Published in final edited form as:

Genes Brain Behav. 2020 July ; 19(6): e12677. doi:10.1111/gbb.12677.

Hippocampal knockdown of α2 nicotinic or m1 muscarinic acetylcholine receptors in C57BL/6j male mice impairs cued fear conditioning

Yann S. Mineur, **Charlotte Ernsten**, **Ashraful Islam**, **Kathrine Lefoli Maibom**, **Marina R. Picciotto***

Department of Psychiatry, Yale University School of Medicine, 34 Park Street, 3rd Floor Research, New Haven, CT 06508, USA

Abstract

Acetylcholine (ACh) signaling in the hippocampus, is important for behaviors related to learning, memory and stress. In this study, we investigated the role of two ACh receptor subtypes previously shown to be involved in fear and anxiety, the M1 mAChR and the α2 nAChR, in mediating the effects of hippocampal ACh on stress-related behaviors. Adeno-associated viral vectors containing short-hairpin RNAs targeting M1 or α2 were infused into the hippocampus of male C57BL/6J mice, and behavior in a number of paradigms related to stress responses and fear learning was evaluated. There were no robust effects of hippocampal M1 mAChR or α2 nAChR knockdown in the light/dark box, tail suspension, forced swim or novelty-suppressed feeding tests. However, effects on fear learning were observed in both knockdown groups. Short term learning was intact immediately after training in all groups of mice, but both the M1 and α2 hippocampal knock down resulted in impaired cued fear conditioning 24 hours after training. In addition, there was a trend for a deficit in contextual memory the M1 mAChR knockdown (KD) group 24 hours after training. These results suggest that α2 nicotinic and M1 muscarinic ACh receptors in the hippocampus contribute to fear learning and could be relevant targets to modify brain circuits involved in stress-induced reactivity to associated cues.

INTRODUCTION

While responses to stress are regulated by multiple neurotransmitter systems, elevated acetylcholine (ACh) signaling in the hippocampus is sufficient to result in a number of stress-induced behaviors (Mineur et al. 2013). Consistent with these findings, ACh levels are increased in the hippocampus during exposure to stressors (Paul et al. 2015). This is consistent with human studies demonstrating that increased ACh signaling by inhibiting its degradation results in feelings of anxiety and depression in healthy subjects (Risch et al. 1981).Thus, hippocampal ACh signaling appears to contribute to adaptive and maladaptive responses to stressors.

*To whom correspondence should be addressed.

Both adaptive and maladaptive responses to stress involve encoding of environmental stimuli that can predict future threats, as can be assessed by fear learning. Stress can potentiate fear conditioning by limiting cognitive flexibility (Raio and Phelps 2015), and also impairs fear extinction (Maren and Holmes 2016). Acetylcholine plays a critical role in fear conditioning both by strengthening the association between sensory stimuli and predicted outcomes (Hasselmo 2006; Hasselmo and McGaughy 2004; Mineur and Picciotto 2019), and by influencing tone-fear association and extinction (Jiang et al. 2016; Wilson and Fadel 2017). However, the role of specific cholinergic receptor subclasses in these process remains is not fully understood.

Acetylcholine signals though two broad classes of cholinergic receptors: muscarinic (mAChRs), which are coupled to either Gi (M2 and M4 subtypes) or Gq (M1, 2, 3) G-proteins (Kruse et al. 2014), and nicotinic (nAChRs) which contain an intrinsic cation channel gated by ACh and generally depolarize the neurons on which they are expressed (Albuquerque et al. 2009). Pharmacological and genetic studies have identified multiple roles for mAChR and nAChR subtypes in the brain. In the hippocampus, M1 mAChRs contribute to cognitive behaviors, such as memory and matching-to-sample problem solving, and engage NMDA receptor signaling (Wess et al., 2007(Anagnostaras et al. 2003). The role of hippocampal M1 receptor signaling in responses to stressful stimuli is still debated, but muscarinic agonists can impair cued and contextual fear conditioning, whereas antagonists can enhance it (for a review see (Wilson and Fadel 2017). Nicotinic agonists are generally thought to augment contextual fear learning (Davis et al. 2006; Gould and Higgins 2003; Gould and Lommock 2003; Kutlu et al. 2016). Lotfipour and colleagues have shown that genetic deletion of α2-containing (α2*) nAChRS in mice impairs fear learning (Lotfipour et al. 2017). While α2 AChRs are expressed at high levels in the interpeduncular nucleus, the effect of constitutive knock out of this subtype on fear conditioning may be specific to their expression in GABAergic interneurons of the *oriens lacunosum moleculare* (OLM) in the CA1 region of the hippocampus (Leao et al. 2012). Stress-induced ACh release engages the hippocampal network by stimulating activity of OLM neurons, which in turn, decreases activity of CA1 pyramidal neurons (Haam et al. 2018). Consistent with this, constitutive knockout of the α2* nAChR subunit prevents nicotine-induced LTP in the CA1 area of the mouse hippocampus (Lotfipour et al. 2017). Thus, α2* nAChRs appear to be ideally located to modulate hippocampal activity in response to stress-induced ACh signaling.

In the current study, we aimed to determine whether M1 mAChRS and α2* nAChRs, two predominant hippocampal cholinergic receptors implicated in modulation of cognitive and emotional behaviors, could contribute to stress-related behaviors and fear learning at baseline and in response to a pharmacological challenge that increases ACh signaling.

METHODS

Animals

Adult male C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Animals were maintained in a controlled environment with a 12-hour light/dark cycle (lights on at 7:00 am) at 22°C± 2°C and housed 5 per cage. All testing occurred during the light phase of the cycle. Food and water were accessible *ad libitum*. Animals were between

10 and 23 weeks of age at the beginning of the study. A total of 43 animals were used and cages were randomly assigned to 3 different groups: scrambled shRNA (control), α2 nAChR knockdown (KD), or M1 mAChR KD.

Since the social defeat test was used as one of the primary outcome measures of stress sensitivity in this study, only male mice were used in these experiments.

shRNA design

Unique short hairpin RNAs (shRNAs) targeting either the mRNA encoding the M1 mAChR (Chrm1) or the α2 nAChR subunit (Chrna2) were designed, constructed and validated using previously published methods (Mineur et al. 2011; Hommel et al. 2003). Briefly, each shRNA was designed either against the mRNA encoding *Chrm1* (GenBank accession NM_001112697.1): 5' to 3': 1 GCAACGCCTCTGTCATGAATCTTC; or against

Chrna2 (GenBank accession NM_144803.2): 5' to 3':

GATCTACCCCGACGTCACCTACTA. All sequences were checked by BLAST to ensure that no other genomic sequences were targeted. shRNAs were then ligated into a pAAV-EGFP-shRNA recombinant vector according to previously described protocols (Benavides et al. 2007; Hommel et al. 2003). Positive clones were identified by PCR and DNA sequencing. Control shRNAs were designed with a scrambled targeting sequence that has no known targets in the *Mus Musculus* genome.

Viral production

Adeno-associated virus 2 (AAV2) production was performed by transfecting Human Embryonic Kidney (HEK) 239 cells with 135 μg each of pAAV-shRNA, pHelper and pAAV-RC plasmids using the standard calcium phosphate method as described previously (Mineur et al. 2011; Hommel et al. 2003; Zolotukhin et al. 1999). 72 hr post transfection, cells were recovered, pelleted and re-suspended in freezing buffer (0.15 M NaCl and 50 mM Tris, pH 8.0), and lysed by repeating 3 freeze-thaw cycles and incubated with Benzonase (50 U/ml final; to remove nucleic acids) for 30 min at 37° C. Cell residues were removed by centrifugation at 37000 g for > 20 min. The clarified lysate was then collected and added to a centrifuge tube containing a 15%, 25%, 40% and 60% iodixanol gradient and centrifuged at 50,000g for 200 min at 10°C. The 40% fraction was extracted, diluted in PBS-MK (1X PBS, 1mM MgCl2 ,2.5 mM KCl), concentrated and purified with a Centricon Plus-20 100K filter column.

Stereotaxic surgeries

Surgeries were performed in a stereotaxic frame (KOPF Instruments, CA, USA) under isoflurane anesthesia (Isoflo® USP, Zoetis, NJ, USA), starting at 4% and maintained at 2%. The breathing of the animals was monitored and reactions to noxious stimuli were checked every 2-minutes. Surgery bilaterally targeted the hippocampus by using the following coordinates from Bregma: anterior/posterior: −1.8 mm, lateral ± 1.2 mm, dorsal/ventral −3.0 mm. These coordinates were based on previous studies showing that viral manipulations of acetylcholinesterase and α7 nAChRs in this hippocampal region alter stress related behaviors (Mineur et al. 2018b; Mineur et al. 2013). Using a 5 μL syringe (Hamilton, Reno,

NV, USA), 1.5 μL of AAV virus (scramble, or α2 KD, or M1 KD) was bilaterally injected into the hippocampus with an infusion rate of 0.2 μL/min. Needle was then kept in place for an additional 6 min to reduce the risk of backflow upon syringe removal. The syringe was lowered an additional 0.1 mm before removal. Mice were marked by ear punches under anesthesia during surgery to prevent additional stress. Postoperative monitoring was performed the following 1 days and animals were then left to recover for at least 5 weeks to allow shRNA expression and target mRNA knockdown (Fig.1A).

Drug injection

Mouse were injected i.p. 30 minutes before each test with 0.15 mg/kg physostigmine (Sigma) mixed at 1.5 mg/ml in PBS.

Behavioral assays

Animals were tested in a battery of behavioral tests sensitive to stress in the order described below.

Light/Dark Box Test—Mice were tested in a 36.5 x 10 x 16 cm box consisting of two opaque Plexiglas compartments with a grid bottom. A 100 W desk lamp was used to illuminate the light side compartment through a transparent Plexiglas cover. The dark side was covered by a black, opaque lid. Test subjects were put in the light compartment facing the corner and could freely cross between the two compartments through a small opening. A crossing was defined as having all four paws in same compartment. Animals were tested for 6 min after first crossing into the dark compartment. Time until first crossing, time spent in light side and number of crossings into light side were measured.

Tail Suspension Test—A paperclip was attached to the tip of the tail (around 4mm) with strong adhesive tape and mice were suspended and recorded for 6 min. An entire cage was tested at one time and the individual mice could not see each other while suspended. Testing was done with lights on, and time immobile and number of bouts were scored.

Forced swim test—2.5 L of water was put in 5L cylindrical beakers around 2 hours prior to testing to let water adjust to room temperature. Animals were put in the testing room 30 min prior to testing. Regular wildtype mice were placed in each beaker for 15 min (day 1) and 5 min (day 2) prior to test subjects in order to provide similar scent cues for all experiments. Animals were carefully put in water with precautions taken to not put nose or ears under water. Time spent immobile and number of bouts immobile were measured.

Marble Burying Test—Animals were left to acclimate for 1 hr in a dimly lit room prior to testing. Regular wildtype mice were put in 29 x 23 x 15 cm plastic boxes with 4 cm new bedding and left to explore for 40 min to provide uniform scent cues for all subjects. 20 dark blue marbles (1 cm diameter) were arranged in a 5x4 grid on top of the bedding. Each mouse was placed in a corner of the box and lid was put on. Animals were tested for 40 min. Marbles were considered buried if 75% or more was covered. After each test, bedding was flattened, and marbles were rearranged in the same grid.

Novelty Suppressed Feeding Test—Animals were weighed and placed in new cages without food in order to prevent them from eating any hidden pieces of food. The goal was to reduce the mice to 85% of their free feeding body weight. Mice were re-weighed after 36-38 h and placed in a dimly-lit testing room for 30 min prior to testing. The tested mouse was placed in a 47 x 46 x 21 cm testing box with 2 cm fresh bedding during the acclimation period. Mice were then individually tested by lowering them gently into a corner of the box with a small piece of food placed on a round piece of white paper in the middle of the box. Time until mice started to bite into the food was recorded. Mice were then removed and placed in a holding tank until the entire cage had been tested. Mice were excluded if they did not initiate feeding within 6 min. After the test, each animal was placed one at a time in their home cage for 5 min with a pre-weighed piece of chow to confirm that they were interested in food. The piece of food was weighed again, and amount of chow eaten in the home cage was recorded.

Social Defeat Paradigm—Male single-housed CD1 mice (retired breeders) were kept in their home cage with bedding unchanged for 2 weeks and screened for aggression. The test subjects were introduced to the aggressor in the CD1 home cage. After the first attack, the two mice were separated by a metal mesh, leaving about 90% of the box for the CD1 mouse. After 10 min, the test mice were put back into their home cage. Defeat was initiated twice a day for 4 days, with a new CD1 mouse for each tested subject each session. On the fifth day, social interaction (SI) was tested. Each test subject was allowed to explore a novel open field environment (38 x 35 x 21 cm) for 2.5 min after which, a novel CD1 mouse was put in a holding tank in one end of the box, and the test subject was left to explore for another 2.5 min. Interaction score was measured as a ratio between time spent in the interaction zone (5 cm around holding tank) with and without a CD1 mouse.

Cued and Contextual Fear Conditioning Test—The fear conditioning protocol was adapted from previously published studies and allows for detection of subtle alterations in hippocampal signaling (Mineur et al. 2007; Park et al. 2016; Phillips and LeDoux 1992; Fig. 4A). Mice were left to acclimate for 30 min in a dimly lit room before testing. Testing was performed in a 43 x 17 x 26 cm shuttle box (Med Associates, Inc., VT, USA) with a metal grid floor and clear Plexiglas walls and top. The bottom of the box was covered with plastic and home cage bedding was added. Walls were covered with paper and tape to introduce some visual cues. Habituation to the test chamber was performed in the morning of day 1. Animals were left in this area for 540 sec in a “safe” context (session 1) with home cage bedding. This session was used to habituate the animals to a safe environment and to limit contextual confounds during the cued recall phase (session 4). The box was then cleaned with water after each mouse. The training session was done in the afternoon on day 1 with an auditory cue (chime tone ~ 75 db) and in a specific “unsafe” context (session 2). Animals were put in the box with almond scent underneath the metal grid. Animals were tested for 540 sec with the following schedule using Med-PC IV software: 160 sec start - 20 sec tone cue with a 0.5 mA foot shock in the last 2 sec - 40 sec resting period - repeated 5 times - 60 sec resting period in the end. A total of 6 shocks were delivered during the training session. In the morning of day 2, contextual recall was assessed. Animals were put back in the grid-floor box (“unsafe context”) with a new scent (vanilla) and left for 340 sec (session 3). The

use of a different odor was used to avoid testing elemental memory (ie, memory of the experience of being tested) as opposed to a specific cued or contextual memory (Fitch et al. 2002). In the afternoon, the cued recall session was performed. Animals were put back in the same part of the box as in session 1 (“safe context”, with plastic bottom and home cage bedding) for 340 sec but with cues delivered as follows: 160 sec rest, 20 sec cue, 40 sec rest (repeated 3 times)(session 4). All 4 sessions were filmed as reference and freezing time was evaluated. During each cue on the training session (day 1), freezing was measured to assess the learning rate of the different groups. Freezing behavior in was measured during the entire context recall session, and only when cues were playing during the cued recall phase. Freezing was defined as no other movements than breathing.

Locomotor Activity—Animals were put in an open field enclosure (48 x 22 x 18 cm) for 20 min. Ambulatory activity was measured by the number of beam breaks (Accuscan), excluding repeated beam breaks due to stereotypic behavior.

Tissue processing and verification of viral infusion/placement

A subset of the animals used for validation of knockdown (see below). All other mice were anesthetized with 0.2 mL Fatal Plus® (Patterson Veterinary Supplies, Inc., Devens, MA, USA) and perfused intracardially with chilled 4% paraformaldehyde (PFA) for 5-10 min after which the brains were removed to determine the infusion site and degres of viral infection. Post-fixation and cryoprotection were done simultaneously by storing the brains in 30% sucrose in 4% PFA at 4°C before slicing with a Leica SM2000R sliding microtome (Leica Biosystems, Wetzlar, Germany) at 40 μm thickness (Mineur et al., 2017). Slices were kept in a solution of 0.02% sodium azide in PBS (pH 7.4) and were then mounted on slides (Superfrost Plus Microscope Slides, Fisherbrand, Thermo Fisher Scientific, Waltham, MA, USA) and examined through an Olympus Fluoview FV10i confocal microscope (Olympus), and infection was confirmed by the detection of green fluorescence in the hippocampus (Fig.1A).

Validation of Hippocampal mAChR M1 Knockdown

Confirmation of hippocampal M1 KD was done by analyzing a subset of animals (5 to 6 animals with confirmed infection) via immunohistochemistry and confocal microscopy (Fig.1B). The scrambled-shRNA infected mice were used as control. Three to four slices from each brain, selected from the dorsal region of the hippocampus where infection was highest, were used for immunostaining, following the same protocol as Wohleb et al. (2016). Slices were washed in PBS (pH 7.4) for 8 min on a shaker and then blocked (3% NDS/0.3% Triton/PBS) for 1 hour at room temperature on a shaker. Slices were washed for another 8 min and then incubated at 4°C overnight on a shaker with an M1 primary antibody (Frontier Institute; mAChR-M1- RB-Af340; 1:1000 dilution). The next day sections were washed and incubated with a conjugated secondary antibody (Alexafluor 647 goat anti-rabbit; 1:1000 dilution) overnight at 4°C on a shaker. Immunofluorescent images were then captured with an Olympus Fluoview confocal microscope scanning at 480 nm and 647 nm.

Three areas of similar surface area (250 μm^2) per slice, positive for infection as visualized by GFP expression, were quantified for integrated density of immunostaining of M1 by

Image J. Results were then averaged per slice and counted as an "n" of 1. Averaged integrated densities were then compared between the control (scramble) and knockdown animals.

Validation of hippocampal α2 nAChR Knockdown

Validation of hippocampal nAChR α2 knockdown was done by qPCR because specific α2 antibodies for immunocystochemistry are not available.

Several mice were heavily sedated with isoflurane (3 x α2 nAChR hippocampal knockdown and 2 x scramble) and quickly decapitated. The brains were quickly removed on ice, and the entire hippocampus was micro-dissected and put in small tubes on dry ice. The RNA was then extracted (RNeasy® Lipid Tissue kit, Qiagen). The RNA was then converted to complementary DNA (cDNA) by reverse transcription (QuantiTect ® Reverse Transcription Kit, Qiagen). The cDNA was diluted 1:6 and a qPCR protocol was run with each sample in triplicate using α2 primers. Primers selective for amplification of TATA-box binding protein (Tbp) were also run in triplicate and this housekeeping gene was used as a control because of its stable expression across conditions. The qPCR protocol was inspired from (Calarco et al. 2018; Mineur et al. 2018a; Wigestrand et al. 2011).

The samples were then processed with Sybr green (Invitrogen) following the manufacturer's recommendations. cDNA was then amplified with a StepOnePlus real-time PCR machine (Applied Biosystems) and cDNA concentration was quantified using $2^{-\Delta\Delta CT}$ as described by (Livak and Schmittgen 2001).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8 and Staview (SAS) softwares. Values are expressed as means ± standard error of the means (SEM). One-way analysis of variance (ANOVA) was used when comparing means between groups, or unpaired two-tailed t-test when comparing two main groups (control baseline vs. control physostigmine), and α was set at 95%. One-way ANOVA with repeated measures was used for fear conditioning learning rate on day 1. When ANOVA reached significance and equal variance was assumed (as defined by Brown-Forsythe test), individual knockdown groups were individually compared to one control group by Dunnett's posthoc tests.

RESULTS

Validation of M1 mAChR and α2 nAChR knockdown

Following analyses of immunofluorescence of brain slices expressing M1 knockdown in the hippocampus, results indicated a decrease of ~ 21% compared to control animals ($F(1,14) = 25.3$, $p = 0.002$). Of note, this number is likely underestimated in neurons, as AAV2 used to carry the shRNAs do not infect glial cells, which are also known to express M1 mAChRs.

For α2 nAChRs, results of the qPCR analyses indicated a decrease of 42.7 % (unpaired t-test from averaged triplicates from full hippocampi: $t(7) = 4.9$; $p = 0.018$); compared to control animals.

Effect of knockdown of M1 mAChRs or α2 nAChRs in the light/dark box and marble burying task.

Behavior in the light/dark box and marble burying task is sensitive to acute administration of anxiolytic drugs (Archer et al. 1987; Bourin and Hascoet 2003). In the light-dark test, there was no difference in the time spent in the light side in or the time to first cross among any of the groups (All F<1; Fig. 1C). As expected, decreasing ACh breakdown by administering physostigmine significantly decreased latency to cross, and decreased time spent in the light side of the light/dark box ($t(22) = 5.58$, $p = 0.022$), but neither M1 nor α2 KD had an effect on these parameters, even though ACh tone was increased (All F<1; Fig. 1D). In the marble burying task, neither M1 or α2 KD changed the number of marbles buried between groups, with or without physostigmine treatment (Fs<1; Fig. 1E and 1F, respectively), while physostigmine administration resulted in a significant decrease in marbles buried.

Effect of M1 mAChRs or α2 nAChRs in the hippocampus in the tail suspension test (TST) and forced swim test (FST).

Behavior in the TST and FST is sensitive to acute administration of antidepressant drugs (Porsolt et al. 1977; Steru et al. 1985). In the TST, both KD had no measurable effects at baseline or following physostigmine treatment (All F<1; Fig. 2A and 2B), although physostigmine increased time immobile, as expected ($t(28) = 2.812$, $p = 0.009$).

In the FST, a significant difference in the time spent immobile during the 15 min test was detected between groups on the first day of testing (ANOVA, $F(2, 39) = 5.01$; $p=0.012$; Fig.2C). Post-hoc analysis revealed that α2 knockdown in hippocampus induced a significant decrease in immobility time compared to the scrambled control animals ($t(28) = 3.08$, $p = 0.007$). In parallel, there was a significant decrease in the number of bouts of immobility (ANOVA, $F(3, 39) = 4.46$; $p = 0.018$), with *posthoc* analysis only reaching significance in α2 KD animals ($t(28) = 2.78$, $p = 0.015$, Fig. 2C). Physostigmine induced a significant increase in the time immobile on both days of the FST, but none of the effects of α2 KD were detected in animals that received physostigmine (ANOVA; F <1; Fig. 2D).

No differences were seen in the number of immobility bouts regardless of treatment group or physostigmine injection (All F<1).

Effects of hippocampal knockdown of M1 mAChRs or α2 nAChRs in novelty suppressed feeding and the social defeat paradigm.

Behavior in the novelty-suppressed feeding task and social interaction test after social defeat is sensitive to chronic administration of antidepressant drugs (Dulawa and Hen 2005). In the novelty suppressed feeding test, there was no difference in the latency to feed between the different treatment groups, (F<1; Fig. 3A). Following physostigmine injection, however, control animals trended towards a greater latency to initiate feeding but this did not quite reach significance (ANOVA, $F (2, 33) = 3.16$, $p = 0.056$) (Fig. 3B).

Modest weight loss was observed after food restriction in animals with the hippocampal M1 KD (% body weight from free-feeding body weight: Scr = 87.65% +/− 0.2449; M1KD = 85.36 +/− 0.6806; α2KD = 86.87 +/− 0.3408; ANOVA $F (2, 38) = 7.137$, $p = 0.003$ with

posthoc t(23) = 3.78, p = 0.001 for M1 KD vs control at baseline and t(21) = 3.12, p = 0.076 following physostigmine administration). However, no differences were seen between groups in the overall amount eaten in home cage in either test (ANOVA with F <1 at baseline and after physostigmine), despite a significant effect of physostigmine (t25) = 3.5, p = 0.002).

In the social defeat paradigm, no differences were observed in the social interaction ratio regardless of hippocampal α2 nAChR or M1 AChR KD, acute physostigmine injection alone, or KD following physostigmine injection (All F<1; Fig. 3C and 3D).

Effects of hippocampal α2 nAChR and M1 mAChR KD in cued and contextual fear conditioning

Behavior in the fear conditioning test is sensitive to muscarinic antagonists and constitutive knock out of α2 nAChRs (Anagnostaras et al. 1999; Gale et al. 2001; Lotfipour et al. 2017). Freezing time was recorded during each of the six cues during training session on day 1, to evaluate learning rate. All animals learned the task at a similar rate as measured by rate of freezing behavior over time (repeated-measures ANOVA, F(5, 205) = 75.5, p < 0.0001), with no effect of α2 nAChR or M1 mAChR KD (two-way repeated-measures ANOVA, F(10, 195) = 1.66, p = 0.093; Fig. 4B).

Contextual (hippocampal-dependent) recall was not different between groups on day 2 (F<1, Fig. 4C); however, following physostigmine injection one hour before testing, freezing time was somewhat increased in the training context, but it did not reach significance in either group; Fig. 4D).

In response to the shock-paired cue, a significant decrease in freezing time was observed (ANOVA F (2, 40) = 5.84, p = 0.006) in both the M1 KD (t(28) = 3.32, p = 0.004) and the α2 KD (t(28) = 2.3, p = 0.005) groups when compared to the scrambled control animals (Fig. 4E). Physostigmine had no effects on freezing on its own, however, following physostigmine treatment, no difference was observed in the KD groups compared to control animals (ANOVA F (2, 35) = 1.14, p = 0.331, Fig. 4F).

DISCUSSION

Elevated ACh signaling in hippocampus as a result of local blockade of its degradation can increase behaviors induced by stress (Mineur et al, 2013). This study therefore investigated whether M1 mAChRs and α2* nAChRs are important for mediating the effects of hippocampal ACh on stress-induced behaviors and fear learning at baseline and following elevation of ACh signaling. Consistent with previous studies, administration of the cholinesterase antagonist physostigmine increased behavioral responses to stress across a number of paradigms. These studies show that hippocampal α2 nAChRs and M1 mAChRs are not responsible for effects of increased ACh tone on behaviors sensitive to anxiolytics, but contribute to a subset of behaviors sensitive to acute antidepressant administration in rodents, as well as to recall of a shock-paired cue after fear conditioning.

In the hippocampus, α2* nAChRs are expressed exclusively on OLM neurons, a group of cells involved in negative feedback to the CA1 output neurons. These neurons are responsive to the elevation of acetylcholine induced by challenging conditions, and are activated during risk taking behavior (Leao et al. 2012; Mikulovic et al. 2018). Conversely, M1 mAChRs are the most highly expressed muscarinic receptors in the hippocampus (Levey et al. 1991) and are involved in fear conditioning (Wilson and Fadel 2017) and learning tasks (Anagnostaras et al. 2003).

The greatest effects of hippocampal α2 and M1 knock down were observed in the fear conditioning test. Although freezing during the learning phase and in the context paired with shock was unaltered by hippocampal α2 or M1 KD, both groups showed a robust decrease in freezing to the shock-paired cue. These effects were not observed following physostigmine administration, suggesting that increasing ACh tone overcomes the effects of decreasing the number of α2* or M1 receptors in the hippocampus. The selective effects of hippocampal α2 and M1 knock down on cued fear conditioning were somewhat surprising because modulating hippocampal signaling is thought to affect contextual, rather than cued, fear conditioning, however responses to shock-paired cues also require hippocampal function (Phillips and LeDoux 1992). One possible explanation is that, whereas dorsal hippocampus is known to be critical for contextual fear conditioning and spatial information rather than emotional valence (Zelikowsky et al. 2014), the ventral hippocampus is thought to be involved in fear and anxiety aspects of fear conditioning, without any effects on contextual memory (Kheirbek et al. 2013). Due to extensive spread of viral infection across hippocampal subfields once the capsule of the hippocampus is pierced (Fig. 1A), even though our target infusion site was the dorsal hippocampus, it is clear that infection included parts of ventral hippocampus as well. We therefore cannot make strong conclusions about selective cholinergic effects on dorsal or ventral aspects of the structure.

Notably, M1 and M3 constitutive knock out mice show specific deficits in contextual fear conditioning (Miyakawa et al. 2001; Patricio et al. 2017). Similarly, scopolamine impairs contextual fear conditioning, and it has been suggested that muscarinic receptors are critical mediators of the contextual fear response (Wilson and Fadel 2017). Hippocampal injections of scopolamine, nicotine, or nicotinic antagonists, all fail to alter cued fear conditioning (Wilson and Fadel 2017). Similarly, whereas contextual freezing is unaffected at baseline in α2 nAChR knock out (α2KO), and male mice do not show a significant change in cued freezing after fear conditioning, they do show a deficit in contextual fear learning (Lotfipour et al. 2013); notably, the deficit in contextual fear learning in these knockout mice could not be rescued with nicotine administration (Lotfipour et al. 2017).

The observation that both M1 mAChR and the α2 nAChR hippocampal knockdown decreased freezing to a shock-paired tone suggests that ACh signaling converging on activity of OLM neurons may be involved in this response. Cued fear learning requires intact amygdala signaling and plasticity (Phillips and LeDoux 1992), but the hippocampus also contributes to cue-fear learning (LeDoux 2003). M1 mAChRs are expressed both pre- and post-synaptically in multiple hippocampal subfields and cell types, and could alter the activity of hippocampal projections, including to the amygdala. Importantly, hippocampal knockdown decreases M1 mAChR expression both in cell bodies of hippocampal neurons

and in their synaptic projections outside the hippocampus, suggesting that the effects of M1 knockdown in hippocampus on cued fear conditioning may be mediated through terminals in amygdala or other brain areas. In contrast, hippocampal knock down of α2* nAChRs would likely decrease the activity of local OLM interneuronal networks, which could indirectly regulate activity of the amygdala. Indeed, activity of hippocampal OLM neurons in CA1 facilitates cued fear association as mice with hypersensitive α2* nAChRs show greater fear learning and increased CA1 plasticity (Lotfipour et al. 2017). Injection of the M1/M3 agonist cevimeline into the basolateral amygdala (BLA) after cued fear conditioning decreased cued fear recall, but a decrease in cued fear conditioning was not seen in constitutive M1 knock out mice (Young and Thomas 2014).

It is possible that the differences observed between published constitutive knock out and pharmacological studies and what we report here is due to the fact that delivery of shRNAs into the hippocampus in adult mice decreases, but not does not eliminate, target protein expression. Further, constitutive knockout mice lack expression of the target gene in all brain areas and tissues, whereas in the current study knock down only occurred in the hippocampus. M1 mAChRs are expressed throughout the brain and body, and the α2 nAChR subunit is expressed at high levels in the interpeduncular nucleus, which is also involved in behavioral responses to stress. Also, constitutive knock out mice lack the target protein throughout development, and compensatory changes in gene expression (Tian et al. 2011; Wigestrand et al. 2011), or adaptative changes in circuitry regulated by ACh may occur (Kleeman et al. 2016) that reflect an important role for ACh signaling through these receptors during development. Finally, details of the fear learning paradigm used could also explain the differences between the current results and those seen in α2KO mice. In this study we pre-exposed mice to the test setting and the training protocol involved 6 CS-US pairings. Conversely, experiments in α2KO mice did not involve a pre-exposure session and only used 2 CS-US pairings (Lotfipour et al. 2013). Thus, it is possible that differences in cued fear conditioning were observed here because animals received more training, resulting in detection of more subtle effects of α2 nAChR knockdown on cued fear memory recall. Thus, these studies taken together suggest a role of ACh signaling through multiple receptor subtypes, and different aspects of fear conditioning are likely to be affected depending on the specific method of receptor manipulation and the paradigms used.

In line with previous studies, administration of physostigmine increased anxiety-like behaviors measured in the light/dark box and the marble burying tests (Mineur et al. 2013); however, neither hippocampal α2 nAChR or M1 mAChR knockdown had effects in these assays, in line with published work demonstrating that hippocampal knock down of the α7 nAChR subtype had no effect on behaviors sensitive to anxiolytic treatment at baseline or following physostigmine administration (Mineur et al., 2017). Thus, ACh signaling in the hippocampus through these nAChR and mAChR subtypes does not appear to be essential for anxiety-like behaviors in male mice. Studies in other rodent models are consistent with this interpretation. In rats, intrahippocampal scopolamine injections did not affect the overall number of crossings or time spent in light side in the light/dark box, although muscarinic blockade did decrease time to the first crossing into the light side (Smythe et al., 1998). Similarly, constitutive M1 KO mice did not show significant changes in behavior in the light/dark box (Miyakawa et al. 2001). In mice, intraperitoneal administration of

dicyclomine (an M1 mAChR selective antagonist) decreased the number of buried marbles at doses starting at 2 mg/kg (Veeraragavan et al., 2011), unlike what we observed with hippocampal M1 KD in the hippocampus, suggesting that M1 receptors outside the hippocampus likely underlie these behavioral changes.

No significant differences were observed between groups in the tail suspension test, although it is worth noting that a wide distribution of the data was observed at baseline, limiting statistical power. Following physostigmine injections, less variability was observed and mice showed more immobility overall, as was previously observed (Mineur et al. 2013), but hippocampal α2 nAChR or M1 mAChR knock down had no effects at baseline or following AChE antagonism. In contrast, in the forced swim test, a significant decrease in time spent immobile was observed following hippocampal α2* nAChR knock down, as was seen at a trend level following knock down of the α7 nAChR subunit in the hippocampus (Mineur et al. (2017). Thus, multiple nAChR subtypes in the hippocampus appear to contribute to regulation of immobility in response to inescapable stress in the forced swim test. Contrary to the widespread distribution of α7 nAChRs in the hippocampus, α2 nAChRs are almost exclusively located on GABAergic OLM interneurons. Thus, it is possible that decreasing ACh signaling through α2 nAChRs, and by extension, decreasing activity of OLM neurons, could disrupt the inhibitory/excitatory balance in the hippocampus and/or disrupt feedback to CA1 during forced swim. Blocking activity of OLM neurons may therefore improve the reactivity to stress induced by forced swim. In contrast to what was observed with hippocampal α7 KD, knocking down α2 nAChR did not prevent the increased immobility induced by physostigmine. As mentioned above, α7 nAChRs are expressed widely throughout the hippocampus, and also mediate the majority of cholinergic signaling in OLM neurons (Leao et al. 2012; Muller and Remy 2014). Thus, α2* nAChRs appear to have greater effect at baseline and only a limited role when ACh levels are increased and additional nAChR subtypes are engaged.

Behavior in the novelty suppressed feeding test is responsive to both chronic antidepressant, and acute anxiolytic, administration, whereas the social defeat test is only sensitive to chronic antidepressant treatment. Hippocampal α2 KD or M1KD did not alter baseline behavior in these tasks or the response to physostigmine significantly. The current data do not suggest a major role for hippocampal α2* nAChRs or M1 mAChRs in the effects of physostigmine on social defeat behavior.

Together with previous studies, the results shown here demonstrate that multiple receptor subtypes are involved in mediating effects of hippocampal ACh signaling on stress-induced behaviors. Thus, modulating ACh signaling with more specific pharmacological compounds might prevent aberrant effects of stress, such as cued-induced fear learning in post-traumatic stress disorder, while preserving critical functions of ACh, such as hippocampus-dependent learning.

ACKNOWLEDGEMENTS

This study was supported by grants MH077681, AA027989 and DA036151 from the National Institutes of Health.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

REFERENCES

Albuquerque EX, Pereira EF, Alkondon M, Rogers SW (2009) Mammalian nicotinic acetylcholine receptors: from structure to function. Physiol Rev 89: 73–120. [PubMed: 19126755]

Anagnostaras SG, Maren S, Sage JR, Goodrich S, Fanselow MS (1999) Scopolamine and Pavlovian fear conditioning in rats: dose-effect analysis. Neuropsychopharmacology 21: 731–44. [PubMed: 10633479]

Anagnostaras SG, Murphy GG, Hamilton SE, Mitchell SL, Rahnama NP, Nathanson NM, Silva AJ (2003) Selective cognitive dysfunction in acetylcholine M1 muscarinic receptor mutant mice. Nature neuroscience 6: 51–8. [PubMed: 12483218]

Archer T, Fredriksson A, Lewander T, Soderberg U (1987) Marble burying and spontaneous motor activity in mice: interactions over days and the effect of diazepam. Scand J Psychol 28: 242–9. [PubMed: 3441771]

Bourin M, Hascoet M (2003) The mouse light/dark box test. European journal of pharmacology 463: 55–65. [PubMed: 12600702]

Calarco CA, Li Z, Taylor SR, Lee S, Zhou W, Friedman JM, Mineur YS, Gotti C, Picciotto MR (2018) Molecular and cellular characterization of nicotinic acetylcholine receptor subtypes in the arcuate nucleus of the mouse hypothalamus. The European journal of neuroscience.

Davis JA, Porter J, Gould TJ (2006) Nicotine enhances both foreground and background contextual fear conditioning. Neuroscience letters 394: 202–5. [PubMed: 16260086]

Dulawa SC, Hen R (2005) Recent advances in animal models of chronic antidepressant effects: the novelty-induced hypophagia test. Neurosci Biobehav Rev 29: 771–83. [PubMed: 15890403]

Fitch T, Adams B, Chaney S, Gerlai R (2002) Force transducer-based movement detection in fear conditioning in mice: a comparative analysis. Hippocampus 12: 4–17. [PubMed: 11918287]

Gale GD, Anagnostaras SG, Fanselow MS (2001) Cholinergic modulation of pavlovian fear conditioning: effects of intrahippocampal scopolamine infusion. Hippocampus 11: 371–6. [PubMed: 11530841]

Gould TJ, Higgins JS (2003) Nicotine enhances contextual fear conditioning in C57BL/6J mice at 1 and 7 days post-training. Neurobiol Learn Mem 80: 147–57. [PubMed: 12932430]

Gould TJ, Lommock JA (2003) Nicotine enhances contextual fear conditioning and ameliorates ethanol-induced deficits in contextual fear conditioning. Behav Neurosci 117: 1276–82. [PubMed: 14674846]

Haam J, Zhou J, Cui G, Yakel JL (2018) Septal cholinergic neurons gate hippocampal output to entorhinal cortex via oriens lacunosum moleculare interneurons. Proceedings of the National Academy of Sciences of the United States of America 115: E1886–E1895. [PubMed: 29437952]

Hasselmo ME (2006) The role of acetylcholine in learning and memory. Curr Opin Neurobiol 16: 710–5. [PubMed: 17011181]

Hasselmo ME, McGaughy J (2004) High acetylcholine levels set circuit dynamics for attention and encoding and low acetylcholine levels set dynamics for consolidation. Prog Brain Res 145: 207–31. [PubMed: 14650918]

Jiang L, Kundu S, Lederman JD, Lopez-Hernandez GY, Ballinger EC, Wang S, Talmage DA, Role LW (2016) Cholinergic Signaling Controls Conditioned Fear Behaviors and Enhances Plasticity of Cortical-Amygdala Circuits. Neuron 90: 1057–70. [PubMed: 27161525]

Kheirbek MA, Drew LJ, Burghardt NS, Costantini DO, Tannenholz L, Ahmari SE, Zeng H, Fenton AA, Hen R (2013) Differential control of learning and anxiety along the dorsoventral axis of the dentate gyrus. Neuron 77: 955–68. [PubMed: 23473324]

Kleeman E, Nakauchi S, Su H, Dang R, Wood MA, Sumikawa K (2016) Impaired function of alpha2-containing nicotinic acetylcholine receptors on oriens-lacunosum moleculare cells causes

hippocampus-dependent memory impairments. Neurobiol Learn Mem 136: 13–20. [PubMed: 27660076]

Kruse AC, Kobilka BK, Gautam D, Sexton PM, Christopoulos A, Wess J (2014) Muscarinic acetylcholine receptors: novel opportunities for drug development. Nat Rev Drug Discov 13: 549–60. [PubMed: 24903776]

Kutlu MG, Tumolo JM, Holliday E, Garrett B, Gould TJ (2016) Acute nicotine enhances spontaneous recovery of contextual fear and changes c-fos early gene expression in infralimbic cortex, hippocampus, and amygdala. Learn Mem 23: 405–14. [PubMed: 27421892]

Leao RN, Mikulovic S, Leao KE, Munguba H, Gezelius H, Enjin A, Patra K, Eriksson A, Loew LM, Tort AB, Kullander K (2012) OLM interneurons differentially modulate CA3 and entorhinal inputs to hippocampal CA1 neurons. Nature neuroscience 15: 1524–30. [PubMed: 23042082]

LeDoux J (2003) The emotional brain, fear, and the amygdala. Cellular and molecular neurobiology 23: 727–38. [PubMed: 14514027]

Levey AI, Kitt CA, Simonds WF, Price DL, Brann MR (1991) Identification and localization of muscarinic acetylcholine receptor proteins in brain with subtype-specific antibodies. J Neurosci 11: 3218–26. [PubMed: 1941081]

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402–8. [PubMed: 11846609]

Lotfipour S, Byun JS, Leach P, Fowler CD, Murphy NP, Kenny PJ, Gould TJ, Boulter J (2013) Targeted deletion of the mouse alpha2 nicotinic acetylcholine receptor subunit gene (Chrna2) potentiates nicotine-modulated behaviors. J Neurosci 33: 7728–41. [PubMed: 23637165]

Lotfipour S, Mojica C, Nakauchi S, Lipovsek M, Silverstein S, Cushman J, Tirtorahardjo J, Poulos A, Elgoyhen AB, Sumikawa K, Fanselow MS, Boulter J (2017) alpha2* Nicotinic acetylcholine receptors influence hippocampus-dependent learning and memory in adolescent mice. Learn Mem 24: 231–244. [PubMed: 28507032]

Maren S, Holmes A (2016) Stress and Fear Extinction. Neuropsychopharmacology 41: 58–79. [PubMed: 26105142]

Mikulovic S, Restrepo CE, Siwani S, Bauer P, Pupe S, Tort ABL, Kullander K, Leao RN (2018) Ventral hippocampal OLM cells control type 2 theta oscillations and response to predator odor. Nat Commun 9: 3638. [PubMed: 30194386]

Mineur YS, Cahuzac EL, Mose TN, Bentham MP, Plantenga ME, Thompson DC, Picciotto MR (2018a) Interaction between noradrenergic and cholinergic signaling in amygdala regulates anxiety- and depression-related behaviors in mice. Neuropsychopharmacology.

Mineur YS, Mose TN, Blakeman S, Picciotto MR (2018b) Hippocampal alpha7 nicotinic ACh receptors contribute to modulation of depression-like behaviour in C57BL/6J mice. British journal of pharmacology 175: 1903–1914. [PubMed: 28264149]

Mineur YS, Obayemi A, Wigestrand MB, Fote GM, Calarco CA, Li AM, Picciotto MR (2013) Cholinergic signaling in the hippocampus regulates social stress resilience and anxiety- and depression-like behavior. Proceedings of the National Academy of Sciences of the United States of America 110: 3573–8. [PubMed: 23401542]

Mineur YS, Picciotto MR (2019) The role of acetylcholine in negative encoding bias: Too much of a good thing? The European journal of neuroscience.

Miyakawa T, Yamada M, Duttaroy A, Wess J (2001) Hyperactivity and intact hippocampus-dependent learning in mice lacking the M1 muscarinic acetylcholine receptor. J Neurosci 21: 5239–50. [PubMed: 11438599]

Muller C, Remy S (2014) Dendritic inhibition mediated by O-LM and bistratified interneurons in the hippocampus. Frontiers in synaptic neuroscience 6: 23. [PubMed: 25324774]

Patricio RR, Soares JC, Oliveira MG (2017) M1 muscarinic receptors are necessary for retrieval of remote context fear memory. Physiology & behavior 169: 202–207. [PubMed: 27940145]

Paul S, Jeon WK, Bizon JL, Han JS (2015) Interaction of basal forebrain cholinergic neurons with the glucocorticoid system in stress regulation and cognitive impairment. Front Aging Neurosci 7: 43. [PubMed: 25883567]

Phillips RG, LeDoux JE (1992) Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. Behav Neurosci 106: 274–85. [PubMed: 1590953]

Porsolt RD, Le Pichon M, Jalfre M (1977) Depression: a new animal model sensitive to antidepressant treatments. Nature 266: 730–2. [PubMed: 559941]

Raio CM, Phelps EA (2015) The influence of acute stress on the regulation of conditioned fear. Neurobiology of stress 1: 134–46. [PubMed: 25530986]

Risch SC, Cohen RM, Janowsky DS, Kalin NH, Sitaram N, Gillin JC, Murphy DL (1981) Physostigmine induction of depressive symptomatology in normal human subjects. Psychiatry research 4: 89–94. [PubMed: 7012883]

Steru L, Chermat R, Thierry B, Simon P (1985) The tail suspension test: a new method for screening antidepressants in mice. Psychopharmacology 85: 367–70. [PubMed: 3923523]

Tian MK, Bailey CD, De Biasi M, Picciotto MR, Lambe EK (2011) Plasticity of prefrontal attention circuitry: upregulated muscarinic excitability in response to decreased nicotinic signaling following deletion of alpha5 or beta2 subunits. J Neurosci 31: 16458–63. [PubMed: 22072695]

Wigestrand MB, Mineur YS, Heath CJ, Fonnum F, Picciotto MR, Walaas SI (2011) Decreased alpha4beta2 nicotinic receptor number in the absence of mRNA changes suggests post-transcriptional regulation in the spontaneously hypertensive rat model of ADHD. Journal of neurochemistry 119: 240–50. [PubMed: 21824140]

Wilson MA, Fadel JR (2017) Cholinergic regulation of fear learning and extinction. J Neurosci Res 95: 836–852. [PubMed: 27704595]

Young MB, Thomas SA (2014) M1-muscarinic receptors promote fear memory consolidation via phospholipase C and the M-current. J Neurosci 34: 1570–8. [PubMed: 24478341]

Zelikowsky M, Hersman S, Chawla MK, Barnes CA, Fanselow MS (2014) Neuronal ensembles in amygdala, hippocampus, and prefrontal cortex track differential components of contextual fear. J Neurosci 34: 8462–6. [PubMed: 24948801]

Key Finding:

Fig. 4. Knock down of M1 muscarinic or α2 nicotinic ACh receptors in hippocampus impairs cued fear conditioning.

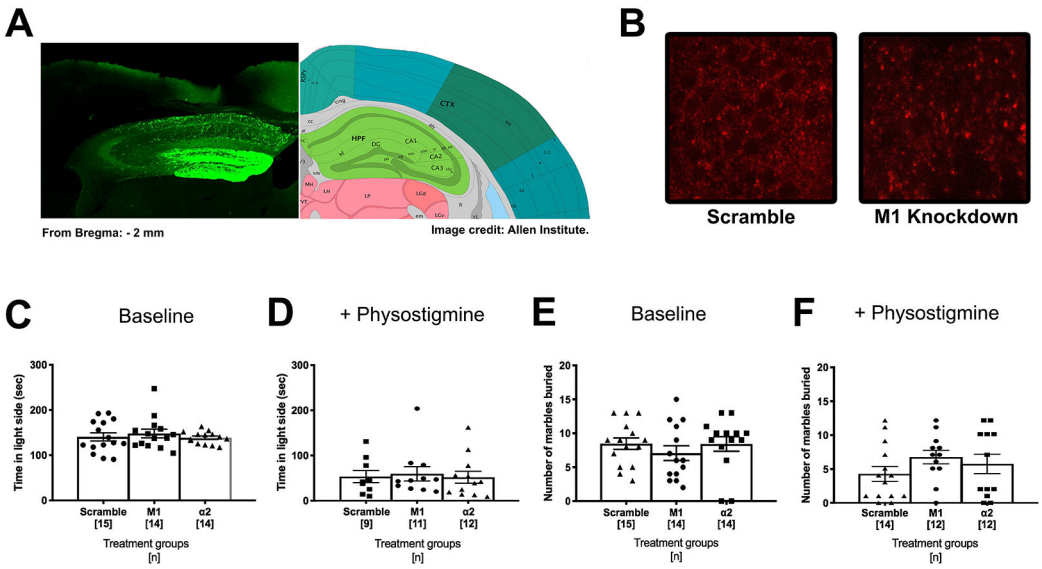

Figure 1:

Demonstration of hippocampal infection, M1 knock down, and effect of hippocampal M1 mAChR and α2 nAChR KD in the light-dark box and marble burying task. **A)** Representative image of viral infection in hippocampus. **B)** Representative images of anti-M1 mAChR immunohistochemistry following infusion of either the scrambled shRNA construct (left) or the vector carrying the shRNA targeting the M1 mAChR into the hippocampus (right). Time spent in the light side of the box in the Light/Dark Test **C)** at baseline or **D)** following physostigmine treatment. Number of marbles buried in the Marble Burying Test **E)** at baseline or **F)** following physostigmine treatment. [n] represents the number of animals per treatment group. Scatterplots represent independent data points for each animal. Data are represented as means +/− SEM.

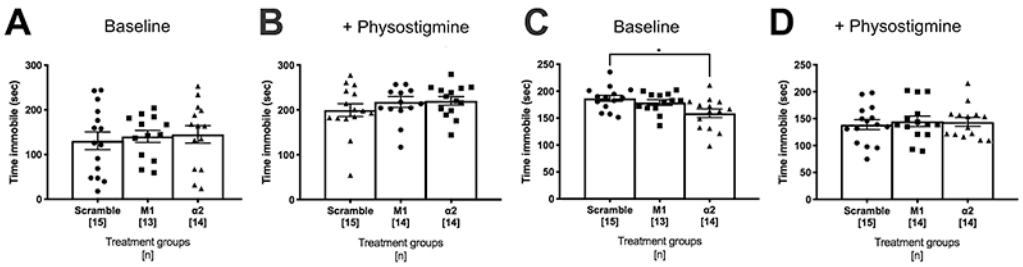

Figure 2:

Effect of hippocampal M1 mAChR and α2 nAChR KD in the tail suspension and forced swim tests. Time spent immobile in the Tail Suspension Test **A)** at baseline or **B)** following physostigmine treatment. Time spent immobile in the Forced Swim Test **C)** at baseline or **D)** following physostigmine treatment. [n] represents the number of animals per treatment group. Scatterplots represent independent data points for each animal. Data are represented as means +/− SEM. * $p < 0.05$.

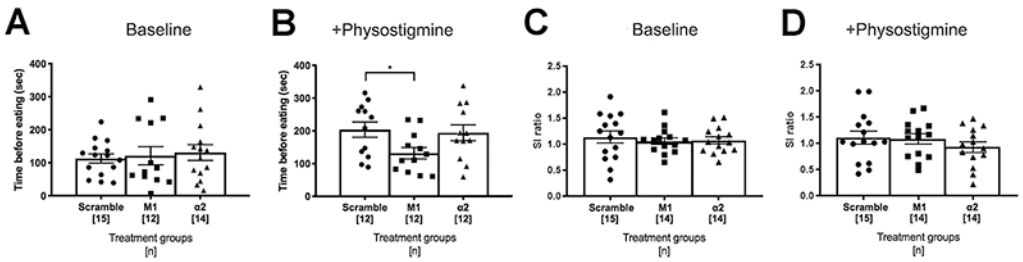

Figure 3:

Effect of hippocampal M1 mAChR and α2 nAChR KD in novelty suppressed feeding and social defeat. Time before first feeding episode in the Novelty Suppressed Feeding Test **A)** at baseline or **B)** following physostigmine treatment. Interaction ratio in the Social Interaction (SI) Test **C)** at baseline or **D)** following physostigmine treatment. [n] represents the number of animals per treatment group. Scatterplots represent independent data points for each animal. Data are represented as means +/− SEM. * $p < 0.05$.

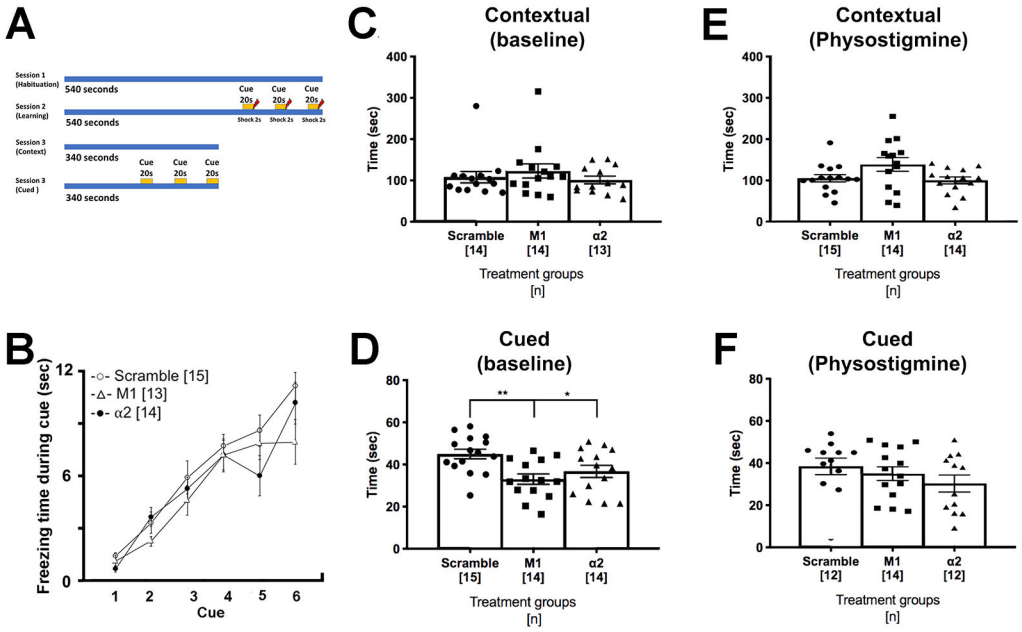

Figure 4:

Effects of hippocampal M1 mAChR and α2 nAChR KD on behavior in a cued and contextual fear conditioning task. **A)** Depiction of the different sessions in the cued and contextual fear conditioning paradigm used here. **B)** Freezing behavior over 6 consecutive 20-sec cues co-terminated by 2-sec shock (learning rate). Time spent freezing in the shock-paired context **C)** at baseline or **D)** following physostigmine treatment. Time spent freezing in response to the shock-paired cue **E)** at baseline or **F)** following physostigmine treatment. [n] represents the number of animals in each treatment group. Scatterplots represent independent data points for each animal. Data are represented as means +/− SEM. * $p < 0.05$; ** $p < 0.05$.