

## Supplementary Materials

for

Mitochondria-dependent post-tetanic potentiation in prelimbic cortico-pontine  
neurons gates extinction learning

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# 1. Supplementary Methods

## *Animals*

The light phase ran from 8 a.m. to 8 p.m., and all experiments were performed during the light cycle. Food and water were freely available to rats until the start of experiments. For mice, 85-90% of the standard body weight of the same aged mice corresponded to approximately 23 - 24 g.

## *Stereotaxic surgery*

Procedures common to all surgeries: Animals were anesthetized with 5% (v/v) isoflurane, the scalp was shaved and the animal was placed into the stereotaxic apparatus (Stoelting, USA), and the scalp was disinfected with 70% (v/v) alcohol and Povidone-Iodine before a circular 1-mm-diameter craniotomy was made. The exposed skull surface was covered with dental cement (Super-Bond C&B, Sun Medical, Japan), and implants were fixed on top of it with another dental cement (Poly-F plus, Dentsply Sirona, USA). Immediately after every surgery the animals recovered under an infrared lamp.

Cannula implantation surgery: The implanted set comprised a guide cannula (26 G), a dummy needle (33 G) and caps (PlasticsOne, USA), and the center-to-center distance between the bilateral guide cannulae was 1.2 mm for rats and 1 mm for mice. Infusions were delivered with a Hamilton syringe driven by an infusion pump, with the injection cannula connected to the syringe via polyethylene tubing (PE50, BD Intramedic, USA). The behavioral session performed 30 min after infusion was trace fear conditioning or the tone test, depending on the experiment.

Cell-type specific optogenetic inactivation surgery: The titers used were as follows: AAV2-retro-EF1a-Cre,  $1 \times 10^{13}$  genomic copies (gc)/mL for CPn inactivation and for the control group and  $2 \times 10^{13}$  gc/mL for COM inactivation; AAV5-CAG-DIO-ArchT-tdTomato,  $1 \times 10^{12}$  gc/mL (CPn) and  $2 \times 10^{12}$  gc/mL (COM, ipsilateral PFC); AAV5-CAG-fDIO-ArchT-GFP,  $2 \times 10^{13}$  vg/mL; AAV2-retro-EF1a-

Flpo,  $2 \times 10^{13}$  vg/mL; and AAV5-CAG-DIO-EGFP-WPRE,  $1 \times 10^{13}$  gc/mL. AAV5-CAG-fDIO-ArchT-GFP was packaged at KIST from the Addgene plasmid, and the control group was prepared for GFP expression in CPn neurons and received virus bilaterally in the pons and PL. Virus was loaded into a Hamilton syringe (7653-01, Hamilton, USA) fitted with a needle (7762-08, 28 G, Hamilton, USA) and injected with an Ultra Micro Pump (UMP3, WPI, USA) connected to a microcontroller (MICRO2T, WPI, USA) over 10 min, and the syringe remained in place for a further 5 min to allow diffusion of the virus. The implanted optic fibers (R-FOC-L200C-39NA, RWD, China) had a 1.25 mm ceramic ferrule, a 200  $\mu$ m core, a 3.5 mm length and 0.39 NA. Behavioral experiments began two weeks after optic fiber implantation.

Shuttle drive surgery: Stereotrodes were made by folding, twisting and heating Formvar-coated nichrome wire (761000, A-M systems, Netherlands). The EIB consisted of a connector (A79026-001, Omnetics, USA) and an open-source printed circuit board (PCB, SCITECH, Korea); it was attached to the 3D-printed body and then glued with epoxy. Individual stereotrode wires went to the channels of the EIB, whereas 28-gauge silver-coated copper wires (TAWG28, SME, Korea) soldered with the skull screws went to its ground and reference holes, all being connected with gold pins (OPES-7013, Open Ephys, Portugal). Before surgery the bundle of stereotrodes was cut with sharp scissors and then gold-plated. After the hair was removed and the scalp incised, the skull surface was cleaned using  $H_2O_2$  before the craniotomy site was marked. The ground screw was placed on the skull of the cerebellar area and the reference screw in the skull of the olfactory bulb area.

Cell type specific in vivo calcium imaging surgery: AAV2-Cre was used at  $1 \times 10^{13}$  gc/mL and AAV-flex-GCaMP6f at  $2 \times 10^{13}$  gc/mL. Virus (1  $\mu$ l) was injected over 10 min at 100 nl/min using a Hamilton syringe and an Ultra Micro Pump with a Micro Controller, and the GRIN lens, 1.0 mm or 0.6 mm in diameter, was implanted one week later. In the first method the lens was sterilized with 70% ethanol and implanted. In the second method the lens was sterilized with UV light, after which AAV-flex-GCaMP6f and silk fibroin were mixed in a 1:1 ratio and 1  $\mu$ l of the mixture was applied to the surface of the lens distal tip and dried at room temperature for at least 1 h. For lens implantation the animals were anesthetized and fixed in the stereotaxic apparatus again.

Superficial cortical tissue was gently aspirated with the tip of a 22-gauge blunted needle connected to a vacuum source, and the craniotomy site was constantly washed with sterile PBS while the lens was lowered. Dexamethasone was given for one week after surgery to avoid surgery-related tissue swelling.

## *Behavior*

Contexts for behavior tests: Context A, the rat conditioning cage (H10-11R-TC, Coulbourn Instruments, USA), was located inside a sound-attenuating box (SCITECH, Korea) fitted with an LED lamp; the speaker module (H12-01R, Coulbourn Instruments, USA) was located on one side of the conditioning chamber, and the footshock was delivered through a floor of stainless-steel bars (H10-11R-TC-SF, Coulbourn Instruments, USA). Context B was hexagonal (32 x 34 x 50 cm), consisted of a white acrylic floor and wall, was located on the floor of an isolated room, and delivered the auditory cue through speakers located on either side; only freezing behavior was monitored there. Context B' was rectangular (36 x 21 x 25 cm) and consisted of a black plastic floor and a glass wall with blue paper on the outside; there, *in vivo* recording of neuronal activities or laser illumination was conducted in parallel with observing freezing behaviors. The parameters used for ezTrack analysis were a motion cutoff of 10 - 25, a freezing threshold of 30 and a minimum freeze duration of 30 samples.

Weak and strong fear conditioning: Weak or strong conditioning was adopted depending on observation interests. In the weak protocol the five CS-US pairings were presented with pseudo-random intertrial intervals of  $180 \pm 20$  s, and in delayed conditioning the footshock (0.5 mA, 1 s) co-terminated with the CS. The tone test on Day 2 after weak conditioning consisted of ten presentations of tones (20 s, 2.5 kHz, 75 dB) in Context B with intertrial intervals of  $90 \pm 20$  s. The strong protocol consisted of eight CS-US pairings with a footshock of 0.7 mA for 1 s; it was adopted because it allowed prelimbic activity related to fear memory retrieval to be observed in a larger number of tone test trials. The eight pairings on Day 1 were preceded by three tone-only habituation trials, which tested whether prelimbic neurons respond to the CS alone before CS and US are associated. The twenty tone presentations on Day 2 were 20 s, 2.5 kHz and 75 dB with

intertrial intervals of  $90 \pm 20$  s. For the optogenetic study and for drug infusion on Day 2, the extinction time course was observed over fifteen or twenty trials of tone presentation in Context B.

Drug infusion into the prelimbic cortex: Each of the bilateral infusion cannulae was connected to its own Hamilton syringe. TPP (218790, Sigma, USA) and Ru360 (557440, Sigma, USA) were purchased from Sigma. TPP/Ru360 and PBS infusion experiments were conducted in a separate cohort using identical behavioral procedures.

Optogenetic inactivation: Before behavior tests, the implanted optic fibers were connected to the 593.5 nm laser (YL593T6-050FC, Lasercentury, China) through a fiber optic patch cable (R-FC-PC-N3-200-L1, 1 m, RWD, China), an optic fiber commutator (R-FC-1x1, RWD, China) and a splitter branch patch cord (FCM-2xZF1.25(F), NA 0.37, Doric, Germany), and the laser intensity was verified with a laser power meter (UT385, UNI-T, China). The tones presented on Day 3 were 20 s, 2.5 kHz and 75 dB.

Operant delayed match to position (DMTP): The apparatus featured two visual stimuli (white squares) displayed above the two corresponding nose-poke holes, and rewards of cherry-flavored Kool-Aid (KHC, Chicago, USA) were dispensed through a lick port located on the opposite side of the visual stimulus; reward delivery was signaled by light illumination. Each session lasted one hour. During the habituation stage, animals underwent reward magazine training and received 2.5  $\mu$ l of reward at 15 s intervals, each delivery accompanied by reward light illumination, and the light was extinguished when the animal licked the reward port. Pretraining began the following day with the presentation of a single visual stimulus; animals received a 2.5  $\mu$ l reward upon poking the nose-poke hole beneath the stimulus, with a 15 s inter-trial interval (ITI), and daily training continued until animals consistently achieved at least 30 trials per hour.

In the match phase, a single visual stimulus (sample) was presented and followed by a 2.5  $\mu$ l reward, after which the visual stimulus reappeared at the same location (choice) and a larger reward (6  $\mu$ l) was provided; incorrect responses, in which the animal poked the opposite nose-poke, resulted in house light illumination for 5 s followed by a 20 s ITI. The 1 s DMTP phase, which was the same as the training session, built upon the match phase with two modifications: a 1 s

delay was introduced between reward light illumination and reward availability during delivery of the sample reward (2.5  $\mu$ l), and two visual stimuli instead of one were presented at the choice stage. In the 4 s DMTP phase the delay was extended to 4 s. The criterion of 30 trials per hour with a 70% success rate was applied at the match, 1 s DMTP and 4 s DMTP stages, and animals were considered ready for experimental testing when they reached it at the 4 s delay interval.

### *Unit recordings and data processing*

The 32-channel RHD recording headstage (C3314, Intan, USA) was connected to the Omnetics connector of the shuttle drive, and the headstage and the RHD USB interface board were connected with a thin serial peripheral interface (SPI) cable (C3213, Intan, USA). The recording session was 34 min during the fear conditioning and 42 min during the tone test. At the end of recording the 30  $\mu$ A current was applied to each stereotrode in order to locate the position of the tip of the stereotrode bundle. Brains were extracted and post-fixed for 24 h in 4% paraformaldehyde-containing PBS solution, and the coronal prefrontal cortex was prepared using a vibratome and imaged with a fluorescence microscope (IX53, OLYMPUS, Japan).

Spike sorting: The 12 and 30 data points before and after the peak of each spike were collected into a spike wavelet matrix. The list of automatically detected spike waveforms was refined by excluding spikelets whose valley/peak amplitude ratio was greater than 0.8 or whose peak amplitude was greater than 1000  $\mu$ V. The three-dimensional matrix of spikelet waveforms (time x spikes x channel) was then subject to principal component analysis to extract three principal features of spikelets per channel, and the extracted features were plotted on the coordinate of six principal components (three principal components x two channels, as a bundle of stereotrodes was used) before a Gaussian mixture model was fitted for clustering. Manual sorting was performed with MatClust software (Mattias Karlsson, 2022).

Classification of CS and PA cells:  $z_f$  and  $z_{isi}$  were calculated for the CS and post-CS periods, respectively. The test for comparing two Poisson means was adopted for  $z_{isi}$  because the distribution of interspike intervals of randomly firing neurons follows an exponential distribution.

$N_1$  and  $N_2$  are the sample sizes of the baseline period and of the CS or post-CS period, respectively, and the comparison was made against baseline.

CS-responsive cells and sequence analysis: The timing of peak activity was determined for each trial, and  $\sigma = 4$  s refers to the standard deviation of the Gaussian filter. The sum of cosine similarity values of population vectors between trials was calculated over the CS and post-CS periods, and in the 1000 shuffled datasets the cell indices were shuffled with the activity time course of each cell left intact.

### *Whole-cell patch recording*

The brain was removed immediately after the rats were sacrificed and then chilled. Prefrontal and visual cortex slices were each cut at 300  $\mu$ m thickness with a vibratome (VT1200S). For recording, slices were transferred to a recording chamber and circulated with standard aCSF using a peristaltic pump (MINIPLUS3, Gilson, USA). Cells were visualized using an upright microscope mounted with differential interference contrast optics (BX61WI, Olympus, Japan), and recordings were made by the whole cell clamp technique. Series resistance was regularly checked by applying a short hyperpolarization (20 ms, 2 mV). For electrical stimulation, a glass pipette of 2-3 M $\Omega$  was filled with aCSF and the monophasic pulses were delivered using a stimulus isolator (A360, WPI, USA). The firing pattern used for PTP induction was derived from *in vivo* electrophysiological recording, and after the patterned stimulation the paired pulse stimulations were resumed. Series resistance compensation, bridge balance and liquid junction potential correction were not applied.

### *In vivo $Ca^{2+}$ imaging and data processing*

On the day of imaging the rats were allowed to acclimate to the experimental room for about 15 min before the Miniscope was docked to the baseplate. The Miniscope and DAQ were connected with a flexible coax cable; the calcium signal was transmitted to the data acquisition system

through the cable and saved on a personal computer, and the frame rate, LED power, gain and focus were controlled using the Miniscope DAQ software. The LED intensity was 2-15 and the gain 2.5-3.5. The behavior of the rats was recorded by a CCD camera, and the calcium imaging and tone presentations were synchronized by Bonsai software (Bonsai Foundation). Because the calcium signal was not recorded during the entire behavioral experiment, recording was started 30 s before the onset of the cue in each trial. Brains were extracted and post-fixed for 24 h in 4% PFA after finishing experiments, and the coronal sections were prepared using a vibratome.

Calcium imaging data processing: The down-sampled videos over five tone trials were concatenated using ImageJ software and saved as one TIF file. The CNMF-E parameters were as follows: the data were filtered with a gaussian kernel with a width of 4 pixels (gSig); neurons were constrained to a diameter of 15 pixels (gSiz); a ring model (bg\_model) was used for the background with a radius of 18 (ring\_radius); neurons with spatial overlap greater than 0.2 (merge\_thr) and centroid distance less than 10 pixels (dmin) were merged; and only ROIs with a minimum peak-to-noise ratio of 15 (min\_pnr) and a minimum spike size of 5 (smin) were extracted. Because not all ROIs classified by CNMF-E could be considered as neurons and some might be dendrites or background fluctuations, all neurons were visually inspected. Further data analysis was performed using the inferred spiking activity (neuron.S), in which a non-zero value was converted to 1 to give the binarized spike activity.

## *Statistics*

The analysis software used was Igor Pro 7 (WaveMetrics, USA), SPSS (IBM, USA), Prism (GraphPad, USA), MATLAB (MathWorks, USA), R (<https://www.R-project.org/>) and RStudio (<http://www.rstudio.com>); the LME model was fitted with the “nlme” package and pairwise comparisons were made with the “emmeans” package (<https://cran.r-project.org/web/packages/emmeans/index.html>). Post hoc comparisons with Bonferroni correction were conducted to investigate differences between groups at each time point. n.s. denotes not significant ( $p > 0.05$ ). All data in the figures are presented as the mean  $\pm$  standard error of mean (SEM).

## 2. Supplemental Figures

*Fig. S1*

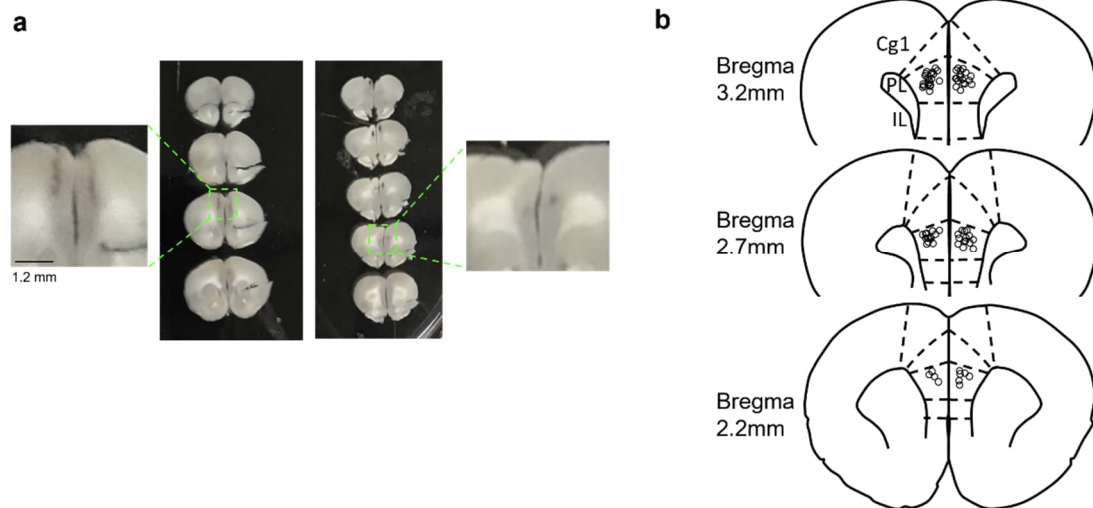


Fig. S1. Bilateral infusion cannula implantation into the PL area. **a**, Representative images of coronal slices of the brain implanted with bilateral cannula. **b**, locations of bilateral infusion cannula of all rats used in the present study.

Fig. S2

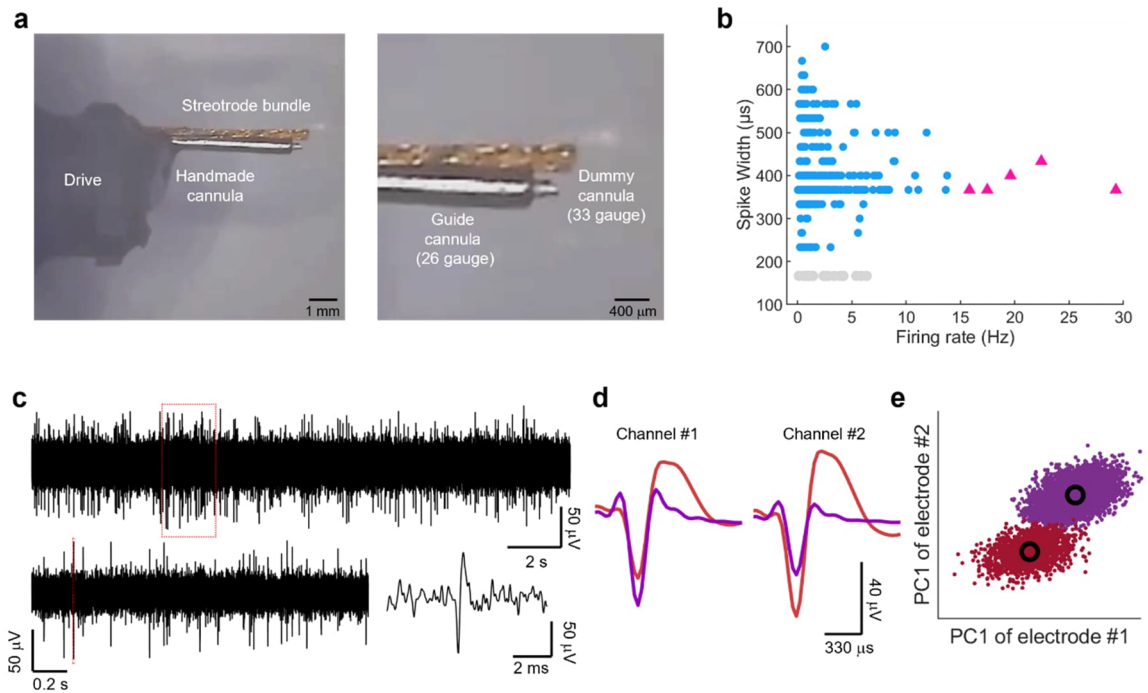


Fig. S2. In vivo electrophysiological recording. a, Modified shuttle drive {Voigts, 2020 #115}. *Left*, Representative image showing a stereotrode bundle and handmade cannula in close proximity. Scale bar, 1 mm. *Right*, Magnified image showing a guide cannula (26 gauge), into which a dummy cannula (33 gauge, same size as infusion cannula) is inserted. The dummy and infusion cannula were set slightly shorter than the stereotrode bundle to ensure an optimal drug delivery at the recording site. Scale bar, 400  $\mu\text{m}$ . b, Scatter plot of sorted spikes on the plane of spike width vs. firing rate for all recording (n = 381). Sorted spikes were classified into putative pyramidal neurons (blue dots) and interneurons (red triangle and gray dots) based on their waveform durations and firing rates. c, Representative traces recorded *in vivo* using a stereotrode at different time scales. The electrophysiological signal was recorded at 30 kHz and band-pass filtered from 0.5 to 5 kHz. The boxed region of upper trace was expanded on time and shown in lower trace. d-e, Representative unit spike waveforms (d, called spike wavelet, 42 data points/wavelet) recorded during TFC. Scale bar, 20  $\mu\text{V}$ , 330  $\mu\text{s}$ . Such Spike wavelets were collected into a matrix of three dimensions (spike x time x channel). The wavelet matrix was then subject to PCA, yielding three principal components at each of two electrodes of a stereotrode. The two spike wavelets shown in d made two separate clusters on the plane of two first principal components from two channels (e). Spikes were sorted by fitting a Gaussian mixture model to the extracted features on the space

of six principal components, and clustering based on split and merge expectation maximization algorithm {Ueda, 2000 #112}.

Fig. S3

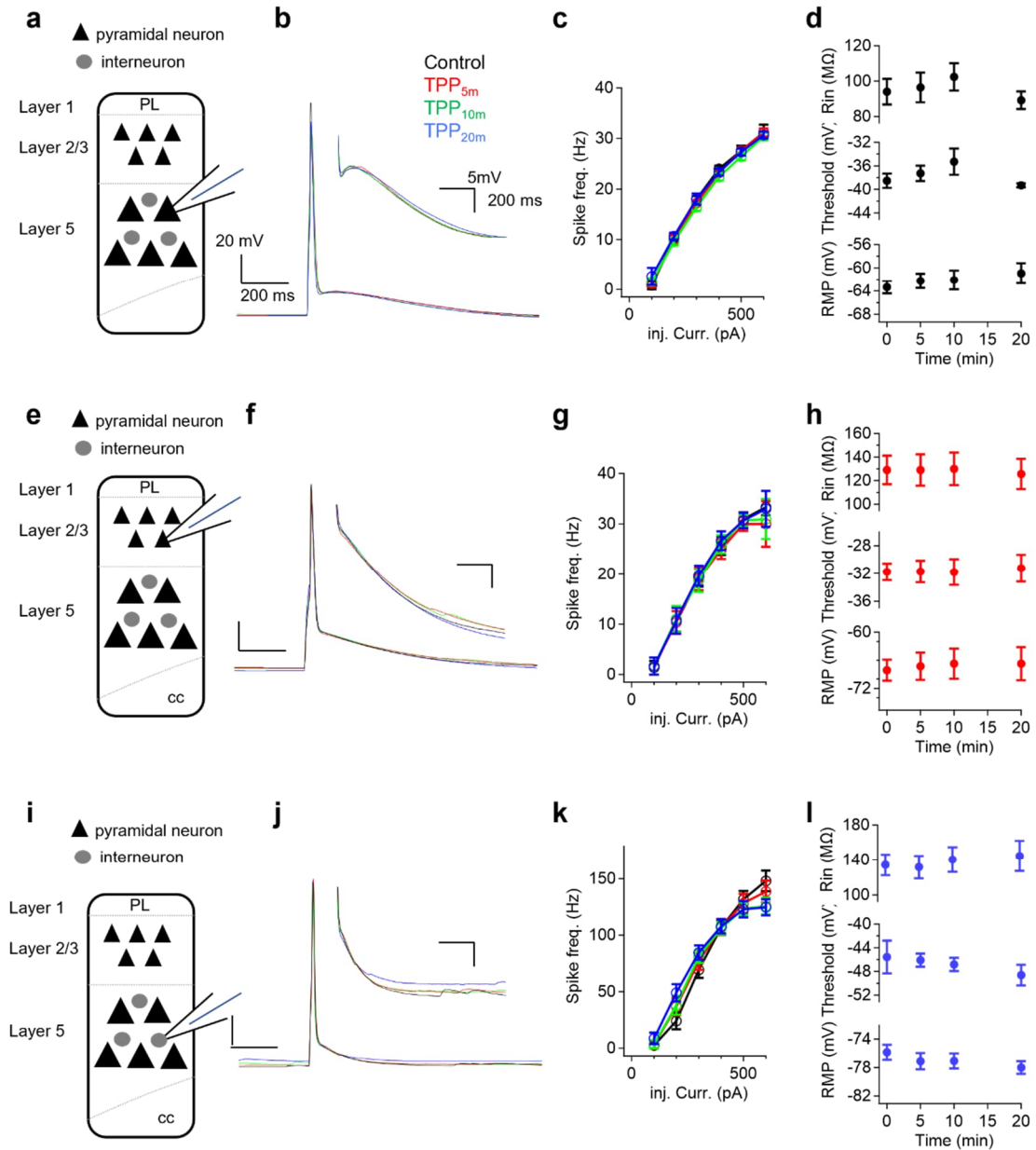


Fig. S3. TPP<sup>+</sup> does not affect intrinsic electrical properties of PL neurons. a-d, TPP<sup>+</sup> effects on L5 pyramidal neurons (PNs) in the PL area. After-depolarization (ADP, *b*), firing frequency-current injection relationship (F-I curve, *c*), Input resistance (*R<sub>in</sub>*, *d*), Resting membrane potential (RMP, *d*) and action potential threshold (AP threshold, *d*) were monitored in L5 PNs over time after TPP<sup>+</sup> application. a, Scheme of experiments showing the location of a patch pipette. b, Representative traces of APs and ADPs (*inset*) over time after TPP<sup>+</sup> application (*black* = control, *red* = 5 min, *green*

= 10 min, *blue* = 20 min after TPP<sup>+</sup> application). c, Averaged F-I curves of L5 PNs (n = 5). Same color codes as in *b*. d, R<sub>in</sub>, RMP and AP threshold were not affected by TPP<sup>+</sup> application (n = 7 for all). e-h, Same as a-d but in L2/3 PNs. Neither of ADP (*f*), F-I relationship (*g*), R<sub>in</sub> (*h*, upper), RMP (*h*, middle) and AP threshold (*h*, lower) was affected by TPP<sup>+</sup> (n = 6 for all). i-l, Same experiments for L5 interneurons (n = 13 for all). All data are mean ± S.E.M.

Fig. S4

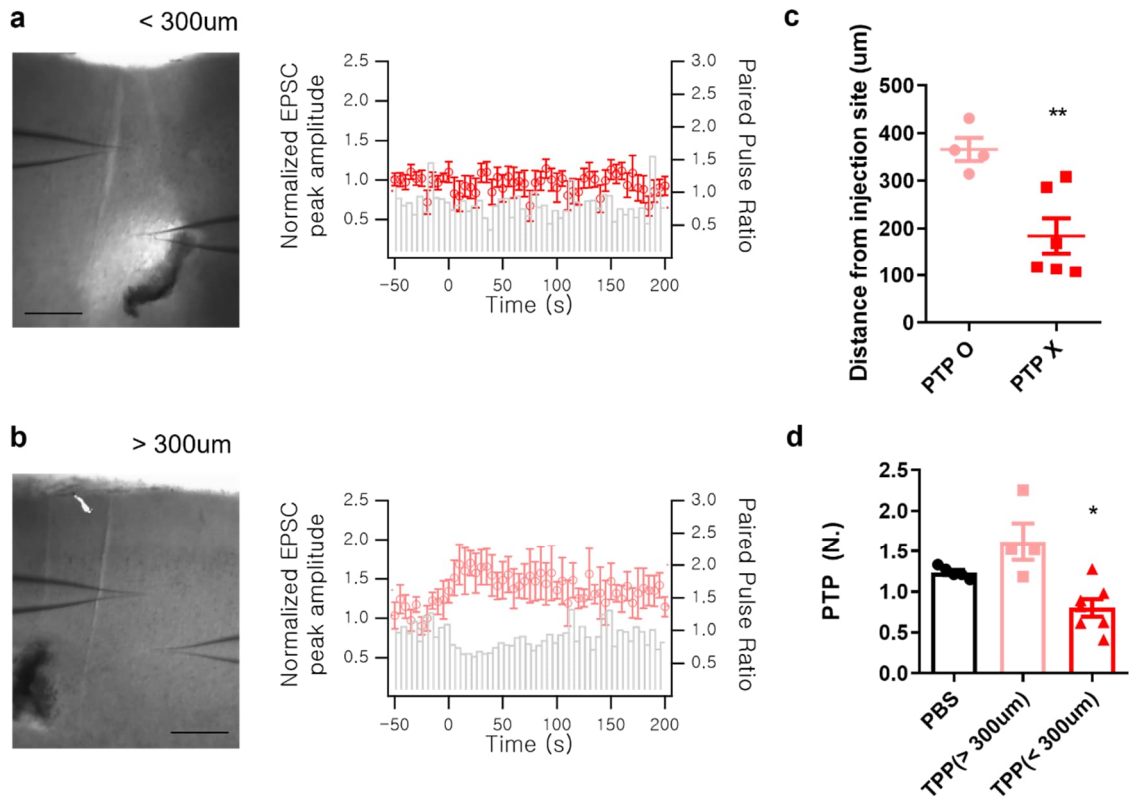


Fig. S4. Post-tetanic potentiation was not induced within the area 300  $\mu\text{m}$  from the injection site but was induced outside the 300  $\mu\text{m}$  area. a, Test for Post-tetanic potentiation (PTP) induction at L2/3-CPn synapses within the area 300  $\mu\text{m}$  from the TPP<sup>+</sup> injection site. *Left*, Representative image of PTP induction experiment within the area 300  $\mu\text{m}$  from the TPP<sup>+</sup> infusion site showing the location of patch clamp pipette, monopolar stimulation glass electrode and PBS injection site. Scale bar, 200  $\mu\text{m}$ . *Right*, Time courses of baseline-normalized EPSC amplitudes before and after tetanic stimulation (TS, 25 Hz for 5 s) applied at  $t = 0$ . PTP was not induced within the area 300  $\mu\text{m}$  from the injection site ( $n = 7$ ). b, Same as a but at L2/3-CPn synapses outside the 300  $\mu\text{m}$  area. PTP was induced outside the area (*right*,  $n = 4$ ). c, Summary scattered plot of distances from injection site vs. occurrence of PTP ( $P = 0.0095$  for PTP o vs PTP x). d, Summary bar graphs for normalized PTP. PTP was not induced within the area < 300  $\mu\text{m}$  from the TPP<sup>+</sup> injection site, while PTP was induced outside the area. For PTP within ( $n=7$ ) vs. outside ( $n=4$ ) the 300  $\mu\text{m}$  area,  $P = 0.0121$ . Mean  $\pm$  S.E.M., \* $P < 0.05$ , \*\* $P < 0.01$ , Mann-Whitney test.

Fig. S5

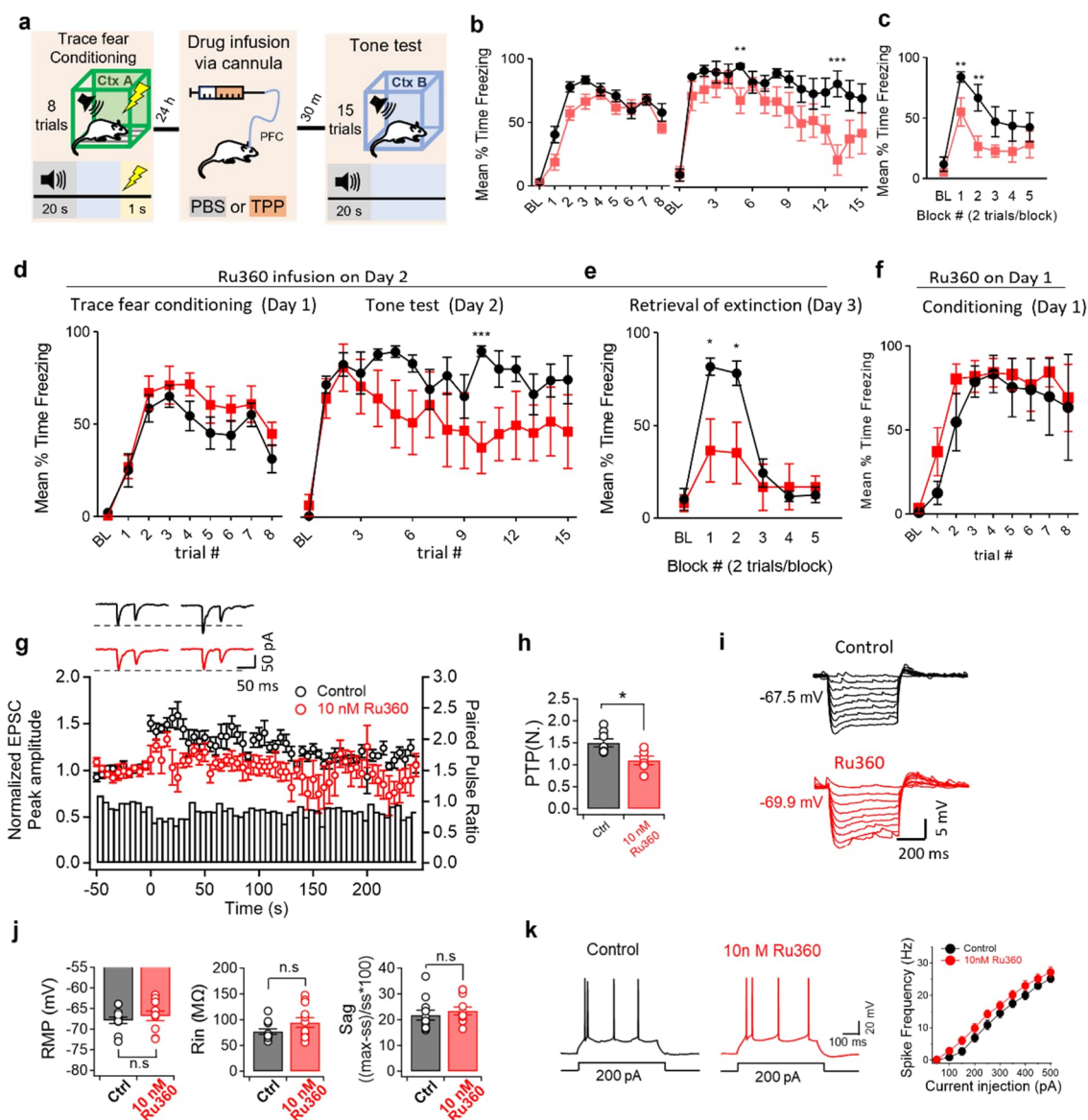


Fig. S5. a-c, Infusion of TPP<sup>+</sup> into the prelimbic cortex prior to the tone test on Day 2 accelerated trace fear extinction similar to TPP infusion on Day 1. a, Schematic of experimental procedure. PBS or 20  $\mu$ M TPP<sup>+</sup> (1  $\mu$ L each) was infused into the prelimbic cortex (PL) 30 minutes before tone tests on Day 2. b, Time courses of freezing behavior during trace fear conditioning (left, day 1) and the tone test (right, Day 2) in PBS and TPP<sup>+</sup>-injected animals (PBS, n=10; TPP<sup>+</sup>, n=8 rats). RM two-way ANOVA (PBS vs. TPP): For conditioning (day 1),  $F_{1,16} = 3.397$ ,  $P = 0.084$ ; For tone test (Day 2),  $F_{1,16} = 5.907$ ,  $P = 0.027$ . c, Mean freezing ratios of PBS and TPP<sup>+</sup>-injected animals as a function of

two trial blocks during tone test (*left*, Day3). RM two-way ANOVA (PBS vs. TPP):  $F_{1,16} = 4.448$ ,  $P = 0.033$ .

d-e, Prelimbic injection of Ru360 30 min prior to the tone test on Day 2 accelerated trace fear extinction learning. d, Time courses of freezing behavior during trace fear conditioning (*left*, Day 1) and the tone test (*right*, Day 2) in PBS (1  $\mu$ L, n=8; black) and 100 nM Ru360 (1  $\mu$ L, n=6; red) injected animals. RM ANOVA revealed a significant effect of group during the tone test on Day 2. [For conditioning (Day 1),  $F_{(1,12)} = 1.567$ ,  $P = 0.234$ ; For tone test (Day 2),  $F_{(1,12)} = 5.924$ ,  $P = 0.032$ ]. e, Mean freezing ratios of PBS and Ru360-injected animals as a function of two trial blocks during tone test (Day 3). RM-ANOVA revealed a significant effect of group during the tone test on Day 3 ( $F_{1,12} = 5.218$ ,  $P = 0.041$ ). f, Ru360 administered on Day 1 had little effect on freezing behavior during trace fear conditioning. Time courses of freezing behavior during trace fear conditioning in rats which received prelimbic Ru360 infusion 30min prior to conditioning. RM two-way ANOVA (PBS vs. Ru360):  $F_{1,3} = 0.303$ ,  $P = 0.620$ .

g-h, Ru360 (10 nM) inhibits post-tetanic potentiation at L2/3-CPn synapses without affecting intrinsic electrical properties of L5 PN. Ru360 effects on L2/3-CPn synapses and L5 CPn neurons in the PL area were assessed 20 min after Ru360 application. g, Time course of normalized EPSC amplitudes before and after tetanic stimulation (TS) at L2/3-CPn synapses in control (*black*) and Ru360 (*red*). TS (25 Hz, 125 pulses) was applied at time zero. *Insets*, representative EPSC traces. h, Summary bar graph for normalized post-tetanic potentiation at L2/3-CPn synapses (*Black*, control, n = 8; *Red*, Ru360, n = 6;  $P = 0.015$ , Mann-Whitney test). i, Representative voltage responses of prelimbic L5 CPn cells to hyperpolarizing current steps (50 pA increments) in control (*black*) and Ru360-treated (10 nM, *red*) conditions. j, Resting membrane potential (*left*, RMP), Input resistance (*middle*,  $R_{in}$ ), and Sag ratio (*right*, Sag) did not significantly differ between Ru360-treated (n = 12; *red*) and control cells (n = 11; *black*) (control vs. Ru360; For RMP,  $P = 0.454$ ; For  $R_{in}$ ,  $P = 0.125$ ; For sag ratio,  $P = 0.508$ ). k, Representative action potential firing responses to depolarizing current step in control (*black*) and Ru360-treated neurons (*red*) (*left*). Averaged firing frequency-current injection relationship (Averaged F-I curve, *right*) in control (PBS, n=12, *black*) and Ru360-treated groups (n=12, *red*). All data are mean  $\pm$  S.E.M. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.005$ .

Fig. S6

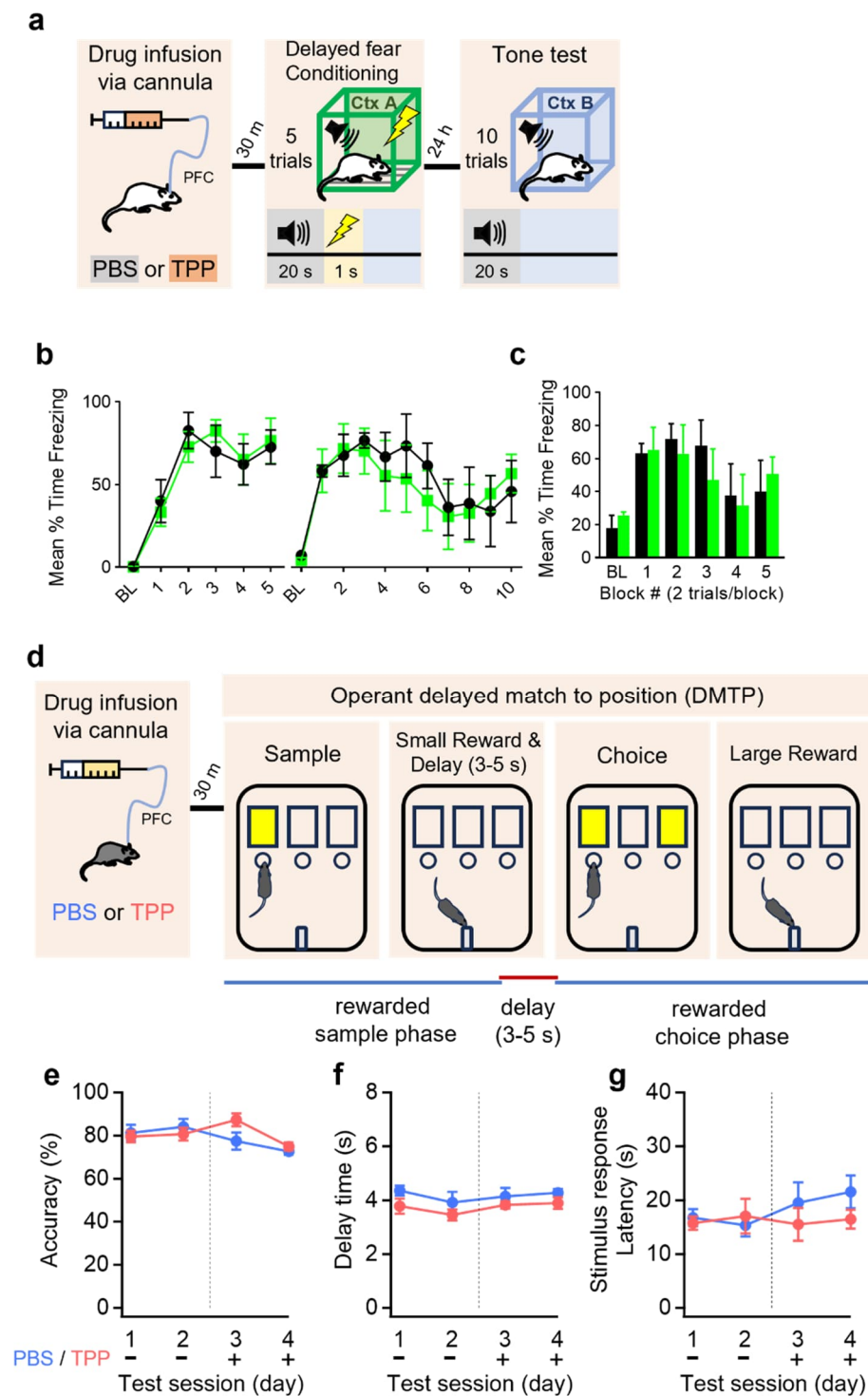


Fig. S6. a-c, TPP<sup>+</sup> infusion into the PL did not affect delayed fear conditioning (DFC) and fear extinction. a, Schematic of DFC experimental procedure. PBS or TPP<sup>+</sup> was infused into the PL 30 min before DFC on Day 1. b, Time courses of freezing ratio of PBS (n=4) and TPP<sup>+</sup> (n=4) animals during DFC (*left*, Day 1) and tone test (*right*, Day 2). RM two-way ANOVA (PBS vs. TPP<sup>+</sup>): For DFC,  $F_{1,6} = 1.162$ ,  $P = 0.981$ ; For tone test,  $F_{1,14} = 444.290$ ,  $P = 0.694$ . c, Mean freezing ratio of PBS and TPP groups during tone test, presented as means of two trial blocks. RM two-way ANOVA (PBS vs. TPP):  $F_{1,6} = 70.232$ ,  $P = 0.874$ .

d-g, TPP<sup>+</sup> infusion into the PL did not affect performance on the working memory task. d, Schematic of operant delayed-match-to-position (DMP) task. Animals were subject to a test session of the DMP task 30 min after drug injection into the PL. Each trial consists of a rewarded sample phase and a rewarded choice phase, separated by a delay of 1 s (training session) or 3-5 s (challenging session). If the performance accuracy reached 70% for 30 trials per hour in the challenging session, the test session, consisted of 30 trials, started on the following day. At 30 min prior to the test session, PBS or TPP was infused into the PL. Performance in the DMP task did not differ between the two groups e, Performance accuracy before and after prelimbic injection of PBS (n = 5; blue) or TPP<sup>+</sup> (n = 6; red circles) during test sessions over four days. PBS (blue circles) or TPP<sup>+</sup> (red circles) was injected on Day 3 (A vertical dashed line). f, Delay time between sample and choice phases of PBS and TPP injected groups. g, Stimulus response latency of PBS and TPP injected groups. All data are mean  $\pm$  S.E.M.

Fig. S7

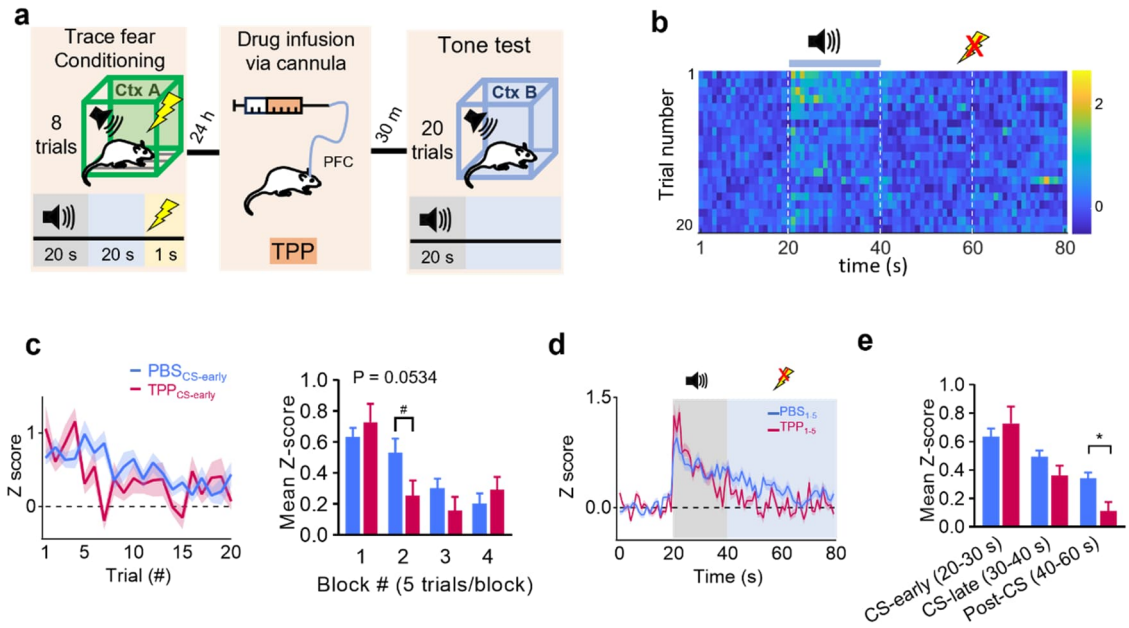


Fig. S7. TPP<sup>+</sup> infusion into the PL prior to tone tests (Day 2) resulted in accelerated trace fear extinction similar to the TPP<sup>+</sup> infusion on Day 1. **a**, Procedure for behavioral tests. **b**, Time course of mean CS cell activities for 20 trials of the tone test. CS cell is defined as the same as Fig. 2. For each trial of the tone test, time series of 1 s binned z-scores were averaged over CS cells (TPP<sup>+</sup>,  $n = 21/77$  neurons from 5 rats). **c**, *Left*, Trial-dependent changes of CS cell activity during first 10 s (CS-early) and late 10 s (CS-late) of tone presentation in the TPP group. *Right*, Mean z-scores of CS cells during CS-early period (20-30 s) across five trial blocks (5 trials/block). LME models (TPP vs. PBS):  $F_{1,12} = 0.480$ ,  $P = 0.0534$ , for trial block 2. **d**, First five trials average of time-dependent mean CS cell activity. Mean z-scores of CS cells of each trial were averaged over five trials and displayed as a function of time. **e**, Mean z-scores of CS cells for three epochs (CS-early, CS-late and post-CS periods) of first five trials in PBS and TPP animals. LME models of 1 s binned CS cell activities (PBS vs TPP): for post-CS period (40-60 s),  $F_{1,12} = 8.467$ ,  $P = 0.0131$ . The PBS data in c-e were reproduced from Fig. 2 for comparison. Post hoc comparison was done using Bonferroni correction to determine the statistical difference between groups at each time point. All data are mean  $\pm$  S.E.M., Shaded areas represent S.E.M., \* $P < 0.05$ , \*\* $P < 0.01$ .

Fig. S8

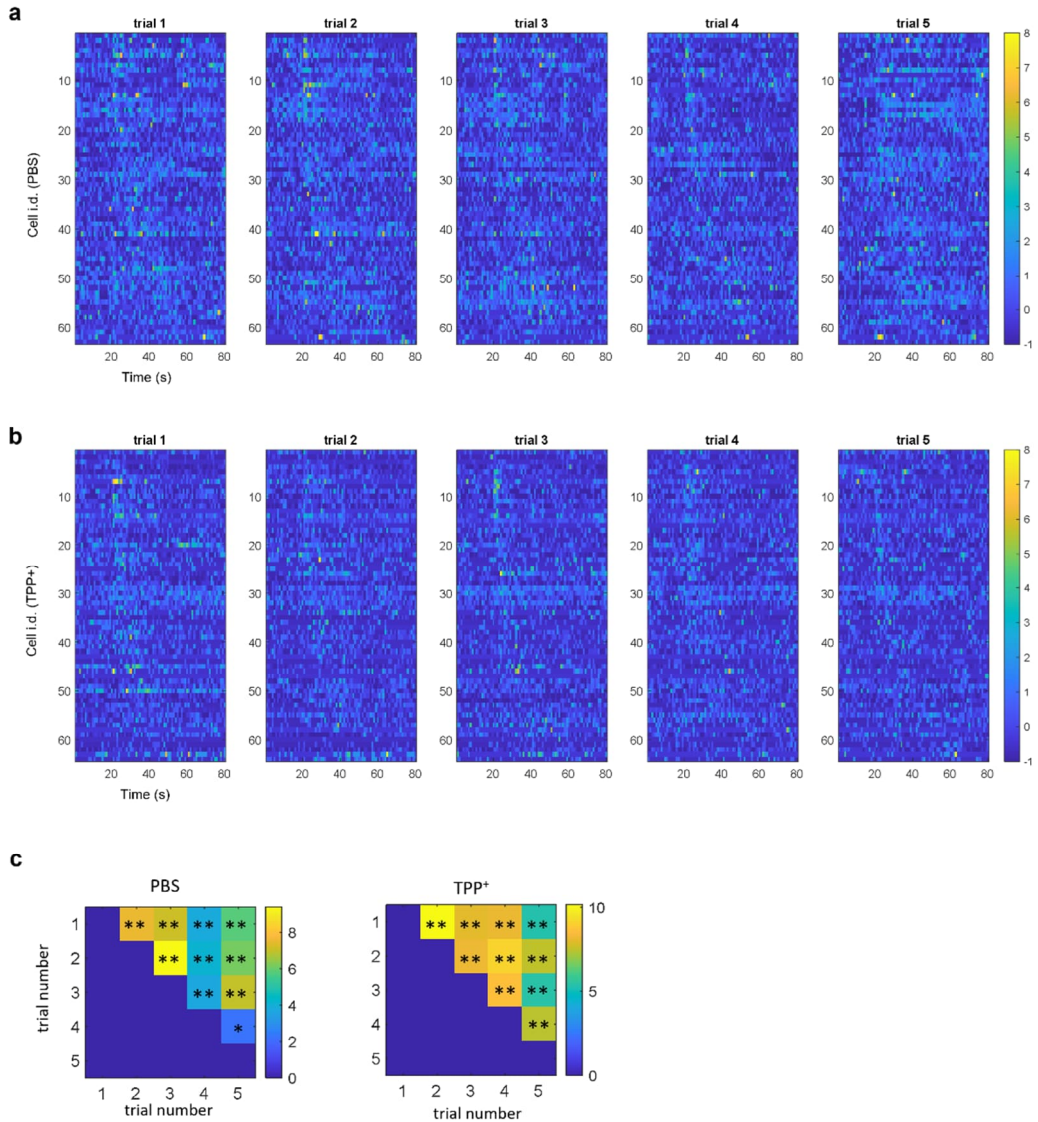


Fig. S8. Firing patterns of CS-responsive cells were consistent between trials. a-b, Firing sequence of CS-responsive cells over first five trials of tone test from PBS(a) and TPP(b)-treated animals. CS-responsive cells include CS cells, PA cells and cells displaying  $z_t > 3.09$  (or  $p < 0.001$ ) at any 1 second bins of CS and post-CS periods. These cells were sorted and numbered according to the peak timing of trial-averaged activity. Heat map of the numbered cell activities in z-score are shown across first five trials of tone test. c, Cosine similarity of population vectors summed over CS and post-CS periods (20 - 80 s) in a and b) between first five trials of tone tests. For each of trial-to-trial, the summed cosine similarity was compared with those calculated from 1000 data

sets, in which cell orders were shuffled. The statistical significance of summed cosine similarity is shown as p values indicated by \* ( $p < 0.05$ ) and \*\* ( $p < 0.01$ ) for PBS (*left*) and TPP (*right*) groups.

Fig. S9

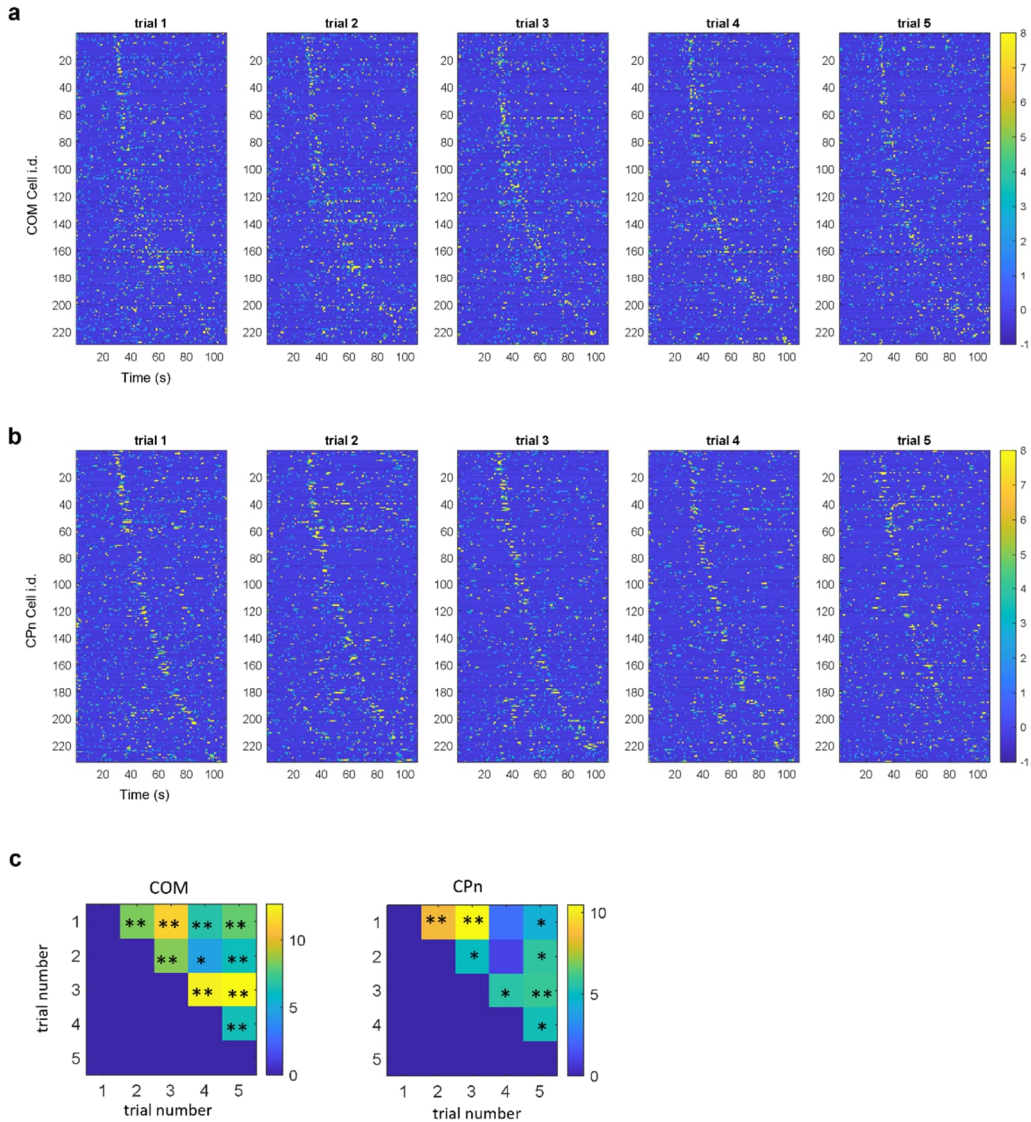


Fig. S9. The same as Fig. S8 but from COM and CPn cells. a-b, Firing sequence of CS-responsive cells over first five trials of tone test from COM (a) and CPn(b) cells of the prelimbic cortex. Heat map of the numbered cell activities in z-score are shown across first five trials of tone test. c, Cosine similarity of population vectors summed over CS and post-CS periods (30 - 100 s in a and b) between first five trials of tone tests. The statistical significance of summed cosine similarity was calculated as in Fig. S8, and shown as p values indicated by \* ( $p < 0.05$ ) and \*\* ( $p < 0.01$ ) for COM (left) and CPn (right) cells.