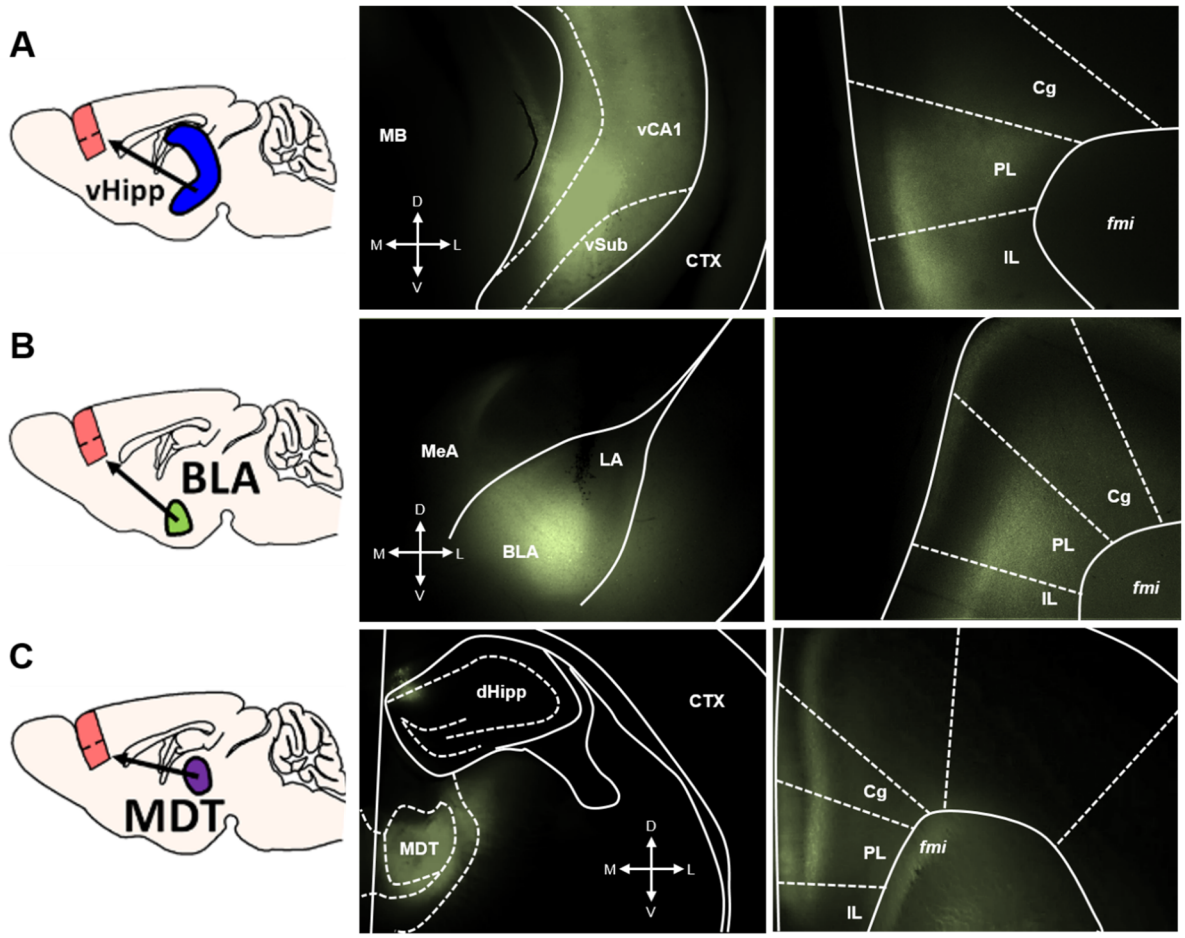
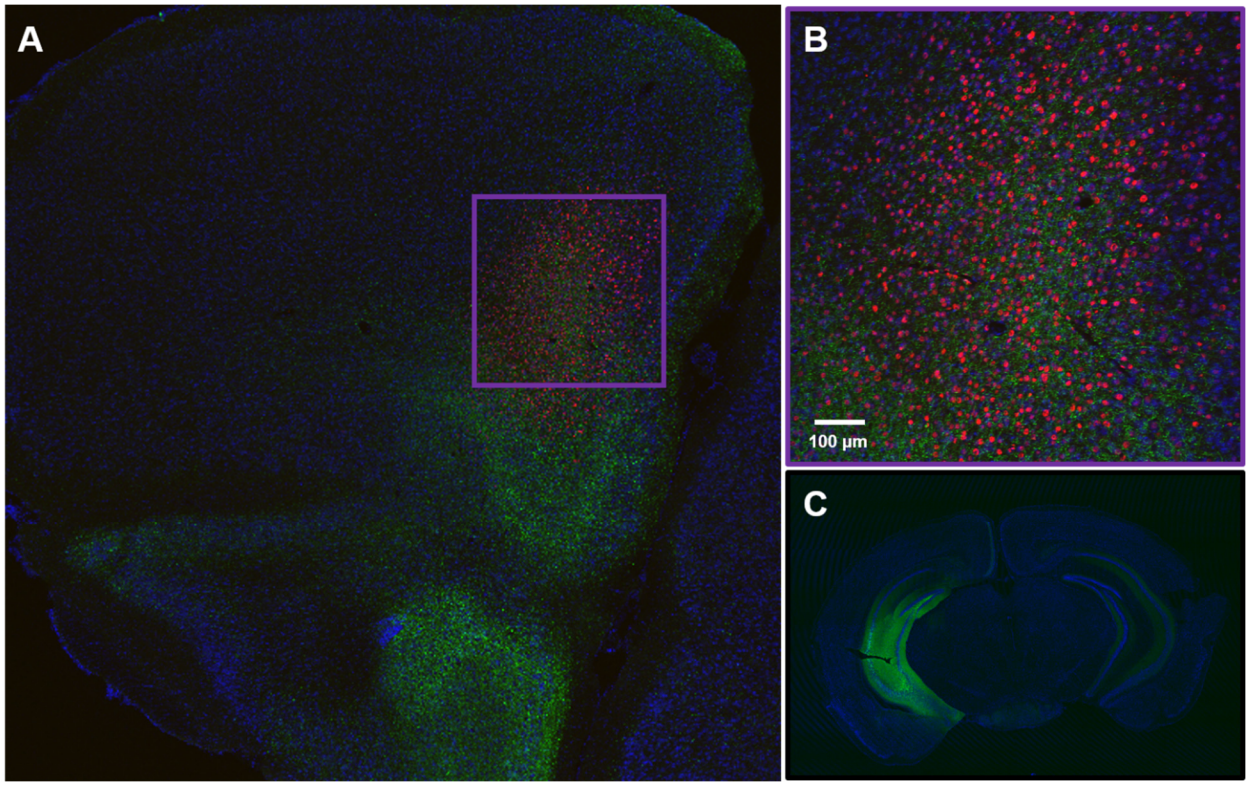
M_1 Muscarinic Receptors Modulate Fear-related Inputs to the Prefrontal Cortex: Implications for Novel Treatments of Posttraumatic Stress Disorder

Supplemental Information

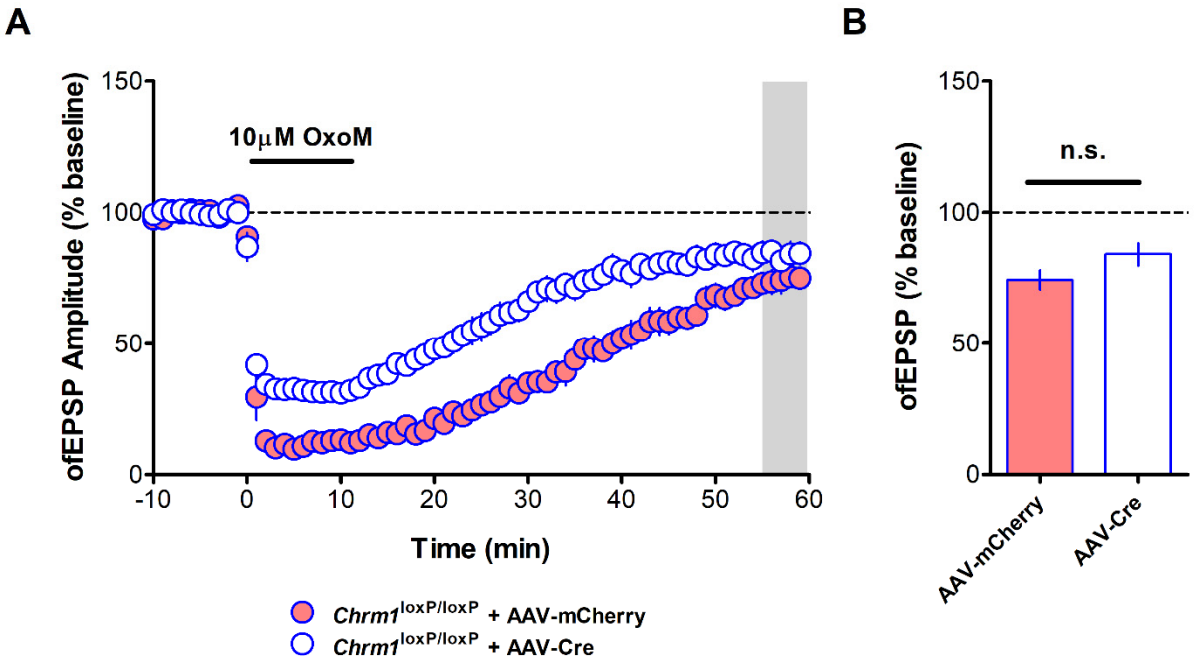
Supplemental Figures



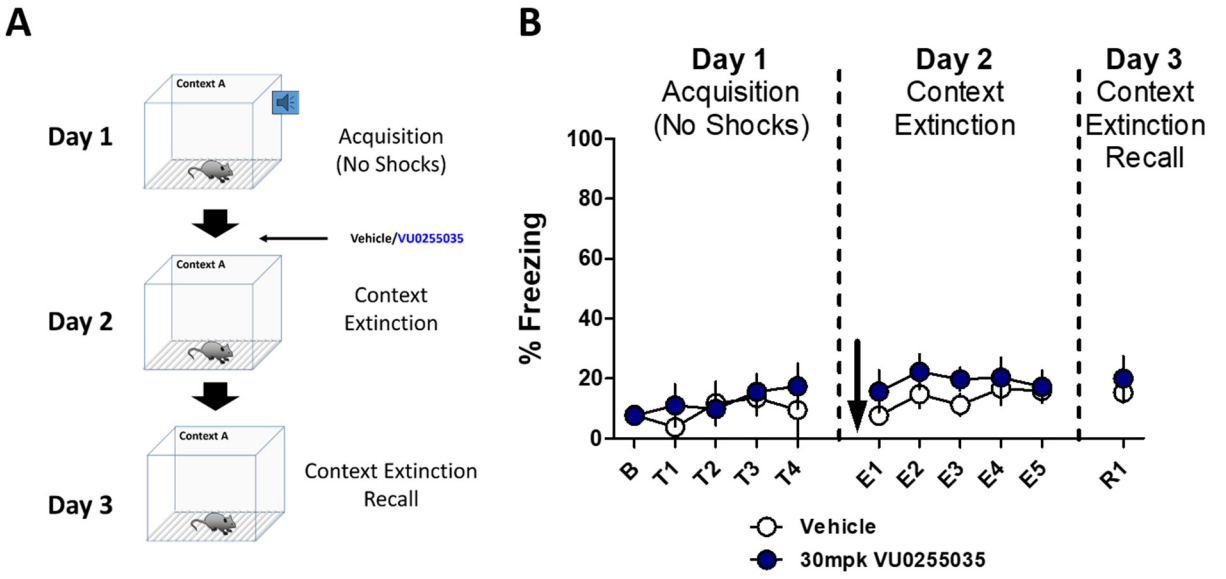
Supplemental Figure S1 (related to Figs 1 and 2): Viral injection sites and terminal expression of eYFP-tagged ChR2 in prefrontal cortex (PFC) slices. (A) *Left,* cartoon of ventral hippocampus (vHipp) projecting to PFC. *Middle,* eYFP expression in ventral CA1 and subiculum. *Right*, corresponding vHipp terminals detected throughout the PFC. **(B)** *Left,* cartoon of basolateral amygdala (BLA) projecting to PFC. *Middle,* eYFP expression in the BLA. *Right,* corresponding eYFP BLA terminals in the PFC. **(C)** *Left,* cartoon of mediodorsal nucleus of the thalamus (MDT) projecting to PFC. *Middle,* eYFP expression in the MDT. *Right,* eYFP MDT terminals expressed throughout the PFC. *MB = midbrain, vCA1 = ventral CA1, vSub = ventral subiculum, CTX = cortex, Cg = cingulate cortex, PL = prelimbic cortex, IL = infralimbic cortex, fmi = forceps minor of the corpus callosum, MeA = medial nucleus of the amygdala, LA = lateral amygdala, dHipp = dorsal hippocampus.*



Supplemental Figure S2 (related to Fig 3): Viral injection sites and expression of mCherry-positive cell bodies and eYFP-positive terminals from the vHipp in the medial PFC of *Chrm1*$^{loxP/loxP}$ mice. Representative confocal images of vHipp and medial PFC slices 5-6 weeks post-viral injection. **(A)** eYFP-positive terminals from the vHipp are detected throughout the medial PFC and mCherry-positive cell bodies of neurons infected with AAV-Cre-mCherry are detected in the prelimbic region of the medial PFC. **(B)** Image containing the prelimbic cortex corresponding to the purple box in A. **(C)** Robust eYFP-staining is observed unilaterally in the CA1 and subiculum regions of the vHipp. *Blue = DRAQ5-stained nuclei*, *Red = mCherry, Green = eYFP*.



Supplemental Figure S3 (related to Fig 3): Incomplete effect of viral-mediated knockdown on muscarinic long-term depression measured using extracellular field recordings in *Chrm1*$^{loxP/loxP}$ mice. (A) Time courses of optically-evoked field excitatory postsynaptic potentials (ofEPSPs) measured in slices from mCherry- or Cre-injected *Chrm1*$^{loxP/loxP}$ mice. The recording electrode was placed in layer V in an area of high mCherry-expression in the prelimbic PFC and brief pulses of 470nm light were used to activate ChR2 in vHipp terminals. Bath application of 10μM OxoM induces a long-term depression of ofEPSPs in both control mCherry- (n = 6) and Cre-infected (n = 6) slices. **(B)** Summary data of the ofEPSP amplitude in the grey shaded region in (A) relative to baseline. Student's t-test, $p > 0.05$.



Supplemental Figure S4 (related to Fig 4): The M_1 antagonist VU0255035 alone does not affect freezing. (A) Schematic depicting the training and testing procedure used. Mice were conditioned in Context A with 5 30s tones, with no foot-shocks. On day 2, mice were administered VU0255035 (30 mpk *i.p.*) or vehicle (20% β-cyclodextrin) and placed in Context A for 15 minutes to assess the effects of VU0255035 on freezing behavior. On day 3, mice were placed back in Context A for 3 minutes to assess the effect of VU0255035 on freezing 24hrs after dosing. **(B)** No effect of VU0255035 was observed within session on Day 2 (Two-way repeated-measures ANOVA: Effect of drug, $F_{1,6} = 0.9034$, $p = 0.379$) or on Day 3 (Unpaired student's test, $p = 0.581$). (N, Veh = 4, 30 mpk = 4)

Supplemental Methods and Materials

Animal Use

C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA), allowed to acclimate to the housing facility for at least one week and were then used for experiments. *Chrm1*$^{loxP/loxP}$ mice were bred in-house and maintained as homozygous breeding pairs. All experiments were performed in mice 8-12 weeks of age. Mice were group-housed (2–5 per cage) on a 12-h light cycle (lights on at 6:00 a.m.). Food and water were available *ad libitum*. All experimental protocols were approved by the Vanderbilt Institutional Animal Care and Use Committee.

Viral Injections

At 4-5 weeks of age, C57BL/6J mice were anesthetized with 3% isoflurane, positioned in a stereotaxic frame (Kopf Instruments, CA) and maintained on 1-2% isoflurane for the remainder of the procedure. Briefly, an incision was made and the skin and muscle atop the skull was pulled to the side. A craniotomy was made above the sites of the injections. Mice were then injected with 0.8µL (ventral hippocampus, vHipp) or 0.4µL (basolateral amygdala, BLA, or mediodorsal nucleus of the thalamus, MDT) of AAV5-CaMKIIa-ChR2-EYFP (UNC Viral Core, NC) per injection site at a rate of 0.1µL/min using a 28G needle attached to a 10µL Hamilton syringe (Hamilton Co., NV). The coordinates for injections relative to Bregma are as follows (in mm): vHipp, AP -3.4, ML ±3.4, DV -4.0; BLA, AP –1.4, ML ±2.9, DV –4.7; MDT, AP –1.2, ML ±0.3, DV –3.0. The needle remained in place for 5 min following injection and was then slowly retracted. The scalp was closed with VetBond (3M, MN) and mice were returned to their home cage.

At 4-5 weeks of age, *Chrm1*$^{loxP/loxP}$ mice underwent a similar procedure with the following modifications. A unilateral injection of 0.8µL AAV5-CaMKIIa-ChR2-EYFP (0.1µL/min) was made into the left vHipp (coordinates above) and a subsequent injection of 0.8µL AAV5-CaMKIIa-Cre-mCherry or AAV5-CaMKIIa-mCherry was made into the ipsilateral PFC (coordinates in mm

relative to Bregma: AP, +1.9, ML -0.3, DV -2.0). All mice that underwent surgery were monitored and administered carprofen (10 mpk) for 48 hours post-surgery.

Electrophysiology

Extracellular field and whole-cell patch clamp recordings were performed as previously reported (1). Briefly, 4-5 weeks (C57BL/6J mice) or 6 weeks (*Chrm1*$^{loxP/loxP}$ mice) following surgery, mice were anesthetized with 5% isoflurane and transcardially perfused with ice-cold NMDG-HEPES artificial cerebrospinal fluid (aCSF) containing (in mM): 92 NMDG, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 0.5 $CaCl_2 \cdot 2H_2O$, and 10 $MgSO_4 \cdot 7H_2O$, titrated to pH 7.3-7.4 with HCl. The brain was rapidly removed from the skull, blocked, and the brain was mounted to the cutting stage of a Vibratome (Leica VT1200S, Leica Camera, Germany). Coronal sections containing the PFC were cut at 400µm (field recordings) or 300µm (whole-cell recordings) in ice-cold NMDG-HEPES aCSF and transferred to 32^{o}C for 10-12 min. Slices containing the injection site (vHipp, BLA, MDT) were obtained to confirm efficient and accurate viral injection then discarded. Following recovery, PFC slices were transferred to a holding chamber containing artificial cerebrospinal fluid composed of (in mM): 126 NaCl, 2.5 KCl, 1.25 Na_2PO_4, 26 $NaHCO_3$, 10 glucose, 2 $CaCl_2$, 1 $MgSO_4$, supplemented with 500µM sodium ascorbate at room temperature for a minimum of 1 hour.

For recording, slices were transferred to a submerged recording chamber (Warner Instruments, CT) and continually perfused with aCSF maintained at 31±1^{o}C using an in-line heater (Warner Instruments, CT) at a rate of 2mL/min. Extracellular recording pipets were pulled from capillary tubes to a resistance of 1-3MΩ, filled with aCSF, and placed approximately 50µm beneath the surface of the slice in layer V prelimbic cortex. Paired 1ms pulses of 470nm light (50ms interpulse interval; LEDD1B, Thor Labs, NJ) were administered through a 40X immersion objective via the epillumination port of an Olympus BX51 inverted microscope to the region around the recording electrode to activate ChR2 at a rate of 0.05Hz. For electrically-evoked fEPSPs, a concentric bipolar stimulating electrode (CBARC57, FHC Inc., ME) was placed in layer II/III

between the recording electrode and the pia surface. 100μs duration electrical paired pulses (50ms interpulse interval) were administered at a rate of 0.05Hz at a stimulation intensity range of 50 to 200μA using a constant-current isolated stimulator (Digitimer Ltd., UK). Extracellular field potentials were amplified using an Axoclamp 700B amplifier, digitized with a Digitdata 1550B, and collected and recorded using Clampex 10.6 software on a PC running Windows 7. All data analysis was performed offline using Clampfit 10.6 (Molecular Devices, CA).

For whole-cell patch clamp recordings in *Chrm1*$^{loxP/loxP}$ mice, 300μm coronal slices of the PFC were obtained as above. Virally-infected pyramidal neurons in PFC layer V were targeted and confirmed by visualization of mCherry fluorescence in response to brief illumination with 545nm light. Pipets pulled to a resistance of 3-5 MΩ were filled with a K-Gluconate-based internal solution (in mm: 125 K-gluconate, 4 NaCl, 10 HEPES, 4 MgATP, 0.3 NaGTP, 10 Tris-phosphocreatine). After obtaining a >1GΩ seal, whole-cell configuration was achieved in mCherry-positive neurons and their cellular identity was confirmed by assessing spiking characteristics in response to a brief (1s) depolarizing current injection in current clamp. Optically-evoked excitatory postsynaptic currents (oEPSCs) were evoked with paired 1ms pulses of 470nm light as above to activate ChR2 at a rate of 0.1Hz. 300 ms of spontaneous EPSCs (sEPSCs) were recorded prior to the first light pulse. Access resistance, membrane resistance, and holding current were monitored throughout the recording. Cells where the access resistance changed more than 20% throughout the recording were excluded from analysis. Electrophysiology experiments were performed in male and female mice and no significant sex differences were observed so the data were combined.

Image Acquisition and Immunofluorescence

Epifluorescence images of the injection sites (vHipp, BLA, or MDT) and terminal expression in corresponding slices of the medial PFC were acquired on an Olympus BX51 inverted microscope through a 10X lens. Slices were briefly illuminated with 470nm light through the epillumination port and images were acquired with a Cool Snap HQ2 camera (Photometrics,

AZ) and Lambda 10–2 shutter (Sutter Instruments, CA) controlled by Metamorph 10.4 software (Molecular Devices, CA).

5-6 weeks post-injection, *Chrm1*$^{loxP/loxP}$ mice were anesthetized with 5% isoflurane, transcardially perfused with ice-cold phosphate-buffered saline (PBS) supplemented with 2g/L glucose followed by 4% paraformaldehyde (PFA) in PBS. Brains were dissected and post-fixed for 24hrs in 4% PFA at 4°C then transferred to increasing concentrations of sucrose-containing PBS (10, 20, then 30% sucrose *w/v*) and then rapidly frozen in chilled (-50°C) 2-methylbutane and stored at -80°C until sectioning. 40µm sections of the PFC and vHipp were cut using a freezing microtome and stored in cryoprotective buffer until staining. Free-floating sections were blocked with 5% normal donkey serum and 0.1% Triton X-100, then incubated overnight at 4°C with a goat anti-RFP (1:1000 in blocking buffer, Cat. No. 43590, Cell Signaling Technology, MA) and chicken anti-GFP (1:2000 in blocking buffer, Cat. No. ab13970, Abcam, UK) primary antibody mixture. Sections were washed, incubated with appropriate secondary antibodies (donkey anti-goat conjugated to Cy3 and donkey anti-chicken conjugated to Alexa488, Jackson ImmunoResearch Laboratories, PA), then incubated with the far-red DNA dye DRAQ5 (Cell Signaling Technology) and mounted on slides using ProlongGold (Thermo Fisher Scientific, MA). Images were obtained at 10X using a confocal microscope (Leica LSM520).

Behavior – Cued and Context Fear Conditioning and Extinction

8-10 week-old C57BL/6J mice underwent classical Pavlovian fear conditioning after at least 1 week of acclimation to the housing facility. An initial cohort of female mice did not acquire sufficient contextual fear conditioning to study extinction so male mice were used for all behavioral studies. Mice were handled and injected with 0.9% saline for at least 2 days prior to fear conditioning to minimize handling and injection stress. The percent of time spent freezing was used as a measure of learned fear and was analyzed using VideoFreeze software (Med Associates, St. Albans, VT).

On day 1, mice were transported to the conditioning room where the house lights were on and allowed to habituate for 1 hour. Mice were individually placed into “context A”, which was a brightly lit conditioning chamber (Med Associates), with a shock grid floor, no wall coverings, scented with 1mL of 10% vanilla extract, and cleaned with 70% ethanol. Following a 2 min baseline period, mice were administered 5 tone-shock pairings that consisted of a 30s tone (90dB, 5000Hz) that co-terminated with a mild foot-shock (0.7mA, 1s duration). Tone-shock pairings were separated by 30 seconds. On day 2, mice were transported to the conditioning room and allowed to habituate for 1 hour with the house lights off and a red light on to illuminate the room. 30 min prior to being placed in the conditioning chamber, mice were injected with vehicle (20% β-cyclodextrin in sterile water), 3, 10, or 30 mpk VU0255035 (formulated at 0.01mL/g body weight; administered *intraperitoneally*). These doses were selected based on previous pharmacokinetic studies with VU0255035 and the top dose of 30mpk is predicted to have good brain exposure but below full occupancy of the M_1 receptor (2). Mice were then placed in “context B” which was a dark conditioning chamber (infrared light used for video monitoring) with a hard white plastic sheet placed over the grid floor, two hard black plastic sheets forming a tent covering the chamber walls, scented with 1mL 10% almond extract, and cleaned with 10% MB-10 solution. Baseline freezing was assessed for 1 min and then a series of 12 30s tones were played, separated by 5s. On day 3, mice were placed back in context B, baseline freezing was assessed for 1 min and then 9 tones were played to assess the consolidation and recall of auditory fear extinction. On day 4, mice were transported to the conditioning room in the same manner as day 1. 30 min prior to being placed in the context A conditioning chamber, mice were injected with vehicle, 3, 10, or 30 mpk VU0255035. The doses were randomized so that a single mouse did not receive the same dose as on day 2. Mice were then placed in context A for 12 min. On day 5, mice were placed back in context A for 3 min to assess the consolidation and recall of contextual fear extinction.

For auditory cued fear conditioning, freezing on days 1, 2, and 3 were measured during the baseline periods and then during each 30s tone. For days 2 and 3, freezing for 3 tones blocks

were averaged together. Mice were excluded from cued extinction analysis if baseline freezing to context B was greater than 30%. For contextual fear conditioning, freezing on days 1, 4, and 5 were measured during the baseline period (day 1), during each tone (day 1), and then in 3 min bins (days 4 and 5).

Behavior – Stress-enhanced fear learning and contextual fear extinction

8-10 week-old C57BL/6J mice were exposed to stress-enhanced fear learning (SEFL) conditioning after at least 1 week of acclimation to the housing facility. For SEFL conditioning on day 1, 4 mice were placed in each conditioning chamber context A (as above) and administered 10 random foot-shocks (0.7mA, 1s duration) over 1 hour. For controls, 4 mice were placed in context A for 1 hour with no shocks delivered. On day 2, mice were transported to the conditioning room and allowed to habituate for 1 hour with the house lights off and a red light on to illuminate the room. Individual mice were then placed in context B (as above, but no hard white plastic sheet over floor grid) and after 2 min of baseline activity, were administered 2 tone-shock pairings. On day 3, mice were transported as on day 2 and SEFL mice were administered either vehicle (20% β-cyclodextrin) or 10 mpk of VU0453595 (formulated at 0.01mL/g body weight; administered *intraperitoneally*). 10 mpk VU0453595 was used as it has previously been determined to reach unbound concentrations in the brain approaching the *in vitro* PAM EC_{50} and has been shown to have behavioral efficacy in multiple paradigms (1). Mice were then placed in context B for 15 min to undergo contextual fear extinction. On day 4 mice were placed back in context B for 3 min to assess the consolidation and recall of extinction.

Compounds

Oxotremorine-M was obtained from Tocris Bioscience (MN) and a stock solution was prepared in diH_2O. VU0255035, VU0364572, and VU0453595 were synthesized in-house. For electrophysiology experiments, stock solutions were prepared in DMSO and diluted in aCSF to a final concentration (≤ 0.1% DMSO). VU0255025 was used for electrophysiology experiments at 10μM, a concentration that is predicted to fully inhibit M_1 in brain slices (1–3) and has been shown

to be selective for M_1 while having no effect on responses that are mediated by activation of other mAChR subtypes, including M_4, M_5, and presumed M_2/M_3 mediated responses (4, 5).

Data Analysis

The number of mice in each experiment is denoted by "N" and the cells or slices by "n". Data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism (La Jolla, CA). A paired or unpaired two-tailed Student's t-test, one/two-way ANOVA, or repeated measures two-way ANOVA with Bonferroni's post-test were used where appropriate. Results of statistical analyses are presented in the figure legends.

Supplemental References

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