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ORIGINAL ARTICLE

Regulation of Cognitive Processing by Hippocampal Cholinergic Tone

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Abstract

Cholinergic dysfunction has been associated with cognitive abnormalities in a variety of neurodegenerative and neuropsychiatric diseases. Here we tested how information processing is regulated by cholinergic tone in genetically modified mice targeting the vesicular acetylcholine transporter (VAChT), a protein required for acetylcholine release. We measured long-term potentiation of Schaffer collateral-CA1 synapses in vivo and assessed information processing by using a mouse touchscreen version of paired associates learning task (PAL). Acquisition of information in the mouse PAL task correlated to levels of hippocampal VAChT, suggesting a critical role for cholinergic tone. Accordingly, synaptic plasticity in the hippocampus in vivo was disturbed, but not completely abolished, by decreased hippocampal cholinergic signaling. Disrupted forebrain cholinergic signaling also affected working memory, a result reproduced by selectively decreasing VAChT in the hippocampus. In contrast, spatial memory was relatively preserved, whereas reversal spatial memory was sensitive to decreased hippocampal cholinergic signaling. This work provides a refined roadmap of how synaptically secreted acetylcholine influences distinct behaviors and suggests that distinct forms of cognitive processing may be regulated in different ways by cholinergic activity.

Key words: Alzheimer's disease, long-term potentiation, Morris water maze, paired associates learning, vesicular acetylcholine transporter, working memory

Introduction

Basal forebrain cholinergic neurons provide input to the entire cortex and hippocampus. In particular, the hippocampus receives most of its cholinergic innervation from neurons in the medial septal nucleus (MS) and vertical limb of the diagonal band of Broca (vdB), whereas the cerebral cortex and the amygdala receive cholinergic inputs from neurons located in the nucleus basalis of Meynert (NBM) (Mesulam et al. 1992; Kitt et al. 1994). Abnormalities in forebrain cholinergic nuclei have been implicated in several cognitive disorders (Bartus 2000; Mesulam 2004), including Alzheimer's disease (AD ; Grothe, Schuster, et al. 2014; Teipel et al. 2014). Moreover, cumulative use of drugs with anticholinergic activity is associated with increased risk for dementia and AD (Gray et al. 2015). However, the relationship between cholinergic dysfunction and maintenance of cognitive abilities in these diseases is not fully understood, due to concomitant pathologies that may contribute to cognitive abnormalities (Mesulam 2013).

Cholinergic signaling is involved in the regulation of hippocampal synaptic transmission and plasticity (Ji et al. 2001;

Leung et al. 2003; Seeger et al. 2004; Ge and Dani 2005; Gu and Yakel 2011). Septal cholinergic activation, either by electrical stimulation or by optogenetics, allows the expression of distinct time-dependent forms of hippocampal plasticity (Gu and Yakel 2011). Pharmacological (Decker and Majchrzak 1992; Anagnostaras et al. 1999; Gale et al. 2001; Wallenstein and Vago 2001; Chudasama et al. 2004; Pichat et al. 2007; Timmermann et al. 2007; Ragozzino et al. 2012) and genetic studies (Anagnostaras et al. 2003; Seeger et al. 2004; Poulin et al. 2010) have shown that modulation of cholinergic receptors influence learning and memory processes. Indeed, both nicotinic receptors (nAChRs) and muscarinic receptors (mAChRs) have been linked with various forms of plasticity (Vidal and Changeux 1993; Gray et al. 1996; Ji and Dani 2000; Seeger et al. 2004; Gautam et al. 2006; Giessel and Sabatini 2010; Zheng et al. 2012). For example, M1 knockout mice exhibit selective cognitive impairments in tasks requiring interactions between the hippocampus and cortex (Anagnostaras et al. 2003), while M2 knockout mice display impairments in working memory, cognitive flexibility, and hippocampal plasticity (Seeger et al. 2004). Moreover, recent evidence shows that activation of M1 mAChRs induces long-term potentiation (LTP), suggesting that M1 mAChRs could play a role in regulating hippocampal plasticity (Dennis et al. 2015). Furthermore, the nAChR β2-subunit knockout mice are impaired in learning the MWM, suggesting that the β2-subunit may mediate effects of ACh on learning and memory (Zoli et al. 1999). However, long-term changes in cholinergic activity, as observed in a number of neurodegenerative diseases, are more complex to model using specific receptor knockouts, given the plethora of subtypes of muscarinic and nicotinic receptors.

One widespread alternative to mimic cholinergic dysfunction is the selective elimination of these neurons using toxins in rodents (Baxter and Bucci 2013; Prado et al. 2013). It is somewhat controversial whether selective 192 IgG-saporin lesion of septohippocampal cholinergic neurons can lead to significant impairments in hippocampal-dependent learning and memory tasks in rodents, with some authors finding little effect (Berger-Sweeney et al. 1994; Baxter and Gallagher 1996; Pizzo et al. 2002; Frick et al. 2004; Parent and Baxter 2004), whereas others find a miriad of deficits (Walsh et al. 1996; Janis et al. 1998; Gil-Bea et al. 2011). In addition, cholinergic neurons have been shown to contain more than one class of neurotransmitter transporters and secrete 2 neurotransmitters (Gras et al. 2008; El Mestikawy et al. 2011; Guzman et al. 2011; Prado et al. 2013; Nelson et al. 2014; Saunders et al. 2015). Therefore, it is difficult to interpret results with toxin lesions for specific contributions of neurotransmitters in neurons that release 2 chemical messengers. Indeed, recent work has shown that some basal forebrain cholinergic neurons can also secrete GABA which acts as a neurotransmitter in the cortex (Saunders et al. 2015).

Genetic targeting of either the vesicular acetylcholine transporter (VAChT; Guzman et al. 2011; Martyn et al. 2012) or choline acetyltransferase (ChAT; Patel et al. 2012) using the Cre/lox system has provided a way for investigating specific contributions of ACh when there is co-transmission (Prado et al. 2013). Decreased VAChT levels severely compromise packaging of acetylcholine (ACh) into synaptic vesicles and thus reduce ACh release by nerve terminals (Prado et al. 2006; de Castro, De Jaeger, et al. 2009). Conversely, overexpression of VAChT enhances ACh secretion proportionally (Song et al. 1997; Kolisnyk, Guzman, et al. 2013) .

The recent development of automated touchscreen behavioral testing for rodents has greatly improved mouse behavioral assessment. Touchscreen tasks were designed using almost identical paradigms and methodologies used in humans, facilitating translational studies between species (Morton et al. 2006; Talpos et al. 2009, 2010; Romberg et al. 2011; Mar et al. 2013). The paired associates learning (PAL) test has been shown to efficiently detect cognitive alterations that are consistently observed in AD (Swainson et al. 2001; Blackwell et al. 2004; de Rover et al. 2011) and schizophrenia (Wood et al. 2002; Barnett et al. 2005). In dementia, PAL has been shown to differentiate between mild cognitive impairment and AD (Blackwell et al. 2004). Here we investigated cognitive performance in mice with deletion of VAChT, a protein required for synaptic release of ACh, in either forebrain cholinergic neurons or selectively in septohippocampal cholinergic neurons. Our experiments reveal that dysfunction in hippocampal cholinergic activity influences synaptic plasticity in vivo and disturbs performance in PAL and working memory, whereas spatial navigation seems relatively preserved.

Material and Methods

Animals

Generation of VAChT$^{flox/flox}$ mice was previously described (Martins-Silva et al. 2011). VAChT$^{flox/flox}$ mice (mixed C57BL/6J × 129/SvEv background, backcrossed to C57BL/6J for 5 generations) were crossed to VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice so that offspring from this mating provided control and test littermates. VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice were generated by crossing VAChT$^{flox/flox}$ with the Nkx2.1-Cre mouse line (C57BL/6J-Tg(Nkx2-1-cre)2Sand/J), purchased from The Jackson Laboratory (JAX stock no. 008661). This line has been previously used to eliminate ChAT from forebrain neurons (Patel et al. 2012). Unless otherwise stated, all control mice used for behavioral studies were VAChT$^{flox/flox}$ littermates. The reporter mouse line Nkx2.1$^{(td\text{-}Tomato)}$ was generated by crossing B6.Cg-Gt(ROSA)26Sor$^{tm9(CAG\text{-}tdTomato)Hze}$/J mice, purchased from The Jackson Laboratory (JAX stock no. 007909) with the Nkx2.1-Cre mouse line (JAX stock no. 008661).

Animals were housed in groups of 3 per cage without environmental enrichment in a temperature controlled room (12:12 light to dark cycles), and food and water were provided ad libitum for most experiments. Animals that underwent touchscreen testing were housed in pairs; food restricted to no more than 85% of their original weight, and they were maintained at the target weight for the duration of behavioral testing. Male mice 3 months old were used for behavioral studies. We followed the ARRIVE guidelines (Kilkenny et al. 2010); hence, mice were randomized for behavioral tests and the experimenter was blind to the genotype. All procedures were performed in accordance with the Canadian Council of Animal Care guidelines at the University of Western Ontario with an approved animal protocol (2008–127).

Immunoflourescence Microscopy

Mice were anesthetized with ketamine (100 mg/kg) and xylazine (25 mg/kg) in 0.9% sodium chloride, and then sacrificed by transcardial perfusion: phosphate-buffered saline (PBS, pH = 7.4) for 3 min and 4% paraformaldehyde for 5 min. Brains were harvested and placed in 4% paraformaldehyde in 1× PBS at 4 °C for 4 h; they were kept at 4 °C until sliced using a vibratome. Brain sections (40 µm) were prepared and free-floating sections in 1× PBS (1 per well in a 24-well plate) were permeabilized with 0.4% Triton X-100 in 1× PBS for 1 h. Non-specific epitopes were blocked using a solution of 1× PBS/0.4% Triton X-100 containing 0.1% glycine (wt/vol), 0.1% lysine (wt/vol), 1% BSA (wt/vol), and 1% normal donkey serum (wt/vol). The primary antibodies used were anti-VAChT (catalog no. 139103; Synaptic Systems), anti-ChAT

(1:200) (catalog no. AB144P, Merck Millipore), and anti-Choline Transporter (CHT1; 1:200), which was kindly donated by Dr R. Jane Rylett, University of Western Ontario, London, Ontario. The primary antibody was incubated in blocking buffer overnight at 4 °C. Sections were then washed 5 times in 1× PBS/0.4% Triton X-100 (10 min each). Hoechst 3342 (Life Technologies, Bibco, Carlsbad, CA, USA) (2–5 µg/mL) and secondary antibodies (1:500; anti-488, catalog no. A-11034, ThermoFisher; 1:500 anti-633, catalog no. A-21082, ThermoFisher) were diluted in blocking buffer and slices were incubated for 1 h at RT. Sections were visualized by Zeiss LSM 510Meta (Carl Zeiss, Oberkochen, Germany) confocal system (63 × objective, 488-nm Ar laser and 633-nm HeNe laser were used for excitation of fluorophores).

Western Blotting

Immunoblotting was performed as previously described (Martins-Silva et al. 2011; Kolisnyk, Al-Onaizi, et al. 2013; Kolisnyk, Guzman, et al. 2013). Antibodies used were anti-VAChT (catalog no. 139103; Synaptic Systems) and anti-Synaptophysin (catalog no. S5768; Sigma-Aldrich).

Training on the PAL Task

Prior to training, both groups of mice (3 months old) were food restricted until they reached approximately 85% of their original weight. Training of the animals to the PAL task was previously described (Talpos et al. 2009). Briefly, the training phase for the mice in the touchscreen chambers involved a habituation session, where they were placed in the chambers with the lights off for 20 min to habituate to the environment for 2 days. Next, mice were put in the chamber with the same parameters as in the habituation phase, but this time a 150 µL reward (strawberry milkshake; Saputo Dairy Products, Canada) was introduced in the reward receptacle. Every time the mouse attended to the reward in the reward receptacle, a tone was played. This 40 min training session was done for the next 2 days until mice completed 36 trials in 60 min (Habituation; Phase 1)

The mice were then trained to associate the reward with a 30 s presentation of training stimuli, which varied in brightness, shape, and pattern, on one of the 3 screens (Initial touch training; Phase 2). Mice were required to touch any of the screens whenever the stimulus was presented to receive the reward, which was paired with a tone. A new trial was automatically initiated once the mice collected the reward. This was done until the mice completed 36 trials in 60 min for 1 day. Next, mice are required to touch the stimulus that is displayed randomly in one of the 3 windows to receive the reward (must touch stimuli training; Phase 3). Mice are only moved to next training after completing 36 trials in 60 min for 1 day. In the next training phase, food is delivered and tray light is turned on. The mouse must nose poke and exit the reward tray before a stimulus is displayed randomly on the screen (Must initiate; Phase 4). This was done until mice completed 36 trials in 60 min for 1 day. Next, animals go to the last phase of the training program required for the PAL task. This training phase is referred to as “punish incorrect” (Phase 5). This phase is similar to the previous one, but if the mouse touched the incorrect screen, that is, one of the blank screens, it was presented with a 5-s time-out. This time-out was accompanied by the presentation of a bright light in the chamber. Criterion to successfully proceed from this training phase was 23 correct responses out of 30 trials in 60 min for 2 consecutive days. Next, both experimental groups were subjected to acquisition training, where 2 stimuli are displayed at the same time during a trial. One will be in the correct location (S+) and the other will be in the incorrect location (S–). Mice were required to touch the correct stimulus (S+) presented on one of the 3 screens to complete a trial and receive the reward. In this acquisition phase, mice were on an unpunished version in which touching the S– was ignored. A completion of a trial was only considered when the mouse touches the S+. Criterion for this training phase is completion of 36 trials in 1 session (1 day). All mice from both experimental groups were able to reach criterion in acquisition training.

PAL Task

After successfully completing the training phase, the mice were placed on a PAL task (dPAL), which involves a different stimulus being presented in each trial. A trial starts in dPAL when the mouse initiates it by touching the food receptacle, which triggers the display of both S+ and S– on the screen. S+ refers to when the stimulus is in the correct location, and S– refers to when the stimulus is in the incorrect location. There were 6 possible trial types and 3 different stimuli were presented (flower, plane, and spider). Within trials, an S+ is the flower presented in the left window, the plane in the middle window, or the spider in the right window. Thus, mice are required to learn to associate a stimulus to its correct location. A response by touching the S– resulted in a 10 s time-out and the chamber light was activated for 10 s, acting as an indication for an incorrect response for the mouse. After 10 s, the next initiation by the mouse was considered a correction error trial, where the same S+ and S– were presented as for the unsuccessful previous trial. The number of correction trials was not counted toward the total number of trials performed per session. An S+ response, however, led to a tone, as well as the reward being dispensed in the receptacle.

Electrophysiology

Animals were anesthetized with urethane (1 g/kg i.p.) and placed in a stereotaxic apparatus. Atropine methyl nitrate was administered (5 mg/kg i.p.) to reduce airway secretions during stereotaxic surgery. Animal body temperature was monitored between 36.5 °C and 37 °C using a feedback controlled rectal thermometer and heating pad. Stimulating electrodes were placed into stratum radiatum at P 1.8, L 2.3 or P 2.5, L2.4 (Franklin and Paxinos 2008) to stimulate Schaeffer collaterals projecting from CA3 to CA1 (Hutchison et al. 2009). A silicon probe, with 16 electrodes separated by 50 µm on a vertical shank, was placed in area CA1 at P 2.2, L 1.8. Laminar profiles of the average (4 sweeps) field excitatory postsynaptic potentials evoked by single pulse stimulation of the Schaffer collaterals at 1.5-2 × threshold stimulus intensity. Current-source density analysis using 100 µm step size was used to determine current sources and sinks. The maximal slope (of 1 ms duration) during the rising phase of the excitatory sink, at its maximum in CA1 stratum radiatum, was used for LTP assessment. After a stable baseline of the excitatory sink slope was established for 30 min (coefficient of variation [SEM/mean] of the sink slopes <0.05), a high-frequency tetanus (100 Hz for 1 s) was delivered at 2–3 times the threshold intensity, and the response was measured for 120 min after the tetanus. For each mouse, the slope of the excitatory sink was normalized by the average value of the baseline, and LTP across mice was averaged and reported as a multiple of the baseline slope.

Rotarod and Neuromuscular Tests

The rotarod task was conducted as previously described (Prado et al. 2006; de Castro, Pereira, et al. 2009). Forelimb and hind

limb grip strength was assessed using a previously described protocol (Prado et al. 2006). The hang-wire experiments were performed as described (Sango et al. 1996).

Morris Water Maze

The spatial version of the MWM was performed as previously described (Vorhees and Williams 2006; Martyn et al. 2012; Kolisnyk, Guzman, et al. 2013). Testing was performed in a 1.5-m diameter pool with 25 °C water. A hidden platform was submerged in a constant location 1 cm below the surface of the water in one of the 4 arbitrarily defined quadrants, and spatial cues were distributed around the pool. Briefly, mice were given four 90-s trials for the duration of 4 days to find the hidden platform, with an ITI of 15 min. The animals were introduced to the pool from different locations within the pool for each trial. Mice that did not find the platform within the 90 s were gently guided to the platform. On the fifth day, spatial memory recall was tested by a 60-s probe trial, where the hidden platform is removed and the amount of time the animal spends in the target quadrant is calculated. To test reversal learning, the hidden platform was relocated to the opposite quadrant, where the animals were given four 90-s trials for 4 days. On the fifth day, the animals were given a 60-s probe trial. Data were analyzed using ANY-Maze video tracking software (Stoelting Co.).

Two-Trial Morris Water Maze

A task used to assess working memory was the 2-trial variation of the MWM. The task was carried out using previously described protocols (Vorhees and Williams 2006; Kolisnyk, Guzman, et al. 2013). The mice were trained on the task over the course of 5 days. During the training period, the mouse was first given a 90-s trial with a 15 s inter-trial interval. Next, the mouse was given a second trial with the same platform location and starting point; this was repeated 3 additional times. After completing the training phase, the mouse was first given a 90 s trial with a 15 s inter-trial-interval. The mouse was then given a second trial with identical platform location and starting point. This was repeated with 4 unique starting location/platform location combinations a day. Mean latency and distance savings ratios were then calculated as previously described (Kolisnyk, Guzman, et al. 2013). Sessions were recorded for both tests and were analyzed using the ANY-Maze video tracking software (Stoelting Co.)

Spontaneous Alterations Y-maze

To assess working memory in the mice, we used the spontaneous alternations Y-maze as previously described (Kolisnyk, Guzman, et al. 2013). Briefly, mice were placed in a symmetrical plastic Y-maze apparatus, and both the number and order of arm entries were recorded. A spontaneous alternation was defined as when the mouse visited all 3 of the arms in a row, without having revisited a previous arm of the maze. Sessions were recorded and analyzed using the ANY-Maze Software.

Stereotaxic Injections of Adeno-Associated Virus

To obtain selective deletion of VAChT in the medial septum, mice were anesthetized with ketamine (100 mg/kg) and xylazine (25 mg/kg) in 0.9% sodium chloride, and 1 µL (titer of ~10^{13} GC/mL) of adeno-associated virus (AAV)8-GFP-Cre- or control virus (AAV8-GFP, Vector BioLabs, Eagleville, PA, USA) was injected into the medial septum/vertical limb of the diagonal band (0.98 AP, 0.1 LL and 4.1 DV) of $VAChT^{flox/flox}$ mice. The injecting micropipette was inserted and left for 2 min to stabilize. After stabilization, a 0.2 µL/min infusion was performed using a micropump followed by a 30 min rest period to allow local diffusion of the virus and avoid virus efflux. The micropipette was then slowly removed and the scalp sutured. A recovery period of 4 weeks was given before behavioral testing to allow transgene expression.

Statistical Analysis

All data are expressed as mean ± SEM. Sigmastat 3.5 software was used for statistical analysis. Comparison between 2 experimental groups was done with Student's *t*-test. When several experimental groups or treatments were analyzed, 2-way analysis of variance (ANOVA) or 2-way ANOVA with repeated measures (RM) were used as required. When appropriate, a Bonferonni *post hoc* analysis test was used.

Results

Deletion of VAChT in Forebrain Projection Neurons

Nkx2.1-driven Cre is expressed in forebrain cholinergic neurons as assessed using a reporter mouse line (see Supplementary Fig. 1A and Table 1). Immunoblot analysis shows that VAChT levels in the prefrontal cortex ($t_{(4)} = 6.162$, $P = 0.0035$), hippocampus ($t_{(4)} = 4.461$, $P = 0.0097$), and striatum ($t_{(4)} = 8.625$, $P = 0.0010$) were severely diminished in $VAChT^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice (see Supplementary Fig. 1B–D). In contrast, VAChT levels remained unchanged in the brainstem of $VAChT^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ compared with controls ($t_{(4)} = 1.040$, $P = 0.3571$, see Supplementary Fig. 1E). Moreover, immunofluorescence imaging indicated decreased VAChT immunoreactivity in the hippocampus of $VAChT^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice compared with controls (Fig. 1A,B). Importantly, these mice presented no neuromuscular deficits (see Supplementary Fig. 2A–C). We have previously shown that reduced VAChT levels proportionally decrease the release of ACh in vivo and in vitro (Prado et al. 2006; Guzman et al. 2011; Kolisnyk, Al-Onaizi, et al. 2013; Kolisnyk, Guzman, et al. 2013).

Forebrain VAChT is Required for Performance in the PAL Task

We tested $VAChT^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice on the PAL task, which requires sophisticated processing of information for proper association of images with specific locations. $VAChT^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice and their matched controls were assessed on the dPAL task using an automated touchscreen system (Fig. 1C and see Supplementary Videos 3 and 4). Prior to being subjected to the PAL task, both experimental groups are trained on a different training sessions (initial touch, must touch stimuli, must initiate, and punish incorrect) to learn how to operate the touchscreen, which includes learning to touch the screen when a stimulus is presented and initiating the task by inserting the head into the reward chamber. In the "punish incorrect" training, when only one stimulus is presented randomly in one of the 3 screens, mice are taught to touch the screen that shows the stimulus. Mice from both experimental groups were able to reach criterion in this phase of the training and no differences were observed between the 2 genotypes ($t_{(12)} = 0.0749$) (Fig. 1D), indicating that VAChT-deficient mice are able to learn that they need to touch the screen when an image is shown. Additionally it argues that VAChT-deficient mice do not present any major visual impairment. During the course of the 9 weeks that mice were tested on the dPAL task, we observed that control mice significantly improved their accuracy performance, while VAChT deletion

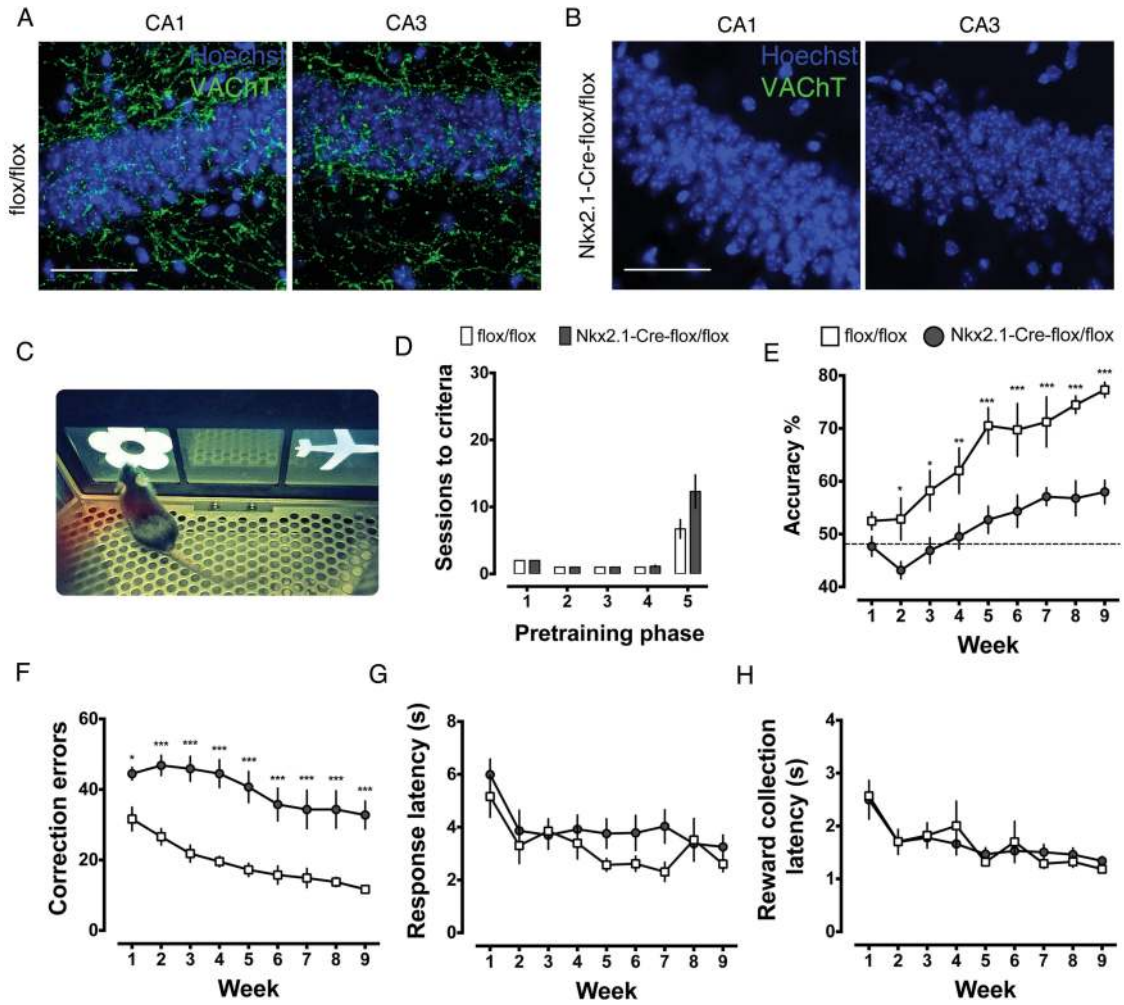

Figure 1. VAChT$^{NKx2.1\text{-}Cre\text{-}flox/flox}$ mice display impairments in the acquisition of dPAL. (*A*) Representative 3-dimensional reconstructed *Z* stack immunofluorescence images of VAChT (green) and Hoechst (blue) in the CA1 and CA3 regions of the hippocampus in VAChT$^{flox/flox}$ ($n = 3$) and (*B*) VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice ($n = 3$) (Scale bar = 100 μm). (*C*) Image depicting a mouse performing the task, where the flower is shown as S+ and the airplane as S−. (*D*) Number of sessions required by both experimental groups to reach criterion during the operant conditioning, pretraining, and training phases. (*E–H*) Data for the acquisition of the dPAL task for VAChT$^{flox/flox}$ ($n = 7$ clear squares) and VAChT$^{NKx2.1\text{-}Cre\text{-}flox/flox}$ ($n = 7$ dark circles) mice. Each week represents 5 testing sessions of 36 trials. (*E*) Mean accuracy; (*F*) Mean correction errors; (*G*) Response latency; (*H*) Reward collection latency (Data are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$).

mutants did not (2-way RM ANOVA shows significant effect of weeks $F_{8,48} = 21.11$, $P < 0.0001$, an effect of genotype $F_{1,6} = 56.94$, $P = 0.0003$, and an interaction effect $F_{8,48} = 2.871$, $P = 0.0074$, Fig. 1*E*). VAChT$^{flox/flox}$ mice (controls) were able to improve performance reaching 77 ± 1% accuracy by Week 9 (Fig. 1*E*). In contrast, peak accuracy performance of VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice in the dPAL task during the same period was 58 ± 2% (Fig. 1*E*). Although VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice were able to perform the 36 trials required in each 1-h session, they failed to associate the stimulus to its correct location. Their poorer performance was also reflected in the number of correction errors performed (Fig. 1*F*). VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice failed to decrease the number of correction errors made over the course of 9 weeks, while control mice improved the number of correction errors performed during the course of the study (2-way RM ANOVA shows significant effect of weeks $F_{8,48} = 12.05$, $P < 0.0001$, an effect of genotype $F_{1,6} = 39.41$, $P = 0.0008$, and an interaction effect $F_{8,48} = 1.224$, $P = 0.0306$, Fig. 1*F*). Correct response latency was not different between the 2 groups over the course of 9 weeks (2-way RM ANOVA shows significant effect of weeks $F_{8,48} = 7.508$, $P < 0.0001$, no effect of genotype $F_{1,6} = 2.437$, $P = 0.1695$, and no interaction $F_{8,48} = 1.195$, $P = 0.3220$, Fig. 1*G*). Furthermore, VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice were no different from controls when the latency to collect the reward was measured, which indicated that motivation was not a factor in their poorer performance (2-way RM ANOVA shows a significant effect of weeks $F_{8,48} = 7.596$, $P < 0.0001$, no effect of genotype $F_{1,6} = 0.0001380$, $P = 0.7681$, and no interaction $F_{8,48} = 0.6061$, $P = 0.7681$ Fig. 1*H*). In summary, VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice were able to learn that they had to touch the screen when the images were shown; however, they failed in making associations, that is, they were unable to assign each image to a specific position.

Hippocampal LTP is Disrupted in Forebrain-Specific VAChT Knockout Mice In Vivo

Formation of associations might depend on lasting increases in synaptic strength. To determine whether VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice have intact synaptic plasticity, we examined LTP of the synapse of the Schaffer collaterals on hippocampal CA1 neurons in anaesthetized mice in vivo. VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice showed decreased LTP which lasted about 90 min post-tetanus delivery while LTP in VAChT$^{flox/flox}$ mice was maintained for 120 min (Fig. 2*A*,*B*). This indicated that the lack of cholinergic signaling disturbs synaptic plasticity in hippocampal CA1 area in vivo.

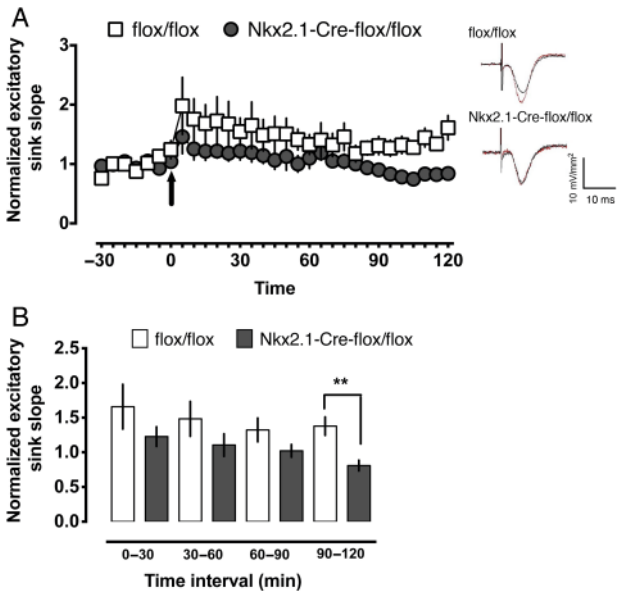

Figure 2. Hippocampal LTP is disrupted in forebrain-specific VAChT knockout mice in vivo. (*A*) Normalized slopes of the excitatory sink recorded at CA1 stratum radiatum (apical dendrites) of VAChT$^{flox/flox}$ (clear squares, $n = 5$) and VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ (dark circles, $n = 6$) mice. Baseline was monitored for 30 min prior to tetanus delivery ($t = 0$), and posttetanic response was monitored for 120 min. A 1-s 100 Hz train, delivered at 2–3 times the threshold intensity (arrow), induced higher and more prolonged potentiation in VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice than VAChT$^{flox/flox}$ controls. Insets show representative current sink time response taken at 80 min (red traces), overlaid on the pretetanus baseline response (black traces), from each genotype. (*B*) Normalized excitatory sink slope averaged across 30-min time intervals (mean ± SEM) in VAChT$^{flox/flox}$ and VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice, with significant difference between mouse groups at 90–120 min ($t_{(9)} = 3.911$, $P = 0.0036$).

To specifically evaluate the contribution of hippocampal cholinergic tone to PAL performance, we stereotaxically injected AAV8-GFP-Cre or AAV8-GFP virus to the medial septum and vertical limb of the diagonal band (MS/VDB) of VAChT$^{flox/flox}$ mice (AAV8-GFP-Cre $n = 13$; AAV8-GFP $n = 7$). Mice were trained on the dPAL task 1 month after viral injection. Following completion of the task, mice were sacrificed to evaluate VAChT protein levels. Given the length of the experiment (≈4 months), and the observation that viral injection was only partially effective to reduce hippocampal VAChT levels (see Supplementary Fig. 6*B*), we did not exclude any mouse from the analysis, even if viral mediated recombination was not effective to eliminate the transporter. Instead, we correlated VAChT levels in the hippocampus from both AAV8-GFP-Cre and AAV8-GFP to their performance on the PAL task.

Performance on the final week of the experiment was positively correlated to VAChT protein levels in terms of response accuracy (Pearson's $r = 0.5208$, CI = 0.1015–0.7829, $P = 0.0186$, Fig. 3*A*) and negatively correlated to number of correction errors (Pearson's $r = -0.6518$, CI = −0.8494 to −0.2940, $P = 0.0018$, Fig. 3*B*). We also evaluated the relationship between hippocampal VAChT protein levels to learning the PAL task. We calculated the rate of learning as the slope of the learning curve of both response accuracy and correction errors across all the weeks of the task. VAChT protein level was positively correlated to the rate of learning of response accuracy (Pearson's $r = 0.5053$, CI = 0.08072–0.7747, $P = 0.0231$, Fig. 3*C*) and negatively correlated to the correction error rate of learning (Pearson's $r = -0.1799$, CI = −0.7982 to −0.1418, $P = 0.0120$, Fig. 3*D*). Importantly, VAChT protein levels did not correlate to mean response latency across the task (Pearson's $r = 0.1349$, CI = −0.3273 to 0.5450, $P = 0.5708$, Fig. 3*E*) or mean reward collection latency across the task (Pearson's $r = -0.1799$, CI = −0.5676 to 0.2731, $P = 0.4352$, Fig. 3*F*), suggesting that response patterns and motivation are unaltered by reduced VAChT levels. Taken together these results show that the less VAChT protein in the hippocampus the worse is the mouse performance in the dPAL task, indicating that dPAL learning is modulated by septohippocampal cholinergic signaling.

VAChT and Spatial Navigation

Given the strong deficit of association of the image with its correct location in the PAL task, it seemed of importance also to evaluate spatial memory in these mice. Spatial memory is widely used to assess information acquisition and storage in the hippocampus, but cholinergic dysfunction has only mild effects in the MWM in mice (Moreau et al. 2008; Martyn et al. 2012). Our data showed that spatial learning on the MWM was relatively normal in VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice compared with controls (see Supplementary Fig. 5*A–C*). On the probe trial of the MWM, both groups of mice spent significantly more time in the target quadrant compared with the opposite quadrant (2-way ANOVA shows a significant effect of quadrant, $F_{3,80} = 39.58$, $P < 0.0001$, and an interaction effect $F_{3,80} = 2.914$, $P = 0.0394$, see Supplementary Fig. 5*D*), *post hoc* analysis revealed that both groups spent significantly more time in the target quadrant. However, VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice had significantly fewer platform crosses compared with littermate controls ($t_{(20)} = 2.795$, $P = 0.0112$, see Supplementary Fig. 5*E*).

To specifically evaluate the contribution of hippocampal cholinergic tone to learning and memory performance in the spatial version of the MWM, we stereotaxically injected AAV8-GFP-Cre ($n = 25$) virus to the MS/VDB in another cohort of VAChT$^{flox/flox}$ mice (see Supplementary Fig. 6*A*,*B*). VAChT$^{flox/flox}$ mice injected with AAV8-GFP ($n = 14$) were used as controls. AAV8-GFP-Cre-injected mice that showed more than 50% of hippocampal VAChT protein levels ($n = 11$) compared with controls were excluded from the analysis (see Supplementary Fig. 6*E*). In AAV8-GFP-Cre-injected mice with reduced hippocampal VAChT levels, VAChT protein in the prefrontal cortex was not changed (97% of AAV8-GFP VAChT levels, $t_{(4)} = 0.453$, $P = 0.665$, see Supplementary Figure 6*C*,*D*). AAV8-GFP-Cre-mediated deletion of VAChT from the medial septum did not significantly alter acquisition of the spatial version of the MWM (Latency, 2-way RM ANOVA shows an effect of days $F_{3,39} = 22.84$, $P < 0.0001$, no effect of Cre virus injection $F_{1,13} = 0.2228$, $P = 0.6447$, and no interaction, $F_{3,39} = 1.302$, $P = 0.2876$, Fig. 4*A*). Similar results were obtained for distance travelled (2-way RM ANOVA shows an effect of days, $F_{3,39} = 23.5$, $P < 0.0001$, no effect of Cre expression $F_{1,13} = 0.3125$, $P = 0.5856$, and no interaction, $F_{3,39} = 1.329$, $P = 0.2787$, Fig. 4*B*). In the probe trial, mice injected with the AAV8-GFP-Cre virus did not differ from controls in terms of preference for the target quadrant (2-way ANOVA shows a significant effect of quadrant, $F_{3,104} = 37.81$, $P < 0.0001$, no effect of Cre expression, $F_{1,104} = 0.6452$, $P = 0.4237$, and no interaction $F_{3,104} = 0.3988$, $P = 0.7541$, Fig. 4*D*) or platform crosses ($t_{(26)} = 0.9547$, $P = 0.3603$, Figure 4*E*). Taken together, these results suggest that decreased levels of hippocampal cholinergic activity do not seem to affect MWM performance.

VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice were also tested on the reversal learning protocol of the MWM. During the course of 4 days, control mice significantly improved in their latency to find the hidden platform in contrast to VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice (2-way RM ANOVA shows a significant effect of days $F_{3,30} = 8.632$, $P = 0.0003$, main effect of genotype $F_{1,10} = 11.17$, $P = 0.0075$, and

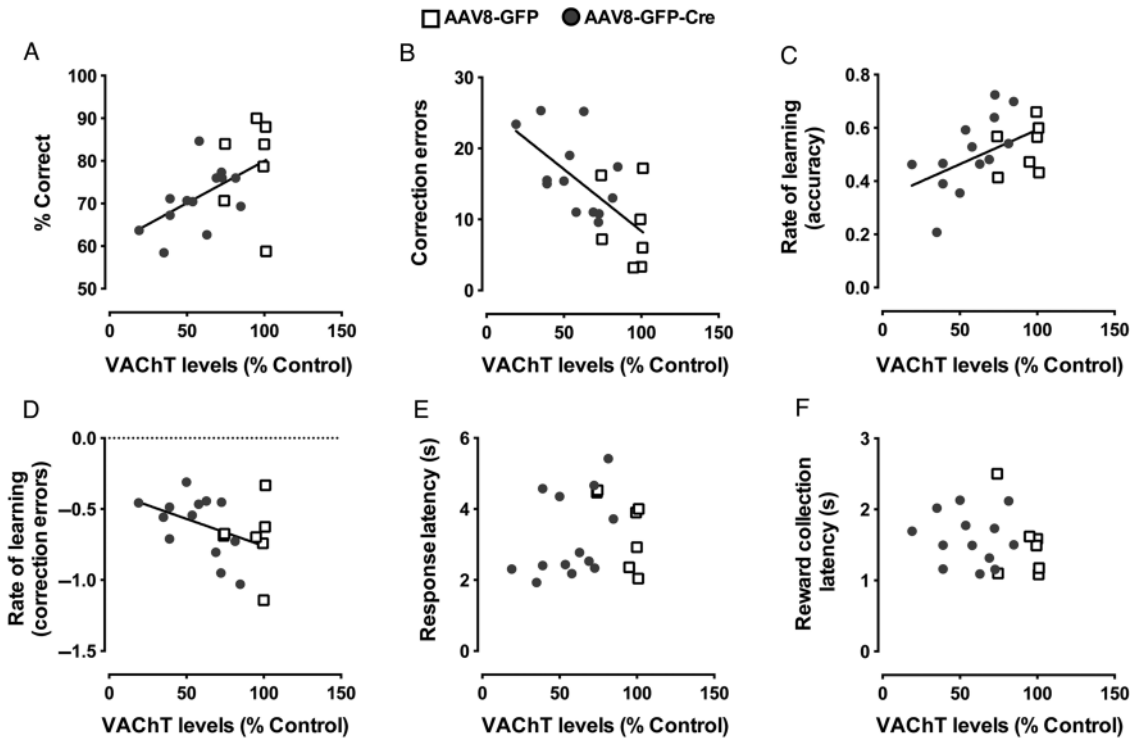

Figure 3. Medial septum AAV8-GFP-Cre-injected mice show deficits in dPAL. (*A*,*B*) Linear regression and correlation between response accuracy ($r = 0.5208$, $P = 0.0186$) and correction errors ($r = -0.5154$, $P = 0.0168$) on Week 9 and hippocampal VAChT protein expression levels for AAV8-GFP (clear squares, $n = 7$) and AAV8-GFP-Cre (dark circles, $n = 13$) injected mice. (*C*,*D*) Linear regression and correlation between response accuracy ($r = 0.4460$, $P = 0.0487$) and correction errors ($r = -0.1799$, $P = 0.0120$) across all the weeks of the PAL task and hippocampal VAChT protein expression levels. (*E*,*F*) The relationship between response latency ($r = 0.1349$, $P = 0.5708$) and reward collection latency ($r = -0.1799$, $P = 0.4352$) across all the weeks of the PAL task and VAChT expression levels.

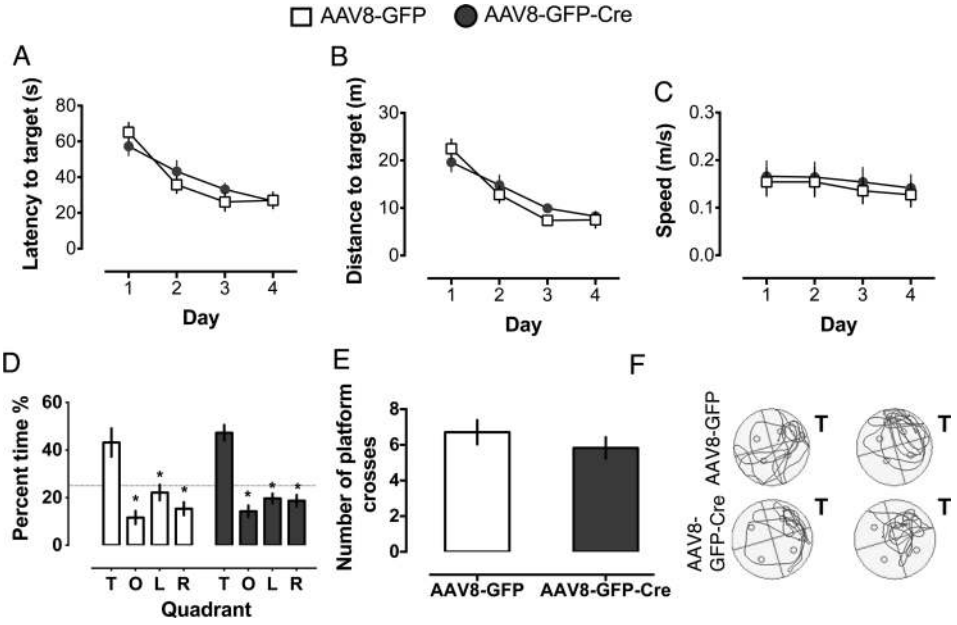

Figure 4. Performance of medial septum AAV8-GFP-Cre-injected mice in the MWM. VAChT$^{flox/flox}$ injected with AAV8-GFP virus (clear squares, $n = 14$) or AAV8-GFP-Cre virus (dark circles, $n = 14$) were tested in the spatial paradigm of the MWM. Data average of four 90-s trials per day were plotted. (*A*) Latency to reach the platform, (*B*) distance to reach the platform, (*C*) speed to reach the platform, (*D*) the percentage of time spent in each quadrant of the pool measured on Day 5 in a 60-s probe trial with the platform removed. (*E*) Number of platform crosses during the probe trial. (*F*) Representative path traces of 2 AAV8-GFP and 2 AAV8-GFP-Cre-injected mice in the probe trial. The target quadrant is in the upper right. Data are mean ± SEM. *$P < 0.05$. T, target; O, opposite; L, left; R, right.

no interaction $F_{3,30} = 1.501$, $P = 0.2342$, Fig. 5A–C). Notably, on the probe trial, control mice spent considerably more time in the target quadrant compared with the other quadrants (2-way ANOVA shows a significant effect of quadrant, $F_{3,80} = 7.226$, $P = 0.0002$, and an interaction effect $F_{3,80} = 3.133$, $P = 0.0301$, Fig. 5D), while VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice visited all quadrants almost equally.

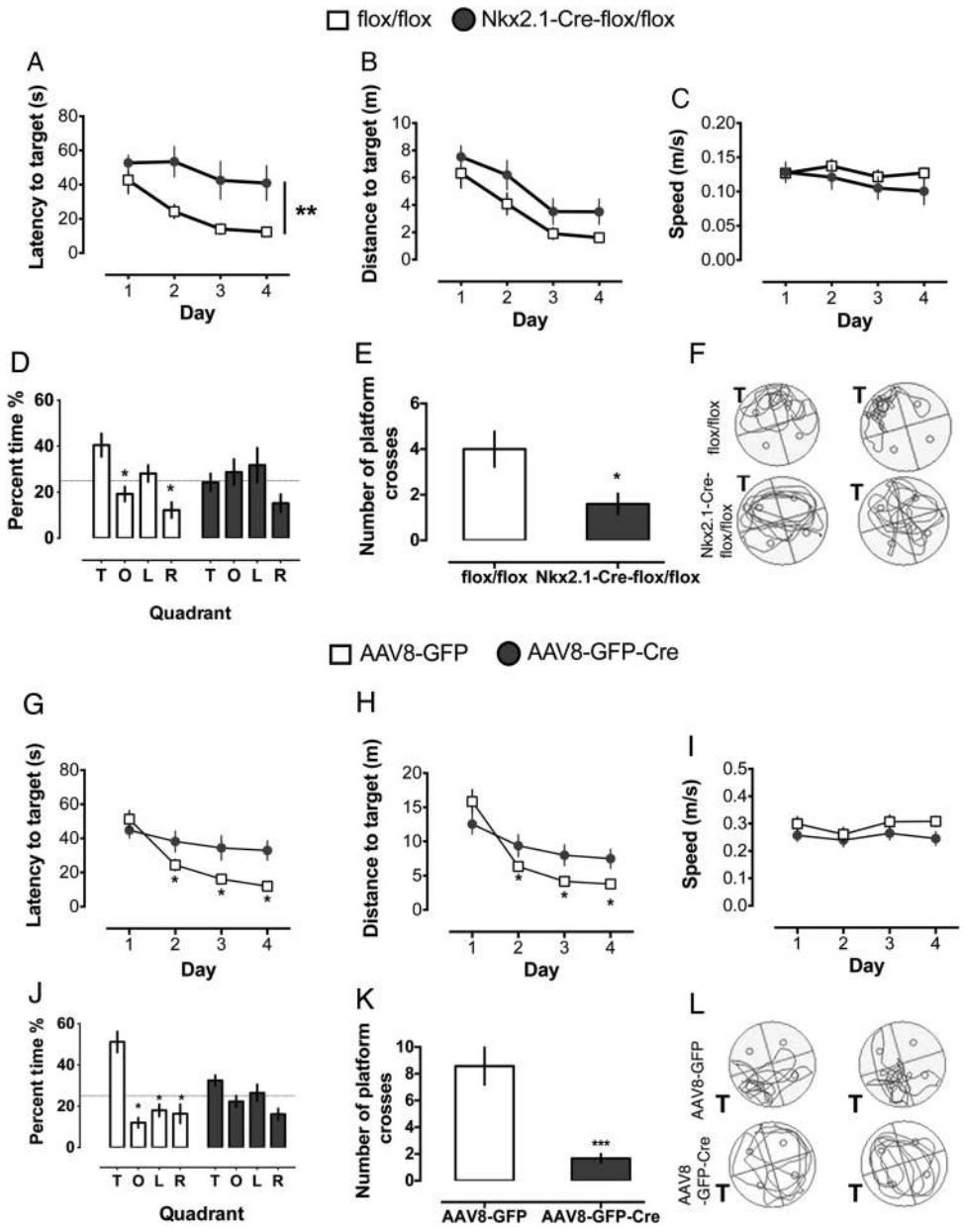

Figure 5. Reversal learning is affected in VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ and medial septum AAV8-GFP-Cre-injected mice. VAChT$^{flox/flox}$ (clear squares, $n = 11$) and VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ (dark circles, $n = 11$) were tested in the reversal paradigm of the MWM. Data average of four 90-s trials per day were plotted. (*A*) Latency to reach the platform, (*B*) distance to reach the platform, (*C*) speed to reach the platform, (*D*) the percentage of time spent in each quadrant of the pool measured on Day 5 in a 60-s probe trial with the platform removed. (*E*) Number of platform crosses during the probe trial. (*F*) Representative path traces for 2 VAChT$^{flox/flox}$ and 2 VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ in the probe trial. The target quadrant is in the upper left. (*G*–*L*) AAV8-GFP (clear squares, $n = 14$) or AAV8-GFP-Cre (dark circles, $n = 14$)-injected mice were tested in the reversal paradigm of the MWM. The data average of four 90-s trials per day were plotted. (*G*) Latency to find the platform, (*H*) distance, (*I*) speed, (*J*) the percentage of time spent in each quadrant of the pool was measured on Day 5 in a 60-s probe trial with the platform removed. (*K*) Number of platform crosses during the probe trial. (*L*) Two AAV8-GFP- and 2 AAV8-GFP-Cre-injected mice in the probe trial. The target quadrant is indicated with a T. Data are mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$. T, target; O, opposite; L, left; R, right.

The number of platform crosses was also higher for control mice compared with VAChT mutants ($t_{(20)} = 2.797$, $P = 0.0111$, Fig. 5*E*). These results indicate that, different from control mice, VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice were unable to extinguish the previously learned position and relearn the new position of the hidden platform.

To account for compromised striatal cholinergic signaling in VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice for the performance in the MWM (see Supplementary Fig. 1*D*), we also tested a mouse line with selective deletion of VAChT in striatal neurons (VAChT$^{D2\text{-}Cre\text{-}flox/flox}$), but spared hippocampal VAChT (Guzman et al. 2011; see Supplementary Fig. 7). Interestingly, VAChT$^{D2\text{-}Cre\text{-}flox/flox}$ mice did not differ from controls (VAChT$^{flox/flox}$) in both acquisition and reversal versions on the MWM (see Supplementary Fig. 7*D*–*H*). These results suggest that deficits seen in reversal learning in VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice are not likely due to impaired striatal cholinergic transmission, but rather a result of hippocampal or cortical deficits or combined cortical hippocampal

dysfunction. To discern among these possibilities, we used virus-injected mice.

Selective reduction of hippocampal cholinergic tone in virus-injected mice also increased latency to find the platform in reversal learning (2-way RM ANOVA shows an effect of days, $F_{3,39} = 21.96$, $P < 0.0001$ and a significant interaction effect, $F_{3,39} = 7.507$, $P = 0.0004$), with *post hoc* analysis revealing that AAV8-GFP-Cre-injected mice performed significantly worse on Day 4 compared with controls (Fig. 5G). During the probe trial, mice injected with AAV8-GFP-Cre virus showed significant impairments, failing to show a preference for the target quadrant (2-way ANOVA shows a significant effect of quadrant, $F_{3,104} = 23.3$, $P < 0.0001$, and an interaction effect, $F_{3,104} = 7.173$, $P = 0.002$, Fig. 5J). *Post hoc* analysis revealed that the AAV8-GFP-Cre mice did not prefer the target quadrant compared with the other quadrants, while the AAV8-GFP-injected controls had a strong preference for the target quadrant. Furthermore, the AAV8-GFP-Cre-injected mice showed a decrease in the number of platform crosses ($t_{(26)} = 0.9547$, $P = 0.0010$, Fig. 5K). These results reveal that disruption of hippocampal cholinergic tone, but not striatal or cortical cholinergic activity, compromises information processing in the MWM reversal learning.

Regulation of Working Memory by Septohippocampal VAChT

To determine whether other cognitive domains of importance in neuropsychiatric disorders that could contribute to the PAL deficits may also be regulated by synaptically released ACh, we evaluated the performance of the VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice on 2 measures of working memory: the working memory version of the MWM and spontaneous alternations in the Y-maze. In the working memory version of the MWM, VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice failed to improve their performance from the first to the second trial resulting in significant impairments in measures of latency savings ($t_{(12)} = 3.580$, $P = 0.0030$, Fig. 6A) and distance savings ($t_{(12)} = 2.852$, $P = 0.0127$, Fig. 6B), suggesting that the VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice have impaired working memory. Similarly, VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice revisited arms in the maze more often than controls resulting in a significant decrease in spontaneous alternations in the Y-maze ($t_{(12)} = 2.674$, $P = 0.0182$, Fig. 6C), suggesting that forebrain VAChT is required for normal working memory performance.

When tested on the working memory MWM test, mice with selective elimination of septohippocampal VAChT by virus injection (same cohort used in the MWM) also showed impaired latency savings ratio ($t_{(26)} = 2.847$, $P = 0.0111$, Fig. 6D) and distance savings ratio ($t_{(26)} = 2.149$, $P = 0.0473$, Fig. 6E). On the spontaneous alternations Y-maze task, AAV8-GFP-Cre-injected mice showed impairments on working memory, measured as a significant decreased rate of spontaneous alternations ($t_{(26)} = 3.347$, $P = 0.0041$, Fig. 6F). It is interesting to note that working memory deficits observed for AAV8-GFP-Cre-injected mice were similar to deficits observed for VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice. Taken together these results indicate that working memory is highly sensitive to hippocampal cholinergic tone.

Discussion

The present work shows that selective inhibition of cholinergic signaling in the hippocampus in mice leads to disruption of synaptic plasticity and specific cognitive impairments. In particular, we show that hippocampal cholinergic signaling is important for the modulation of cognitive tasks shown to be impaired in schizophrenia and dementia, including the PAL task. Interestingly, some hippocampal-dependent tasks appear to be more sensitive to decreased cholinergic signaling than others. Our results provide a comprehensive map of cholinergic-regulated hippocampal cognitive processing that may be useful to understand similar deficits in humans with cholinergic deficiency.

Notably, we report novel data indicating the importance of cholinergic signaling in regulating the PAL task. Clinically, the PAL task has been suggested as a potential cognitive marker of decline in psychosis (Wood et al. 2002). Significant impairments

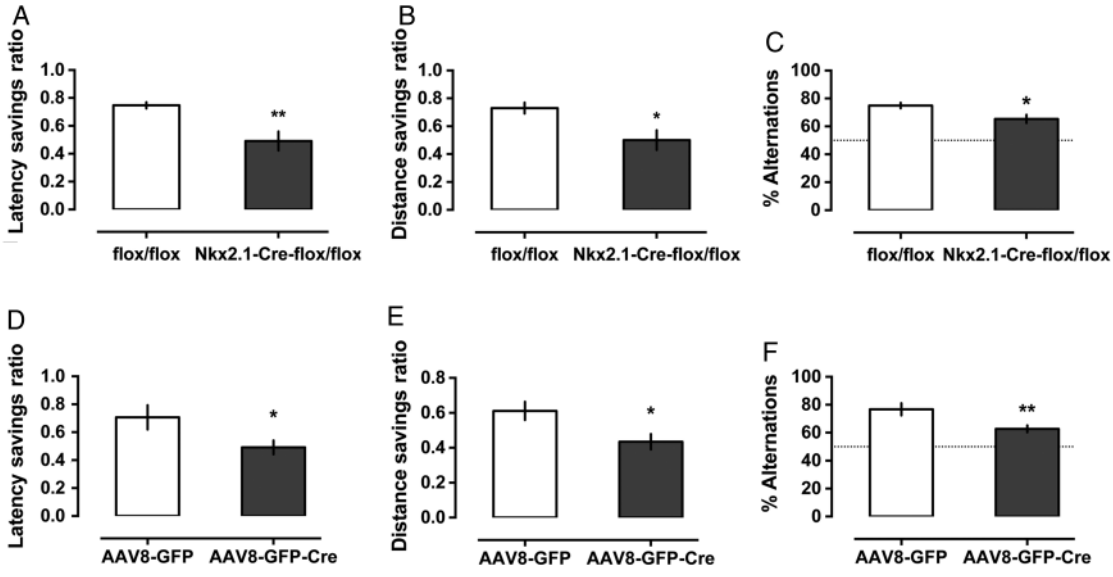

Figure 6. Working memory depends on hippocampal cholinergic tone. (*A*) Latency savings ratio and (*B*) distance savings ratio for VAChT$^{flox/flox}$ (clear, $n = 7$) and VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ (dark, $n = 7$) mice in the working memory version of the MWM. (*C*) Spontaneous alternations in the Y-maze for VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$. (*D*) Latency savings ratio and (*E*) distance savings ratio for AAV8-GFP (clear, $n = 14$) and AAV8-GFP-Cre (dark, $n = 14$) mice in the working memory version of the MWM. (*F*) Spontaneous alternations in the Y-maze for virus-injected mice. Data are mean ± SEM. *$P < 0.05$, **$P < 0.01$.

in PAL have been observed in patients with schizophrenia with a positive correlation between failure on the PAL task and negative symptoms (Barnett et al. 2005). Additionally, hippocampal activation during PAL is changed in patients with mild cognitive impairment compared with aged-matched controls (de Rover et al. 2011). Hence, PAL has also been considered a sensitive task for predicting cognitive decline in AD (Swainson et al. 2001; Blackwell et al. 2004).

Nonetheless, whether cholinergic signaling is required for acquisition of the task has not been clearly established. Systemic administration of donepezil, a cholinesterase inhibitor, improved post-acquisition PAL performance in mice, an effect that was attenuated with administration of muscarinic antagonists (Bartko, Vendrell et al. 2011). Similar results have been observed in monkeys where both mecamylamine (nicotinic antagonist) and scopolamine (muscarinic antagonist) induced deficits in PAL performance (Taffe et al. 2002; Katner et al. 2004). These results suggest that cholinergic signaling might be relevant for PAL. Also, rats previously trained in PAL that received injections into the dorsal hippocampus of either scopolamine or mecamylamine and that were re-tested did not show deficits in performance, suggesting that hippocampal cholinergic signaling might not modulate recall in this task (Talpos et al. 2009). Our results indicate that disruption in forebrain cholinergic tone disturbs PAL learning. Additionally, our data suggest that dysfunctional hippocampal cholinergic signaling may decrease PAL performance, as performance of mice in the PAL task correlates with hippocampal VAChT protein levels. Importantly, these deficits occurred in the absence of alterations in latency to touch the screen or to collect the reward, indicating that motivation was not a factor in the poorer performance of mice with lower cholinergic tone. Interestingly, mice deficient for the M1 receptor presented no differences compared with controls in their acquisition of the PAL task (Bartko, Romberg et al. 2011), suggesting that nicotinic and/or other muscarinic receptors might be involved in mediating learning in this hippocampal-dependent task.

Performance in PAL, as well as in other paired-associated tasks, may depend on intact hippocampal function in humans and rats (Talpos et al. 2009; de Rover et al. 2011). For example, short-lasting inactivation of the rat hippocampus using lidocaine (non-selective Na+ channel blocker) significantly impairs performance postacquisition of the PAL task, suggesting that the hippocampus is required at least for performance in this task (Talpos et al. 2009). In addition, human fMRI studies have shown bilateral BOLD activation of the hippocampus during the encoding phase of the PAL task (de Rover et al. 2011). Interestingly, subjects with memory deficits showed decreased hippocampal activation with increased memory demand, whereas healthy controls showed the opposite (de Rover et al. 2011). Moreover, PAL performance correlates with hippocampal volume loss in schizophrenia and mild cognitive impairment (MCI) (Keri et al. 2012). Intriguingly, recent reports indicate that mice with hippocampal lesions are still able to acquire the PAL task (Delotterie et al. 2015; Kim et al. 2015). One possible explanation (Kim et al. 2015) regarding these findings is that with a functional hippocampus the task is acquired in a hippocampal-dependent manner, but with a dysfunctional hippocampus, the task can be learnt using an alternative hippocampal-independent strategy. For example, mice with hippocampal lesions could have used the dorsal striatum to acquire the task (Delotterie et al. 2015). Indeed, the development of such behavioral plasticity has been shown in rats with unilateral hippocampal lesions (Zou et al. 1999). Our findings that forebrain VAChT-deficient mice seem unable to acquire the task, whereas decreased VAChT levels in the hippocampus decrease acquisition performance, suggest that the hippocampal cholinergic tone may facilitate acquisition of the PAL task. However, it is unlikely that only one brain region is involved in such a complex task.

The mechanisms by which ACh tone facilitates PAL performance are not fully understood. It is possible that cholinergic tone is required for specific types of synaptic plasticity. Indeed, hippocampal LTP in vitro is disturbed in a different mouse line lacking forebrain VAChT (Martyn et al. 2012). We corroborated this finding in vivo in VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice and demonstrated that in the absence of VAChT expression, hippocampal LTP is compromised, suggesting that disturbances of synaptic plasticity might contribute to the deficit. To note, previous studies have shown that levels of VAChT are correlated to levels of ACh release (Prado et al. 2006; de Castro, Pereira, et al. 2009, reviewed in Prado et al. (2013)); an increase in VAChT levels increases ACh release whereas decreased levels have the opposite effect (Song et al. 1997; Prado et al. 2006; Kolisnyk, Guzman, et al. 2013). VAChT is decreased in AD (Parent et al. 2013). These results suggest that correlating levels of VAChT detected with PET ligands (Efange 2000) to performance in the PAL test (Harel et al. 2013) might provide a potential biomarker of remaining cholinergic function and cognitive reserve.

We showed that acquisition of the spatial version of the MWM and recall of platform location was mildly affected in VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice, while AAV8-GFP-Cre-injected mice did not show any deficit in this behavioral task. Similarly, impairments in the spatial version of the MWM have been observed in rats with combined lesions of MS/VDB and nucleus basalis magnocellularis (NBM) cholinergic neurons produced by immunotoxin 192 IgG-saporin (Pizzo et al. 2002), while rats with immunotoxin lesions restricted to MS/VDB did not show any impairment (Berger-Sweeney et al. 1994; Baxter and Gallagher 1996; Pizzo et al. 2002; Frick et al. 2004). Interestingly, rats with 192 IgG-saporin lesions restricted to NBM also did not show behavioral impairments in the MWM (Pizzo et al. 2002). These data suggest that forebrain cholinergic signaling is necessary for reference spatial learning and memory assessed using the MWM; however, it seems that both the cortical and hippocampal cholinergic projections need to be compromised to produce a severe spatial deficit. Thus, providing that cortical cholinergic projections are intact, hippocampal cholinergic activity is not absolutely required for this behavioral task. It remains to be established whether GABA or glutamate, which could potentially be co-released with ACh (Guzman et al. 2011; Saunders et al. 2015) in both the hippocampus and cortex, may contribute to regulation of spatial memory by cholinergic neurons.

In contrast to the reference memory test, both VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ and AAV8-GFP-Cre-GFP-injected mice when tested in the MWM reversal learning task presented extensive deficits, suggesting a prominent role for hippocampal cholinergic signaling in reversal learning. The impairments seen in VAChT-deficient mice in reversal learning could relate to the loss of muscarinic presynaptic inhibition of excitatory feedback within cortical circuits (Hasselmo and McGaughy 2004), which would slow the extinction of a previously learned strategy (Hasselmo et al. 2002; Hasselmo 2006). To note, the findings with VAChT$^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice recapitulated the deficits seen in reversal learning in a different mouse line with deficient forebrain cholinergic tone we generated previously (Martyn et al. 2012; Kolisnyk, Al-Onaizi, et al. 2013). Interestingly, rats with 192 IgG-saporin lesions restricted to NBM also show behavioral flexibility impairments (Cabrera et al. 2006). Taken together, these results suggest that both NBM-cortical and septohippocampal cholinergic

signaling might be critical for the mediation of this form of cognitive flexibility.

The most common form of LTP underlying hippocampal synaptic plasticity in spatial memory depends on the activation of NMDARs (Collingridge et al. 1983; Martin et al. 2000; MacDonald et al. 2006). Intracerebroventricular administration of a NMDAR antagonist (AP5) significantly impaired performance of rats during reversal testing in the MWM (Morris et al. 1990). Moreover, genetically modified mice with deletion of the GluN2B subunit of NMDARs in the CA1 region of the hippocampus exhibited impairments in reversal learning (von Engelhardt et al. 2008). Similarly, mice with corticohippocampal deletion of GluN2B present deficits in hippocampal synaptic plasticity, highlighted by abolished long-term depression (LTD), a partial deficiency of LTP, and memory impairments (Brigman et al. 2010). The impairments observed in $VAChT^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice in LTP and reversal learning suggest that long-term cholinergic signaling may regulate NMDAR-mediated synaptic plasticity required for reversal learning in the MWM.

Both prefrontal cortex and hippocampus have been implicated in working memory (Yoon et al. 2008). A number of studies indicate that cholinergic neurotransmission is crucial for modulation of working memory in various behavioral tasks (Levy et al. 1991; Baxter et al. 1995; Furey et al. 2000; Hironaka et al. 2001). Whether cholinergic modulation of working memory is dependent on ACh acting on prefrontal cortex, hippocampus, or in both structures simultaneously is not known. Our results show that deficits in the working memory version of the MWM task and the Y-maze alternating task are equally severe in both forebrain VAChT mutants ($VAChT^{Nkx2.1\text{-}Cre\text{-}flox/flox}$ mice) and hippocampus VAChT mutants (AAV8-GFP-Cre-injected mice), suggesting that hippocampal cholinergic tone is vital in regulating information processing in working memory tasks. Taken together, these results suggest that ACh may exert important roles in working memory via modulation of hippocampal function. Whether these working memory deficits somehow contribute to the poor performance in PAL remains to be established.

Imaging studies involving volumetric measurement of basal forebrain cholinergic nuclei in humans reveal a drastic decrease in the volume of basal forebrain neurons in AD and MCI patients, in comparison to healthy elderly controls (Grothe et al. 2010, 2012; Grothe, Ewers, et al. 2014; Teipel et al. 2014). Given that individuals with dementia may present long-term changes in cholinergic tone, our mouse lines and approaches may be directly relevant to understand molecular, cellular, circuitry, and behavioral consequences of cholinergic malfunction. The present work is relevant to understand how drug-induced cholinergic dysfunction or degenerative changes in cholinergic neurons contribute to cognitive alterations in several neuropsychiatric disorders (Severance and Yolken 2008; Scarr et al. 2009). In summary, hippocampal cholinergic activity does not seem to be critical for spatial reference learning and memory, but has fundamental roles on working memory, reversal learning, and paired associates learning. As PAL performance may be dependent on cholinergic integrity, it is tempting to speculate that the PAL task could be used to identify individuals with cognitive dysfunction linked to cholinergic abnormalities.

Supplementary Material

Supplementary material can be found at http://www.cercor.oxfordjournals.org/ online.

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Notes

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