *$P < 0.001$ in a two-tailed t test; **$P < 0.010$ in a two-way ANOVA post hoc test; ***$P < 0.001$ in a two-way repeated measures ANOVA. L, left; O, opposite; R, right; T, target.

Selective Elimination of VAChT in the Mouse Forebrain. Deficits in synaptic plasticity in the hippocampus have been linked to spatial memory impairments in the MWM (5). To avoid the muscular dysfunction deficit in VAChT KD^{HOM} mice (20) we produced a forebrain-specific knockout mouse line using our recently generated floxed VAChT mouse line [VAChT$^{flox/flox}$ (22)] and a Six3-Cre mouse line, which expresses the Cre recombinase enzyme (Cre) under the control of the Six3 promoter, broadly active in the ventral forebrain (26). We crossed the Six3-Cre line

with a reporter mouse line (Rosa26-YFP) and determined that Cre is expressed in forebrain cholinergic neurons in this line. Although Cre was expressed in noncholinergic neurons, this would not affect these neurons, because VAChT expression would be only relevant in cholinergic neurons in the brain. About 85% of all basal forebrain cholinergic neurons and ~58% of striatal interneurons expressed Cre (Fig. S2 and Table S1).

We intercrossed Six3-Cre mice to VAChT$^{flox/flox}$ to selectively eliminate VAChT from the forebrain (VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$). VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice were born in expected Mendelian ratios and appeared normal. Biochemical analysis of VAChT expression revealed that VAChT mRNA and protein were eliminated in the forebrain of these mice. In contrast, high-affinity choline transporter (CHT1) and choline acetyltransferase (ChAT) were unaffected (Fig. S3).

Synaptic Plasticity Is Impaired in VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ Mice. To further determine that elimination of VAChT, and therefore ACh release (19, 20), affects synaptic plasticity, we studied LTP in VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice. Recordings from VAChT$^{flox/flox}$ slices showed a robust potentiation of fEPSPs indicative of synaptic plasticity (Fig. 3*A*; 176.2 ± 16.1, $n = 7$). Consistent with results obtained with VAChT KD^{HOM} slices, slices obtained from VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice did not show any potentiation (Fig. 3*A*; 109.9 ± 11.1, $n = 10$, $P = 0.0032$ in a Student t test). Analysis of synaptic transmission using input-output relationship (I/O) and paired-pulse facilitation indicated no change between VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ and control mice (Fig. S4). Hence, in two lines of mice with VAChT deficiency LTP is impaired.

Forebrain Cholinergic Tone Is Involved in Spontaneous Locomotion. We have previously reported the importance of VAChT in locomotor activity (22). Hyperactivity is not a consequence of decreased cholinergic tone in the striatum, because selective removal of striatal VAChT does not cause hyperactivity (19). Other brain regions that influence locomotor activity are the basal forebrain and cholinergic neurons projecting from the brainstem. Importantly, whereas basal forebrain VAChT is eliminated in VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice, brainstem VAChT is preserved (Fig. S3), affording a way to test for the role of the basal forebrain in regulating locomotion. We found that VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice were also hyperactive compared with controls (Fig. 4, $P = 0.0092$ in a two-tailed t test). These results suggest that forebrain cholinergic neurons regulate locomotor activity in mice.

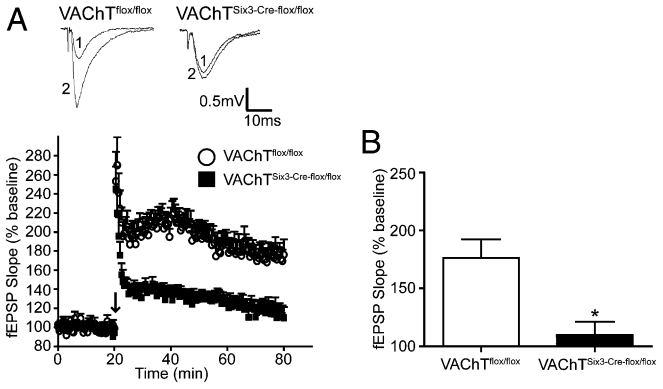

Fig. 3. Plasticity of glutamatergic synaptic transmission in the hippocampus of VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice. (*A*) Field EPSPs recorded in slices derived from VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ (black, $n = 10$) and VAChT$^{flox/flox}$ (white, $n = 7$) mice. LTP was induced by four 500-ms, 100-Hz trains of stimuli delivered 20 s apart (timing indicated by arrow). Insets show representative traces taken from the time points indicated in the graphs. (*B*) Increased fEPSP slope after potentiation (60 min). *$P = 0.0032$ in a Student t test.

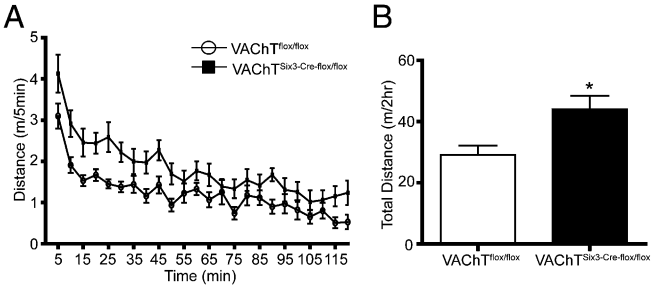

Fig. 4. Locomotor activity of VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice. (*A*) Horizontal activity in an open field for VAChT$^{flox/flox}$ (white, $n = 14$) and VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ (black, $n = 14$) mice was measured over time and (*B*) cumulatively over 2 h. All data are plotted with SEM. *$P = 0.0092$ in a two-tailed t test.

In individuals with dementia, hyperactive behavior is often associated with changes in anxiety and depressive behaviors (27). Moreover, patients undergoing cholinergic drug treatments have shown changes in mood (28). We therefore examined psychiatric-like behaviors in VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice. Depressive-like behavior was not observed in VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice; they performed similar to controls in both the forced swim and tail suspension tests (Fig. S5 *A* and *B*). Similarly, these mutant mice showed no evidence of anxiety in the dark–light transition test and elevated plus maze compared with controls (Fig. S5 *C* and *D*). Consistent with locomotion data, an increase in general activity was observed in VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice in the dark–light transition test (Fig. S5*C*). Hence, despite hyperactivity, we did not observe behavioral manifestations related to psychiatric dysfunction in mice with reduced cholinergic tone.

VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ Mice Show Impairment in Spatial Memory Acquisition. Because VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice showed impaired synaptic plasticity similar to that of VAChT KD^{HOM} mice, we tested their spatial memory in the Barnes maze (Fig. 5*A*). Although latency to find the target hole was not significantly different for VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice compared with VAChT$^{flox/flox}$ controls over the 4-d training phase of the task, mutant mice walked for longer distances and made significantly more errors before finding the target hole [Fig. 5*A*, $F_{(3,90)} = 0.309$, $P = 0.05$ and $F_{(3,39)} = 0.517$, $P = 0.015$, respectively]. Similar to VAChT KD^{HOM} mice, investigation of the target quadrant in the day 5 probe trial was significantly longer compared with the other quadrants for both control and mutant mice [Fig. 5*A*, $F_{(3,120)} = 6.703$, $P < 0.01$]. Additionally, the latency to find the target hole on the probe trial was no different between groups, suggesting that VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice are capable of learning and remembering locations in this spatial memory task. Together, these data support the notion that mice with decreased VAChT have an impairment of acquisition in the Barnes maze but are still able to learn a spatial memory task.

To further examine the importance of ACh in spatial memory, VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ and controls were tested in the more widely used MWM, a demanding paradigm that challenges spatial memory (1). VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice showed no neuromuscular dysfunction; both grip strength and motor performance in the rotarod test were normal compared with controls (Fig. S5 *E* and *F*). This suggests that VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice are physically fit and could be used in the more physically challenging MWM task.

In the hidden platform (spatial) version of the MWM, no differences were observed between genotypes in the latency to find the escape platform in the 4-d training period, and both mutant and control mice improved in the time it took to find the platform over training (Fig. 5*B*). However, the distance required for VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice to find the platform was significantly longer compared with controls [Fig. 5*B*, $F_{(3,77)} = 1.824$, $P < 0.001$]. Closer examination of path traces in the MWM

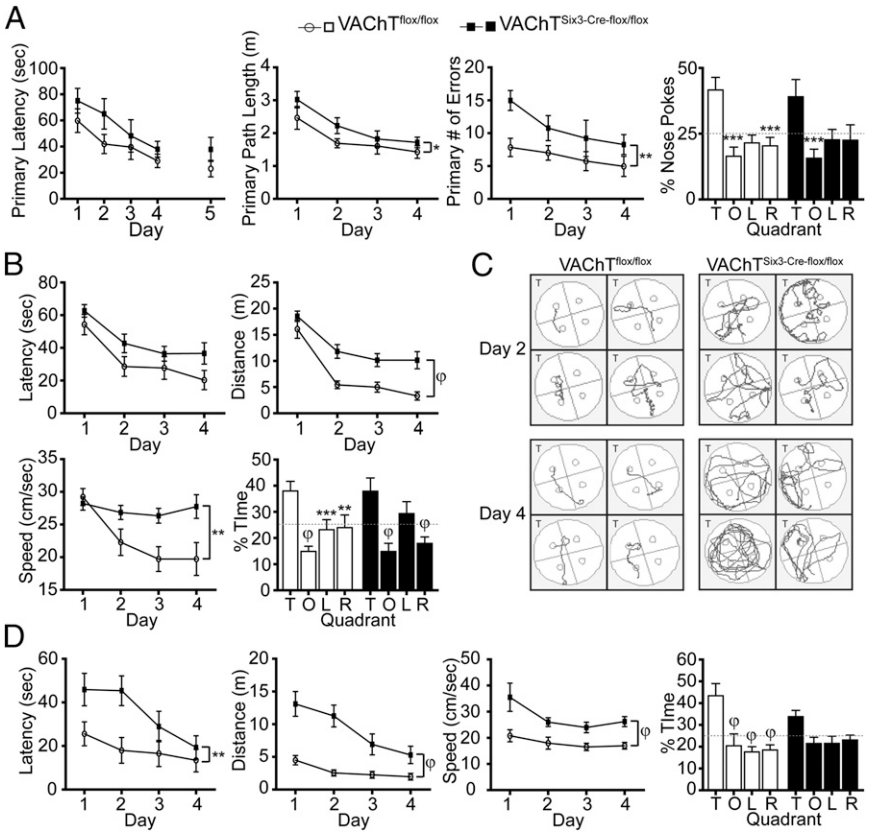

Fig. 5. VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice have impaired spatial memory. (*A*) VAChT$^{flox/flox}$ (white, $n = 9$) and VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ (black, $n = 11$) mice were subject to the Barnes maze paradigm. The average of four 3-min trials per day is plotted. Primary latency and the percentage of nose pokes per quadrant were measured on day 5 in a 90-s probe trial. (*B*) VAChT$^{flox/flox}$ (white, $n = 14$) and VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ (black, $n = 15$) mice were subject to the MWM paradigm. The average of four 90-s trials per day is plotted. The percentage of time spent in each quadrant was measured on day 5 in a 60-s probe trial with the platform removed. (*C*) Representative path traces for four VAChT$^{flox/flox}$ mice (*Left*) and four VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice (*Right*) in the MWM. Trial 3 for days 2 (*Upper*) and 4 (*Lower*) in the training period are shown. The target quadrant for each trace is in the upper left. (*D*) VAChT$^{flox/flox}$ (white, $n = 14$) and VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ (black, $n = 15$) mice were subject to reversal training in the MWM. The average of four 90-s trials per day is plotted. The percentage of time spent in each quadrant was measured on day 5 in a 60-s probe trial with the platform removed. All data were plotted with SEM. $^{*}P = 0.05$; $^{**}P < 0.05$; $^{***}P < 0.01$; $^{\varphi}P < 0.001$. L, left; O, opposite; R, right; T, target.

revealed that VAChT$^{flox/flox}$ controls learned the location of the platform as early as the second day of training. However, even after 4 d of training VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mutants showed no improvement in efficiency to find the escape platform (Fig. 5*C*). VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ were significantly faster swimmers during several of the trials [Fig. 5*B*, $F_{(3,77)} = 6.607$, $P < 0.05$], which may explain the discrepancy between latency and path length over the training period. Hence, by swimming fast in several directions VAChT-mutant mice could compensate for the longer distance taken in finding the platform. When spatial memory retrieval was investigated on the day-5 probe trial, time spent investigating the target quadrant was significantly longer compared with the other quadrants for both control and mutant mice [Fig. 5*B*, $F_{(3,108)} = 12.882$, $P < 0.001$ compared with the opposite quadrant].

To further investigate the participation of ACh in acquisition and retrieval of spatial memory, we tested the ability of VAChT-mutant mice to extinguish their initial acquisition of the platform position and to learn a new goal position. To test this memory flexibility we used the spatial reversal protocol in the MWM (29). Similar to the results in the initial MWM experiments, VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice demonstrated significantly longer path length and increased swim speed compared with controls [Fig. 5*D*, $F_{(3,81)} = 3.613$, $P < 0.001$ and $F_{(3,69)} = 1.546$, $P < 0.001$, respectively]. VAChT$^{flox/flox}$ controls showed remarkable memory flexibility when challenged to learn a new position of the escape platform. In contrast, VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice were significantly impaired in this task [Fig. 5*D*, $F_{(3,81)} = 2.893$, $P < 0.05$] and were not able to find the platform by using the alternative strategy to swim around the pool. Importantly, on the day-5 probe trial, control mice retained their ability to remember the new position of the platform [Fig. 5*D*, $F_{(3,108)} = 1.706$, $P < 0.001$], whereas VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mutants could not (Fig. 5*D*). Together, these data suggest that forebrain cholinergic tone is important for spatial acquisition as well as for extinction and relearning of a task, suggesting that forebrain ACh contributes to behavioral flexibility.

A role for cholinergic neurons in spatial memory has previously been suggested (11, 12, 15, 16), but the precise role of ACh in this process has yet to be revealed. Using two distinct lines of genetically modified mice, we provide strong evidence that decreased cholinergic tone affects LTP. The molecular mechanisms related to the deficit in LTP in VAChT-deficient mice are not understood but might be related to modification of downstream signaling cascades that contribute to the induction of NMDAR-dependent synaptic plasticity. Although some studies showed no alteration in LTP after cholinergic degeneration (13), the results presented here agree with other experiments showing regulation of LTP by cholinergic neurotransmission in vivo (7) and in slices (6, 8). These findings support the importance of using gene-targeting based strategies in studying cholinergic neurotransmission and avoid the inconsistency seen when using immunolesion methods. Indeed, activation of ACh receptors, including muscarinic (30) and nicotinic receptors (9) can facilitate LTP, and this seems to be related to temporal activation of these inputs before activation of glutamatergic transmission (9). Specifically, activation of cholinergic input before hippocampal stimulation results in LTP facilitated by the α7-nicotinic receptor, whereas muscarinic receptors are responsible for facilitating LTP when cholinergic input occurs poststimulation (9). It is likely that these two mechanisms are impaired in VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice. Additionally, M1- and M2-muscarinic receptor–deficient mice present a deficit in synaptic plasticity (31, 32). Hence, we conclude that cholinergic input to the hippocampus regulates glutamatergic synaptic plasticity. Future experiments are needed to dissect the exact mechanism involved in this process.

Our experiments showed that VAChT-deficient mice have a learning deficit; spatial acquisition during training was disturbed and they had difficulties using spatial cues to find the platform.

Additionally, VAChT deficiency affected the ability of mice to extinguish a previous location and learn a new platform location. Accompanying the learning impairment was a deficit in the induction of LTP; this further supports the hypothesis that deficits in LTP contribute to the impairment in spatial memory (33). Importantly, knockout mice for the M2 muscarinic receptor also show reduced hippocampal LTP, a similar pattern of learning deficit in the Barnes maze, and impaired reversal learning (32). These data strengthen our finding that basal forebrain cholinergic input is essential for the generation of robust LTP at the Schaffer–CA1 synapse and is also necessary for learning and behavior flexibility. Furthermore, they suggest that ACh regulation of hippocampal LTP and spatial learning relies heavily on M2 receptor activity (32). Therefore, we conclude that forebrain cholinergic deficiency can affect spatial learning, but this depends on the task and on the demand.

Intriguingly, despite deficits in spatial learning, spatial memory retrieval was preserved in VAChT-deficient mice. This may seem paradoxical given the extent to which early phase LTP was reduced in these mice. However, it is important to recall that although LTP was reduced, LTD was preserved in VAChT-deficient mice. Indeed, accumulating evidence suggests that LTD also plays an important role in learning and memory (34). LTD seems to be particularly important in tasks requiring the learning of a new context, in conjunction with the rapid extinction of information that is no longer relevant (33). Importantly, efficient learning seems to require proper balance between LTP and LTD (33, 34). VAChT-deficient mice show preserved LTD but impaired reversal learning. Although this finding might seem contradictory with the potential role of LTD in learning and memory, the consequences of interfering with synaptic plasticity on cognition are not straightforward. Further experiments using a broader range of plasticity-inducing stimulation frequencies to determine whether there is any shift in the threshold for eliciting LTD/LTP may help us to better understand this process.

It should be pointed out that behavioral and cognitive outcomes show a high degree of complexity involving a multitude of molecular, neuroendocrine, and gene–environment interactions. Although our data suggest that impaired synaptic plasticity contributes to the deficit in spatial memory observed in VAChT-deficient mice, they do not exclude the possibility that decreased cholinergic tone also interferes with other processes that affect cognition and behavior. To note, it is possible that the hyperactivity observed in VAChT-KD^{HOM} and VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice is not related to impaired hippocampal LTP, because this behavior has been described in mice presenting normal LTP as well as impaired or enhanced LTP (33, 35, 36). However, based on the discussion above, we cannot discard a role for impaired synaptic plasticity in hyperactivity. Increased locomotor activity was observed in different behavioral tasks including the locomotor box, dark–light test and the MWM. In all these tasks VAChT-deficient mutants were tested in a novel environment. These data are consistent with previous findings showing that ACh release in the cortex and hippocampus is dramatically increased upon exploration of a novel environment (37). Moreover, these results support the hypothesis that release of ACh in the brain is normally required to "turn down" neuronal circuits controlling locomotion (22). Interestingly, deficiency in cholinergic signaling has recently been shown to decrease cortical expression of the heterogeneous nuclear ribonucleoproteins hnRNP A/B family. Reduced expression of these hnRNA splicing factors induces alternative splicing impairments, dendrite loss in primary neurons, and cognitive impairments in mice (38). Further studies will be necessary to investigate whether expression of hnRNA splicing factors is affected in VAChT-deficient mice and contributes to cognitive dysfunction.

Adaptive changes in brain neurochemistry during development in response to the decreased VAChT expression might also contribute to the observed cognitive and behavioral deficits observed in VAChT mutant mice. However, recovery of memory deficits in VAChT KD^{HOM} mice by cholinesterase inhibitors (20) suggests that the cognitive deficits are mainly due to decreased cholinergic tone. There is also a possibility that VAChT-deficient mice are more susceptible to inflammation and some of the behavioral changes are disease-related. Recent studies show that the cholinergic system has an important role in regulating cytokine production to prevent damaging inflammation (39, 40). In fact, VAChT KD^{HOM} mice have been shown to develop increased inflammatory immune response when infected with parasites (41), indicating that the cholinergic anti-inflammatory reflex in these mutants is affected. However, prompt rescue of different cognitive and muscular deficits of VAChT KD^{HOM} mice by cholinesterase inhibitors (20) strongly suggests that inflammation does not play any important role in these behavioral and cognitive deficits. Moreover, VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice reproduce the cognitive deficits observed in VAChT KD^{HOM} mice. Because VAChT ablation in VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice is restricted to the forebrain, they may not present deficits in the cholinergic anti-inflammatory reflex. Furthermore, the mRNA expression level for the proinflammatory cytokines TNF-α, IL-1, and IL-6 in the cortex, hippocampus, brainstem, and striatum of VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice and littermate controls is very similar (Fig. S6), suggesting that elimination of VAChT expression in the forebrain does not lead to brain inflammation.

In summary, by using VAChT-deficient mouse lines we determined that selective elimination of hippocampal cholinergic tone regulates LTP. We also found that these mice are hyperactive and show deficits in spatial memory. By defining the specific roles of ACh in learning and memory through targeting of VAChT, our experiments provide unique insights on cognitive functions that can be targeted to compensate cholinergic deficiency.

Materials and Methods

Animals. Generation of homozygous VAChT knockdown mice (VAChT KD^{HOM}) was previously described (20). VAChT$^{flox/flox}$ mice (Fig. S1) were also previously reported (22) and are kept in a mixed C57BL/6J x 129/SvEv x NMRI background (predominantly C57BL/6J background). Note that VAChT KD^{HOM} and VAChT$^{flox/flox}$ mice were generated from independent constructs (20).

Six3-Cre mice (Six3) were a gift from Guillermo Oliver, St. Jude Children's Research Hospital, Memphis, TN. VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ mice were generated by crossing VAChT$^{flox/flox}$ (mixed C57BL/6J x 129/SvEv background, backcrossed to C57BL/6J for five generations) with the Six3-Cre mouse line (NMRI background, backcrossed to C57BL/6J for 5 generations). We then intercrossed VAChT$^{Six3\text{-}Cre\text{-}flox/wt}$ mice to obtain VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ and subsequently bred VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ and VAChT$^{flox/flox}$ to obtain all of the mice used in the present study. Unless otherwise stated, all control mice used were VAChT$^{flox/flox}$ littermates without the Cre transgene. Rosa26-YFP mice (B6.129 × 1-Gt(ROSA)26Sor$^{tm1(EYFP)Cos/J}$, stock number 006148) were obtained from Jackson Laboratories.

Animals were housed in groups of three or four per cage without environmental enrichment in a temperature-controlled room with 14:10 light–dark cycles, and food and water were provided for ad libitum consumption. All procedures were conducted in accordance with the National Institutes of Health (NIH) guidelines and the Canadian Council of Animal Care (CCAC) guidelines at the University of Western Ontario with an approved institutional animal protocol (2008–127). Male mice older than 12 wk were used for behavioral studies.

Immunofluorescence, qPCR, and Western Blotting. Immunofluorescence experiments were performed as previously described (22). For RNA analysis, sample processing and quantitative real-time PCR (qPCR) was performed as previously described (19). Primer sequences are available upon request. Protein extraction and Western blotting was performed as previously described (20).

Electrophysiology. Field excitatory postsynaptic potential fEPSPs were recorded in hippocampal slices from 21- to 40-d-old mice. Brains were isolated and processed as described in ref. 34. For VAChT KD^{HOM} and control mice, slices were stimulated with one train of 100 Hz (1-s duration) to produce LTP or

one train of 1 Hz (600-s duration) to produce LTD. For VAChT$^{Six3\text{-}Cre\text{-}flox/flox}$ and control mice, LTP was induced with four trains of 100 Hz (500-ms duration each) delivered 20 s apart. This "plasticity-inducing" phase was followed by 1 h of baseline stimulation. All field data are expressed as mean ± SEM. Statistical difference between means was determined by a Student *t* test.

Locomotor Activity. All behavioral experiments were performed between 0900 and 1600 hours in the light cycle, as previously described (21).

Barnes Maze. The Barnes maze consists of a white, circular platform (92 cm in diameter) with 20 equally spaced holes (5 cm in diameter; 7.5 cm between holes), elevated 105 cm above the floor (San Diego Instruments). All tests were performed between 0900 and 1600 hours in the light cycle, as previously described (25).

Morris Water Maze. The MWM was performed as previously described (29). Briefly, mice received four consecutive training trials, during which the hidden platform was kept in a constant location with a 15-min intertest interval. Mice were placed at a different starting location within the pool for each trial, which consisted of a swim followed by a 10-s platform sit. Spatial reversal was tested by relocating the platform to the opposite quadrant and administering another set of four trials per day for four consecutive days followed by a 1-min probe trial on the fifth day (29). All data were recorded using a video camera and analyzed using ANY-Maze video tracking software (Stoelting Co.).

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