Supporting Online Material

Materials and Methods

Subjects

cfos-htTA mice (*11*) were bred with tetO-hM_3D_q mice (*12*) to produce double transgenic experimental animals. Control animals were single transgenic siblings that underwent all of the same experimental conditions. Mice were housed socially (2-4 animals per cage), allowed free access to food and water, and maintained on a 12 hour light-dark cycle (unless tested between 6:30 and 7:00 AM). To avoid introducing additional stress from handling to read ear tag numbers, all animals were uniquely labeled with a permanent marker on the tail 2 days before the beginning of an experiment.

Context Conditioning

For context B (ctxB), mice were placed in chambers (30 cm length x 24 cm width x 25 cm height) with a black and white checked-pattern back wall, clear polycarbonate top, stainless steel side walls, and a stainless steel grid floor. One light in each chamber was on to allow motion detection by a digital camera, and the room lights were off. A round wintergreen scent dish was hung from the door of the chamber. When placed into and removed from the chamber, mice were handled with rubber tipped forceps.

For context A (ctxA), mice were first placed into a square plastic box (18 cm length x 18 cm width x 18 cm height) with opaque white walls and floor, and then the box was slid into the context B chamber. The back wall of the chamber was changed from black white checked-pattern to solid white. In addition to a light in the chamber, six room lights mounted at the corners of the room walls and ceiling were on as well. No scent dish was used for context A. When placed into and removed from the box, mice were hand handled.

Pre-exposure to a novel context off of doxycycline was performed between 7 AM and 11 AM and again between 4 PM and 7 PM. Fear conditioning was performed between 7 AM and 12 PM. Testing began between 6:30 AM and 7:30 AM and lasted 2 to 6 hours depending on the number of mice in the experiment and whether or not they were perfused after testing.

During fear conditioning, mice were given 3 minutes 18 seconds to explore the chamber, and were then administered four 0.8 mAmp shocks 2 seconds in duration with an intershock interval of 78 seconds. After the final shock, mice remained in the conditioning chamber for an additional minute before being placed back in their home cage.

Behavior in each context was recorded using a digital camera and motion was quantified and analyzed. The bout length was 1 second, and the threshold for freezing behavior was 10 (determined by eye by 2 students blind to experimental conditions and animal genotype). Freezing scores for context retrieval were calculated by dividing the test session into 1 minute bouts and averaging together all three minutes for each animal.

Injections

All injections were intraperitoneal (IP). Clozapine-N-oxide (CNO) was dissolved in dimethylsulfoxide (DMSO) and then diluted in 0.9% saline solution to yield a final DMSO concentration of 10%. Saline solution for injections also consisted of 10% DMSO. Not more than 1 uL of DMSO per 1 gram mouse was injected into animals (*23*). 0.5 mg/kg CNO was injected into mice 28 minutes before behavioral assays. This dose of CNO resulted in behavioral signs of seizure activity (*24*) in approximately 20% of mice during fear conditioning, which were excluded from behavioral and histological data analysis.

Immunohistochemistry

Sixty minutes after behavioral testing, mice were transcardially perfused with 0.9% saline for approximately 1 minute followed by 4% paraformaldehyde (PFA) for 6 to 9 minutes. Brains were post-fixed in 4% PFA overnight, sectioned with a thickness of 100 µm on a vibratome, and then stained while free-floating. All sections were blocked for 1 hour at room temperature in 0.8% TX-100 and 10% NGS in PBS followed by 1 hour at room temperature in 0.3% TX-100 and 10% NGS in PBS. Polyclonal rabbit anti-HA primary (Rockland),1:450 dilution, was used to label the HA tag on the hM_3D_q receptor (*12*). Polyclonal rabbit anti-cfos (Calbiochem), 1:700 dilution, was used to label the cfos protein. Primary antibodies were diluted in 0.3% TX-100 and 10% NGS in PBS, incubated at 4^{o}C for 48 hours, and rinsed 3 times for 15 minutes in PBS. Cy3-conjugated goat anti-rabbit (Jackson Immuno Research), 1:500 dilution in 0.2% TX-100 and 10% NGS in PBS was then applied to the sections for approximately 2 hours at room temperature followed by 3 rinses for 15 minutes in PBS. Finally, topro3 iodide (Molecular Probes, Eugene, OR), 1:1000 dilution in PBS, was applied to the sections for 15 minutes at room temperature to label cell nuclei. The sections were then rinsed for 10 minutes in PBS, mounted with coverslips on glass slides, sealed with clear nailpolish, and stored at 4^{o}C.

Confocal Microscopy

Confocal microscope was used to collect cfos and hM_3D_q receptor images. PMT, laser power, gain and offset were kept constant between experimental groups. cfos image stacks consisted of 8 slices separated by 10 µm steps.

cfos Quantification

Image J was used to count total number of cells (topro3 positive cells) and cfos positive cells in CA1, CA3, dentate gyrus, lateral amygdala, basalateral amygdala, and central amygdala. Percent cfos positive cells are reported with respect to total topro3 positive cells to account for differences in selected region sizes. Every other image slice in the z-stack was cell counted yielding cell numbers for 3 different positions along the z-axis. The z-slice with the highest percentage of cfos positive cells was used for further analysis and statistics. All quantification was performed blind to experimental group.

hM_3D_q receptor- cFos co-expression

For co-expression experiments (S3), a c-fos transgene with a GFP linked reporter was used to quantify cfos expression by measuring endogenous fluorescence (*11*).
Mice were injected with CNO and one hour 28 minutes later were perfused and the brains were fixed and stained for HA as decribed under *immunohystochemistry*.

***In vivo* electrophysiology**

For electrophysiological recordings, a total of 12 mice (6 single positive controls and 6 double positive experimental) were used. To mirror the behavioral study, mice were first fear conditioned and returned to their home cage overnight. 24 hours after fear conditioning, the mice were anesthetized with ketamine-medetomidine-atropine and prepped for electrophysiological recording. The head was placed in a stereotaxic frame, the skull was exposed, and a small hole (1 mm in diameter) in the skull was drilled above the left dorsal hippocampus (A, −2 mm from the bregma; L, 1.5 mm from the midline). A bundle of 4 tetrodes comprised of spun 17micron platinum-iridium wire or a 16 channel silicon probe were slowly advanced into the hippocampus and allowed to settle for a period of 10 minutes before recording. Tetrodes were targeted to the CA1 pyramidal layer, while the sites of the multi-channel silicon probe were positioned to span the hippocampal layers. After reaching the desired depth and rest, a 5 or 10 minute baseline period was recorded followed by injection of CNO and another 50+ minutes of recording. During the recording sessions, neurophysiological signals were amplified, bandpass filtered (0.1 Hz to 6 kHz), and acquired continuously at 32 kHz on a 32-channel system. In the case of the silicon probes, the site closest to the CA1 pyramidal layer was analyzed. Data were analyzed using a combination of custom-written MATLAB software and the MATLAB-based toolbox Chronux. Multi-unit spiking activity (MUA) was quantified by first filtering the fully sampled data (200Hz to 6kHz), then setting a threshold of 5 s.d. above the mean and counting all events over the threshold. The mean MUA (in spikes/second) was calculated for 30 to 40 minutes after CNO injection and then compared to 4 minutes of pre-injection baseline using a Wilcoxin signed-rank test.

Supplementary Data

Brain Region	$hM3D^{fos}$ (mean %)	control (mean %)	x fold increase	P value
CA1	40.8	16.2	2.5	0.0129
CA3	23.5	10.5	2.2	0.0188
DG	46.5	2.24	20.8	0.0067
LA	18.5	8.18	2.3	0.0491
BLA	21.9	8.3	2.6	0.0026
CA	24.8	5.8	4.3	0.0074