Decreased acetylcholine release delays the consolidation of object recognition memory

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Abstract

Acetylcholine (ACh) is important for different cognitive functions such as learning, memory and attention. The release of ACh depends on its vesicular loading by the vesicular acetylcholine transporter (VACHT). It has been demonstrated that VACHT expression can modulate object recognition memory. However, the role of VACHT expression on object recognition memory persistence still remains to be understood. To address this question we used distinct mouse lines with reduced expression of VACHT, as well as pharmacological manipulations of the cholinergic system. We showed that reduction of cholinergic tone impairs object recognition memory measured at 24 h. Surprisingly, object recognition memory, measured at 4 days after training, was impaired by substantial, but not moderate, reduction in VACHT expression. Our results suggest that levels of acetylcholine release strongly modulate object recognition memory consolidation and appear to be of particular importance for memory persistence 4 days after training.

Keywords:
Acetylcholine
Consolidation
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VACHT

1. Introduction

Memories may last days, months, or even a lifetime. In fact, the mechanisms that underlie the formation of new memories have been broadly explored [1–3]. However, the neurochemical basis underlying changes in the strength of synaptic connections necessary for memories to persist is still not precisely understood.

Most studies probing long-term memory (LTM) formation [1,2,4] or persistence in rodents [5,6] are carried out in aversive tasks, both because they are rapidly acquired and because their post-training consolidation [1,4] and post-consolidation steps [5,6] are well defined. In recent years, object recognition memory (OR) has become a critical task to investigate learning and memory in rodents without aversive stimuli [7–10]. So far, most studies have shown that despite the obvious differences between this type of task and fear-motivated tasks, both recruit hippocampal and neocortical circuits. Regarding the hippocampus, its participation on OR is still debated. However, recent evidence suggests that, while the hippocampus may not be the only brain structure to participate in OR [11–14], it certainly plays a role in it. Hippocampal lesions [7], as well as the inhibition of ribosomal [15] or mTOR-dependent [16] protein synthesis in the hippocampus impair OR memory. Further, OR consolidation is accompanied by LTP of the CA3-CA1 synapse and can be occluded by a preceding LTP of this synapse [17].

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Several authors have shown that the cholinergic system modulates OR memory. For example, cholinergic terminals in the perirhinal cortex play a major role in memory acquisition [11,12]. Moreover, muscarinic receptors in the insular cortex were shown to participate in OR memory [13]. Conversely, approaches using electrolytic [18] or chemical [19] lesions in cholinergic neurons either failed or found delayed effects on memory, suggesting an indirect effect of ACh. Thus, studies using alternative tools to precisely affect ACh release would improve the knowledge about the role of ACh on OR memory consolidation.

We have recently described an animal model of cholinergic deficiency: the vesicular acetylcholine transporter (VACHT) knock-down mice [VACHT KD, 20]. Heterozygous and homozygous VACHT KD mice (VACHT KD<sup>HET</sup> and VACHT KD<sup>HOM</sup>) have 40% and 70% decrease in the VACHT expression, which is the protein responsible for ACh uptake into synaptic vesicles and thereby a key regulator of ACh availability for release [21–23]. Release of ACh in these mice is proportional to the levels of transporter [20,24]. Therefore, these mice represent a good model to investigate the contribution of endogenous release of ACh on cognitive functions. In fact, VACHT KD<sup>HET</sup> mice present object recognition memory deficits that can be reversed by administration of acetylcholinesterase inhibitors during a pre-training period [20,25]. However, these experiments have not tested the role of ACh on memory consolidation.

Here we used VACHT mutant mice to explore the role of ACh release on OR memory consolidation. We found that moderate reduction of ACh release affects 24 h-LTM, but surprisingly it did not affect memory tested after 4 days. In contrast, further reduction of ACh release in VACHT KD<sup>HOM</sup> mice impaired memory retention at 4 days. Our results present novel evidence that memory consolidation can be modulated by changing the levels of ACh release.

2. Materials and methods

2.1. Animals

For this work we used VACHT KD<sup>HET</sup>, VACHT KD<sup>HOM</sup> and VACHT<sup>WT/DEL</sup>, the latter a heterozygous VACHT knockout mouse line with 50% decrease in VACHT expression. Extensive description of the mutants can be find in the papers [20,26], respectively. Reduction of 40–50% in VACHT expression causes similar decrease in ACh release in the brain [20]. Animals were housed in groups of three to five per cage in a temperature-controlled room with 12:12 light-dark cycles, and food and water were provided ad libitum. Every possible effort was made to minimize animal suffering and all procedures were conducted in accordance with NIH guidelines for the care and use of animals. All animal protocols are approved by the Institutional Animal Care and Use Committees at the Federal University of Minas Gerais.

2.2. Object recognition

The object recognition test is based on differential spontaneous exploration of novel and familiar objects [8]. The apparatus used was an open box made of PVC 50 cm × 35 cm × 25 cm (height) surrounded by a video camera and a light. Two objects made of glass or plastic were used. Their weight was such that they could not be displaced by mice or they were fixed with tape under the object. As far as we could ascertain, they had no natural significance for mice and they had never been associated with reinforcement. Initial tests showed that mice did not have any preference for the objects used.

The general procedure consisted of three different phases: a familiarization phase, a training phase and a test phase. On the 1st day, mice were individually submitted to a single familiarization session of 10 min, during which they were introduced into the empty arena. On the second day (24 h later), animals were submitted to a single 10 min training session during which two identical objects (A1 and A2) were placed in symmetrical positions from the center of the arena and each object was 15 cm from the side walls. After a delay during which mice returned to their home-cage, they were reintroduced into the arena for 10 min (test phase) and exposed to two objects, a familiar object and a novel object (A and B), placed at the same locations as during the training phase. The role (sample or new object) as well as the position of the two objects during the test session was interchanged between mice and across sessions. To control odor cues, the apparatus was cleaned with 90% ethanol or ventilated between each session and animal. If a mouse had a total exploration time of less than 10 s (sum of exploration of both objects), they were discarded. All session were performed during the first part of the light cycle and mice were acclimated to the room for at least 15 min before the beginning of each session.

All the injections were performed after the training session to avoid any effect of injections during the training and the effect was observed only on consolidation.

2.3. Experiment 1: long-term memory

To examine long-term memory (LTM) of mice with reduced expression of VACHT, they were tested 24 h after training. In addition, mutant mice were treated with an acetylcholinesterase inhibitor (galantamine, i.p. 1 mg/kg) or with NaCl (9%) just after training to reverse the deficit of memory and confirm the importance of ACh in memory consolidation (see Fig. 1a).

2.4. Experiment 2: effect of increased training sessions

Previously experiments have shown that VACHT KD<sup>HET</sup> mice can perform like WT mice after numerous days of training in accelerated rotarod [25]. To see if mutant mice are able to form memory trace after several training sessions, the mice received one training (10 min) per day for four days with the same object and were tested 24 h after the four training sessions (Fig. 2a).

2.5. Experiment 3: effect of training duration

In the previous experiment, several different parameters were changed in comparison to the LTM experiment. One of these parameters was the total time that mice were exposed to the object during training, as this time was changed from 10 min to 40 min. In order to test if the duration of the training session could influence memory formation, the mice were trained during a unique 40 min training session and tested 24 h after training for 10 min (Fig. 3a).

2.6. Experiment 4: effect of object

To determine object importance in the 4 trial experiment, mice underwent one trial (the second day) and, during 3 days they were placed in an open field for 10 min without an object and were tested on the 6th day for 10 min (Fig. 3c).

2.7. Experiment 5: effect of delay between trial and test sessions

In this experiment, we tested whether the delay between training and test sessions was important for the formation of memory in mice which had reduced ACh release. Animals were trained for 10 min on the second day, returned to their home cage for the next 4 days, and were tested on the 6th day (Fig. 4a). VACHT KD<sup>HET</sup> mice and WT were also tested on the 10th day, so 8 days after the training (Fig. 4d).

2.8. Statistics and data collection

The basic measure was the total time spent by mice exploring an object during the session. Exploration of an object was defined as follows: directing the nose at a distance <1 cm to the object and/or touching it with the nose [8]. Result was expressed as mean ± SEM and we used SigmaStat 3.1 software for statistical analysis. Statistical analysis was performed using the two-way analysis of variance (ANOVA) with repeated measures and when appropriate, a Tukey post hoc comparison test was used.

3. Results

Our initial experiments evaluated how moderate levels of decreased VACHT, and consequently decreased ACh release would affect OR memory consolidation. VACHT KD<sup>HET</sup> mice have impairment in long-term memory formation (Fig. 1b): A two factor ANOVA revealed no effect for genotype (F<sub>1,14</sub> = 1.038, P = 0.326), a significant effect for object (F<sub>1,14</sub> = 25.824, P < 0.001) and interaction between the two factors (F<sub>1,14</sub> = 15.262, P < 0.01). Post hoc analysis indicated that WT mice explored the new object longer during the second exposure (P < 0.001), whereas VACHT KD<sup>HET</sup> mice explored the new object and the familiar object similarly (P = 0.420). The impairment in LTM was confirmed with an independent line of VACHT-modified mice, VACHT<sup>WT/DEL</sup> mice, which presents 50% decrease in VACHT expression (Fig. 1c). VACHT<sup>WT/DEL</sup> mice also showed a deficit of LTM and the two factors ANOVA revealed no effect of genotype (F<sub>1,18</sub> = 0.291, P = 0.596), a significant effect of object (F<sub>1,18</sub> = 34.629, P < 0.001) and interaction between the genotype × object (F<sub>1,18</sub> = 24.550, P < 0.001). A Tukey post hoc test showed a difference between exploration for the two objects by WT mice (P < 0.001), whereas no difference was observed for VACHT<sup>WT/DEL</sup> mice (P = 0.556). The long term memory deficit was
Fig. 1. Pharmacological and genetic down-regulation of cholinergic neurotransmission affect consolidation of OR LTM. (a) Scheme of the protocol for investigation of LTM. (b) Time of exploration for the object during the training and the test session. VACHT KD\textsuperscript{HET} mice (n = 8) present a deficit in long term memory compared to WT (n = 8). (c) Object recognition LTM for VACHT\textsuperscript{WT/WT} (n = 12) and VACHT\textsuperscript{WT/DEL} (n = 8) mice. (d) Injection of saline (n = 7) or galantamine (n = 8, 1 mg/kg, i.p.) after the training in VACHT KD\textsuperscript{HET} galantamine is able to reverse LTM deficit in this mice. (e) Injection of galantamine (n = 6, 1 mg/kg, i.p.) or saline (n = 6) after training in VACHT\textsuperscript{WT/DEL}. (f) Object recognition LTM for VACHT KD\textsuperscript{HOM} mice (n = 6) and WT (n = 9). (g) Injection of saline (n = 6) or galantamine (n = 5, 1 mg/kg, i.p.) after the training in VACHT KD\textsuperscript{HOM}. All the Results are expressed as mean + SEM of total exploration time. The letters A and B represent, respectively, the familiar and new object. ***Significant difference between the new object (A) and the familiar object (B) p < 0.001 with a two way repeated measure ANOVA.

Also observed with VACHT KD\textsuperscript{HOM} mice (Fig. 1f), which show further reduction of ACh release (around 70%) [20,24]. The two way ANOVA showed no effect of genotype (F\textsubscript{1,13} = 0.04, P = 0.845), a significant effect of object (F\textsubscript{1,13} = 11.125, P < 0.01) and interaction between the genotype × object (F\textsubscript{1,13} = 7.153, P < 0.05). A Tukey post hoc test showed a difference between exploration for the two objects by WT mice (P < 0.001), whereas no difference was observed for VACHT KD\textsuperscript{HOM} mice (P = 0.677).

The importance of ACh releasing to OR memory consolidation was assessed with injection of galantamine just after training, which reversed the LTM impairment in VACHT KD\textsuperscript{HET} mice (Fig. 1d). A two factor ANOVA showed no effect of treatment
on exploration \( F_{1,13} = 1.201, P = 0.293 \), a significant effect of object \( F_{1,13} = 8.517, P < 0.05 \) and interaction between object and treatment \( F_{1,13} = 5.939, P < 0.05 \). VAChT KD\textsuperscript{HET} mice injected with galantamine explored the new object longer \( P < 0.01 \) than those given saline \( P = 0.747 \). We repeated this experiment with VAChT\textsuperscript{WT/DEL} mice injected with galantamine (Fig. 1e). A two factors ANOVA revealed no effect of treatment \( F_{1,9} = 0.5853, P = 0.4815 \), a significant effect of object \( F_{1,9} = 9.094, P < 0.05 \) and interaction between object \& treatment \( F_{1,9} = 9.217, P < 0.05 \). VAChT\textsuperscript{WT/DEL} mice injected with galantamine explored the new object longer \( P < 0.001 \) than saline-injected VAChT\textsuperscript{WT/DEL} mice, which showed similar exploration for both objects \( P = 0.889 \). Galantamine injection just after the training, allowed VAChT KD\textsuperscript{HOM} mice to form memory when tested at 24h (Fig. 1g). A two factors ANOVA revealed no effect of treatment \( F_{1,9} = 5.392, P = 0.05 \), a significant effect of object \( F_{1,9} = 28.622, P < 0.001 \) and interaction between object \& treatment \( F_{1,9} = 26.340, P < 0.001 \). VAChT KD\textsuperscript{HOM} mice injected with galantamine explored the new object longer \( P < 0.001 \) than saline-injected VAChT KD\textsuperscript{HOM} mice, which showed similar exploration for both objects \( P = 0.875 \). These results suggest that increasing the availability of ACh in VAChT-mutant mice, during the early phase of memory consolidation, reverses LTM deficit.

It was demonstrated that the active exploration of objects increases ACh release and improves memory consolidation in the radial maze [27]. Moreover, we found that increased training improves the response of VAChT KD\textsuperscript{HET} mice in motor learning, suggesting that overtraining might mitigate memory deficit [25]. In order to test if OR memory can also be rescued by increasing the exposure of mutant mice to the stimuli objects, we trained mice with the same objects for 4 consecutive days and tested them for OR memory retention one day later (Fig. 2a). As shown in Fig. 2b and c, after 4 training sessions, VAChT KD\textsuperscript{HET} mice behave as WT mice and spend more time exploring the new object [A two-factor ANOVA revealed no effect for genotype \( F_{1,18} = 0.564, P = 0.462 \), a significant effect for object \( F_{1,18} = 38.766, P < 0.001 \) and no interaction between the two factors \( F_{1,18} = 1.112, P = 0.306 \)]. Therefore, after 4 training sessions VAChT KD\textsuperscript{HET} mice do not show memory deficit.

Next, we tested if the exposure to the object for a longer period is the main factor allowing hypochoolinergic mice to recognize the object after the 4 training sessions. We submitted mice to a single, but longer, period of training (Fig. 3a) and observed a genotype-dependent difference in the total exploration time during the test \( F_{1,18} = 5.355, P < 0.05 \), but not during the training \( t_{18} = 0.723 P = 0.479 \) session. We also observed a significant effect of object \( F_{1,18} = 39.329, P < 0.001 \) and an interaction between the two factors \( F_{1,18} = 32.988, P < 0.001 \). Post hoc Tukey analysis showed an increase in exploration of the novel object compared to the familiar object in WT mice \( P < 0.001 \), whereas the VAChT KD\textsuperscript{HET} mice exhibited similar exploration for both objects \( P = 0.714 \), which suggests that the time the mice were exposed to the object does
not affect memory encoding. In conclusion, overtraining by 40 min exploring the objects did not reverse the deficit of VACHT KD\textsuperscript{HET} mice in OR memory (Fig. 3b).

Next, we tested if the number of training sessions is the factor allowing memory consolidation in mutant mice. To test that, we trained mice once and then re-exposed them to the box (context) for 3 consecutive days (Fig. 3c). Surprisingly, mutant mice remembered the familiar object at 96 h (Fig. 3d) even without being re-submitted to the object. The two way ANOVA yielded no effect of genotype ($F_{1,18} = 0.732, P = 0.403$), a significant effect of object ($F_{1,18} = 49.001, P < 0.001$) and no interaction between genotype and object ($F_{1,18} = 0.313, P = 0.583$).

In order to test if the re-exposure is the main factor allowing the OR memory persistence for 4 days, we trained mice once and then tested them 4 days later (Fig. 4a). When the interval between the trial and the test was extended to 4 days instead of 24 h, VACHT KD\textsuperscript{HET} mice showed intact OR memory (Fig. 4b), similar to control mice. In fact, a two-factor ANOVA revealed no effect of genotype ($F_{1,135} = 0.492, P = 0.488$), a significant effect of object ($F_{1,135} = 43.479, P < 0.001$) and no interaction between these two factors ($F_{1,135} = 0.497, P = 0.485$). To test if the level of cholinergic tone could influence memory retention for 4 days, we used VACHT KD\textsuperscript{HOM} mice. Fig. 4c shows that 4 days OR memory is impaired in VACHT KD\textsuperscript{HOM} mice. In fact, a two-factor ANOVA revealed no effect of genotype ($F_{1,12} = 0.353, P = 0.563$), a significant effect of object ($F_{1,12} = 7.950, P < 0.05$) and interaction between these two factors ($F_{1,12} = 13.864, P < 0.01$). Post hoc Tukey analysis showed an increase in exploration of the novel object compared to the familiar object in WT mice ($P < 0.001$), whereas the VACHT KD\textsuperscript{HOM} mice exhibited similar exploration for both objects ($P = 0.561$); this suggests that a higher decrease in cholinergic tone impairs memory retention for 4 days.

In order to test if a longer delay between test and training will allow VACHT KD\textsuperscript{HOM} mice to form object memory, we tested animals 8 days after training (Fig. 4d). The VACHT KD\textsuperscript{HOM} mice did not recognize the new object. However, with such a long delay, even the WT mice did not retain memory (Fig. 4e). In fact, the ANOVA revealed no effect of genotype ($F_{1,19} = 2.118, P = 0.180$), or object ($F_{1,19} = 0.115, P = 0.742$) and no interaction between these two factors ($F_{1,19} = 0.186, P = 0.676$) was observed.

4. Discussion

We used both pharmacological approaches and genetically modified mice to investigate the modulation by cholinergic tone on OR memory consolidation. We showed that moderate decrease in the cholinergic tone impaired OR memory retention after 24 h, but not after 4 days. However, the VACHT KD\textsuperscript{HOM} mice, which have a more drastic reduction of ACh, showed 24 h and 4 days memory impairment.

The apparent “return” to normality of memory at 4 days for VACHT KD\textsuperscript{HET} mice may simply reflect a much slower build-up of memory to regular LTM levels in the animals with decreased ACh tone, so that it would take 4 days instead of one to encode the trace. Such an explanation would be in line with recent observations of a very protracted ACh intervention in other forms of memory [28]. Unfortunately, we were not able to see if the VACHT KD\textsuperscript{HOM} mice would be able to remember the object after a longer delay (8 days) because this memory is absent even in the WT mice.

We cannot reject the possibility that the 4 days normal retention of object memory in VACHT KD\textsuperscript{HET} mice involves other neurotransmitter system. However, if the ACh level is not important to drive 4 days memory consolidation, VACHT KD\textsuperscript{HOM} mice should present intact memory 4 days after as WT mice did. However, we
observed the opposite. The impairment of the 4 days memory in the VACHt KD hom mice suggests that the ACh is also involved in the 4 days memory formation. Still, we cannot rule out that drastic, but not moderate, decrease in VACHt expression compromised other neurotransmitter system. Therefore, the 4 days memory deficit observed in VACHt KD hom mice could be an indirect consequence of decreased synaptic ACh levels.

It has been recently observed that systemic injection of muscarinic receptor antagonist impairs the STM, while antagonist of nicotinic receptor blocks LTM [29]. Since our heterozygote mice have general decrease of VACHt we should observed impairment for both STM and LTM. We found here that decreasing cholinergic tone impaired memory at 24 h after training, and we previously reported impairment of STM with VACHt KD het [20], which is in accordance with the work of Tinsley and other previous works [11,12,25,30–32].

Furthermore, our results with the acetylcholinesterase inhibitor, galantamine, suggest that the deficit observed in the hypocholinergic VACHt-mutant mice is due to decreased ACh release during the post-training period and not due to any developmental problem. In fact, in previous experiments we also showed that galantamine administration, 30 min prior to the test session, does not rescue deficits of the OR memory in VACHt KD het mice [25].

We demonstrated that a moderate decrease in cholinergic tone does not affect memory retention after 4 days. It has been demonstrated that exploration of objects increases ACh release in the hippocampus [27]. However, a longer training was not sufficient to reverse the LTM deficit observed in VACHt KD het mice, perhaps because synaptic vesicles filling by VACHt could not cope with the demand in these mice.

According to the systems consolidation theory, the hippocampus stores experiences for a short period of time before the information is transferred to the cortex for durable storage [reviewed in 33, and 34]. It is likely that multiple brain regions participate in object recognition memory. Indeed, the perirhinal and the insular cortex have an important role in OR memory consolidation whereas the hippocampus has a role for object location or the context in which the object is presented [35]. However, other experiments do suggest that the hippocampus is important for object recognition memory consolidation [7,17,35]. Importantly, ACh influences LTM in the hippocampus [36]. Our experiments do not discriminate the role of these different regions, but rather indicate that cholinergic deficiency affects the way OR memory is processed.

Recently, Sarter et al. reviewed the evidence in favor of ACh acting not only synthetically, on receptors across the synaptic gap, but at least in part also at a distance, by volume transmission [37]. In this case, impaired storage of ACh in vesicles could be expected to lengthen considerably the time it takes to reach an appropriate concentration of the neurotransmitter in another distance. In the case of a more drastic reduction in VACHt expression, as observed in VACHt KD hom mice, we could expect that volume transmission would be particularly affected.

The “biphasic” hypothesis concerning memory modulation by ACh propose that during the waking phase, high levels of ACh would be necessary for long term memory formation at the hippocampal level. In contrast, during the sleep phase, a lower level of ACh would permit building a second memory trace, at the cortical level [38,39]. The suppression of the high level of ACh and the decreasing amplitude between the two phases could explain why the consolidation may be slower in mutants and why these mice present a memory deficit at 24 h, but not at 4 days after the training. Therefore, we may suggest that VACHt KD het mice are able to build the second trace at the cortical level only during the sleep. This assumption is partially supported by the fact that VACHt KD het mice showed 4 days memory when the interval between days was given (Figs. 3d, 2b and 4b), independently of context or objects exposure. Nevertheless, if the decrease in ACh release is more drastic, as observed in VACHt KD hom mice, it is possible that the second phase of ACh release, occurred during the sleep, is also compromised. Thus, the memory formation at cortical level would be impaired, which could explain why VACHt KD hom mice do not show novel object preference 4 days after the training.

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