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30 Abstract

31 Dysfunction of NCLX, a major mitochondrial calcium efflux pathway, is one of the earliest signs of
32 neurodegenerative disease. Previously we have shown that layer 2/3 (L2/3) to corticopontine
33 (CPn) synapses of the prelimbic area of medial prefrontal cortex are specifically affected by NCLX
34 inhibition, resulting in inhibition of mitochondria-dependent post-tetanic potentiation (mitoPTP).
35 We investigated cognitive consequence of NCLX inhibition in the prelimbic area (PL), focusing on
36 trace fear conditioning, a form of short-term memory task. The PL is essential for trace memory
37 formation and interferes with extinction learning. Conditioned stimulus (CS) inputs to the trace
38 fear conditioned rats triggered bursting of layer 2/3 pyramidal neurons, which readily induces
39 mitoPTP at synapses onto CPn neurons. In rats undergoing extinction training, two
40 mechanistically distinct mitoPTP inhibitors, tetraphenylphosphonium (TPP^+) and Ru360,
41 accelerated extinction learning without affecting trace fear memory formation. Optogenetic
42 inhibition of CPn neurons, but not commissural (COM) neurons, phenocopied these effects. In
43 vivo electrophysiological and Ca^{2+} imaging studies revealed that, among CS-responsive cells, CPn
44 but not COM cells displayed persistent activity after the CS offset, and that TPP^+ reduces the
45 persistent activity of CPn neurons. These findings identify not only the cognitive consequences of
46 NCLX inhibition but also a cell type- and local circuit-specific mechanism in the PL that selectively
47 gates extinction learning by regulating mitoPTP.

48

49 Key words

50 Mitochondria, NCLX, Post-tetanic potentiation, Prelimbic cortex, Cortico-pontine neurons, Trace
51 fear conditioning, Extinction learning

52

53 Introduction

54 Presynaptic terminals are exposed to high calcium concentration ($[Ca^{2+}]$) during high frequency
55 synaptic transmissions. Mitochondria play a key role in Ca^{2+} homeostasis of presynaptic terminals
56 by buffering high $[Ca^{2+}]$ ¹. Mitochondrial dysfunction has been implicated in a variety of
57 neurodegenerative diseases². Presynaptic mitochondria absorb Ca^{2+} during high frequency
58 stimulation via mitochondrial Ca^{2+} uniporter (MCU), and the absorbed calcium is released into
59 cytosol via mitochondrial Na^+/Ca^{2+} exchanger (mNCX or NCLX) after cessation of tetanic
60 stimulation. Such mitochondrial Ca^{2+} release elevates presynaptic basal $[Ca^{2+}]$, which in turn
61 increase vesicular release probability^{1, 3, 4}. Because post-tetanic elevation of presynaptic $[Ca^{2+}]$ is
62 sustained for tens of seconds, it results in post-tetanic potentiation, a form of short-term
63 plasticity in which synaptic weight increases for a comparable time span after tetanic stimulation³.
64 ⁴. We refer to this form of PTP as mitochondria-dependent post-tetanic potentiation (mitoPTP).

65 NCLX, a major mitochondrial Ca^{2+} efflux pathway, is inhibited by mild mitochondrial
66 depolarization⁵, which is one of the earliest signs of pre-symptomatic Alzheimer model mice⁶.
67 Moreover dysfunction of NCLX has been found in knock-out mice of PINK1, a gene linked to
68 Parkinson disease (reviewed in ref. ⁷). Two cardinal functions of mitochondria are ATP supply and
69 buffering high $[Ca^{2+}]$. Different from impaired ATP supply, which exerts neuron-wide effects due
70 to intracellular high diffusion rate of ATP⁸, altered Ca^{2+} buffering results in synapse-specific effects
71 because Ca^{2+} is under tight spatial regulation and only half of boutons are harboring
72 mitochondria⁹. Therefore, it is crucial to address which kinds of synapse type and cognitive
73 functions are impaired by dysfunction of NCLX to understand the earliest signs of
74 neurodegenerative disorders.

75 Prelimbic pyramidal neurons in layer 5 (L5) are categorized by their projection targets:
76 commissural (COM) and corticopontine (CPh) neurons, which project to the contralateral
77 prefrontal cortex and the pontine nuclei, respectively¹⁰. We previously found that PTP occurs
78 selectively at two types of local synapses targeting prefrontal CPh cells: one is from L5 COM cells
79 and the other from L2/3 pyramidal neurons¹¹. Among these two types of synapses, only the latter
80 (L2/3-CPh synapses) exhibited robust presynaptic mitochondrial Ca^{2+} release after tetanic

stimulation. Notably, mitoPTP did not occur in other neocortical areas including the infralimbic cortex¹¹.

Conceptually PTP, by itself or by supporting persistent activity, can maintain task-relevant information during the delay period of short-term memory tasks, in which requires associative learning between events that are temporally separated by a delay period^{12, 13}. The prelimbic cortex (PL) is essential for short-term memory tasks, and impedes the extinction of previously learned associations^{14, 15}. A subpopulation of neurons in the PL persistently fire during the delay period^{14, 16}, which may convey information about the preceding event, thereby bridge events that are temporally separated. The information carried during a delay period is different, however, depending on the cell-types^{16, 17}. For example, intra-telencephalic cells encode task-relevant information during a delay period of a short-term memory task, but pyramidal tract cells do not despite that they display robust persistent activity¹⁷. The role of persistent activity that is not directly relevant to a task performance is still elusive. One of possibilities is that persistent delivery of already learned information may hinder the modification of existing associations. Therefore, a circuit-specific approach to determine how persistent activity contributes to extinction learning will uncover the dynamic function of the PL. The current understanding of PL local circuitry, however, lacks a causal relationship between PTP and persistent activity and their roles in associative learning and its extinction.

In this study, we tackled this issue using tetraphenylphosphonium (TPP⁺), which inhibits PTP at prelimbic L2/3-CPh synapses by blocking post-tetanic mitochondrial Ca²⁺ release with little adverse effects on intrinsic electrical properties, presynaptic Ca²⁺ current and mitochondrial potential^{1, 4, 11}. Convergent approaches combining in vivo electrophysiology, cell-type specific calcium imaging and optogenetic manipulations revealed that mitoPTP contributes to the persistent activity of prelimbic CPh neurons and interferes with extinction learning, but not associative learning.

107 Materials and Methods

108 *Ethical statement*

109 All procedures were approved by the Seoul National University Institutional Animal Care and Use
110 Committee (SNU-200831-3-3, SNU-090115-7-9).

111 *Animals*

112 Sprague Dawley rats (21-56 days; main study) and C57BL/6J mice (16-18 weeks; working memory)
113 were housed at 21 °C and 65% humidity under a 12-h light/12-h dark cycle. Rats had food and
114 water ad libitum, mice were food-restricted to 85-90% of age-matched weight.

115 *Stereotaxic surgery*

116 Cannula implantation surgery: Bilateral guide cannulae (PlasticsOne, USA) were implanted into
117 the PL (rat: AP 2.3, ML ± 0.6 , DV -3.4; mouse: AP 1.8, ML ± 0.5 , DV -1.6) and cemented, locations
118 are illustrated in Figure S1. Two weeks later, TPP⁺ or PBS was infused (1 μ l/side, 100 nl/min, 10
119 min) through a 33 G cannula left in place 5 min, and behavior was tested 30 min later.

120 Cell-type specific optogenetic inactivation surgery: For CPn inactivation, AAV2-retro-EF1a-Cre
121 (Addgene #55636) and AAV5-CAG-DIO-ArchT-tdTomato (Addgene #28305) were bilaterally
122 injected into the pons and PL, respectively. For OOM inactivation, AAV2-retro-EF1a-Cre with
123 AAV5-CAG-fDIO-ArchT-GFP (Addgene #124640) and AAV2-retro-EF1a-Flpo (Addgene #55637)
124 with AAV5-CAG-DIO-ArchT-tdTomato were injected into contralateral and ipsilateral PFC,
125 respectively; controls received AAV5-CAG-DIO-EGFP-WPRE (Addgene #51502). Under 5% (v/v)
126 isoflurane, rats were craniotomized and virus (1 μ l) was injected at 100 nl/min (pons: AP -7, ML
127 ± 1 , DV -10; PL: AP 2.3, ML ± 0.6 , DV -3.4), leaving the syringe 5 min. Two weeks later, optic fibers
128 (RWD, China) were bilaterally implanted into the PL for 593 nm illumination.

129 Shuttle drive surgery: An in vivo electrode drive (shuttle drive) was built according to Voigts et al
130 (2020) with modifications¹⁸, combining 16 stereotrodes of nichrome wire, a 3D-printed body and
131 an electrode interface board (EIB). Wires and skull screws were connected to the EIB with gold

132 pins. For TPP infusion experiments, a 26-gauge stainless tube was passed through the EIB and
133 body (Figure S2a). Bundles were gold-plated to 200–300 k Ω .

134 Rats were anesthetized, fixed in a stereotaxic apparatus and disinfected as above. The craniotomy
135 site was marked (PL: AP 2.3, ML 1.6, DV -3.5, Angle 16°) and the stereotrode bundle was slowly
136 lowered to 3.5 mm. Ground and reference screws were placed over the cerebellum and olfactory
137 bulb, and skull and drive were fixed with dental cement.

138 Cell type specific in vivo calcium imaging surgery: Lenses were implanted in two ways. 1) AAV2-
139 Cre was injected into the pons or contralateral PFC and AAV-flex-GCaMP6f (Addgene #100833)
140 into the PL, and a GRIN lens (Inscopix, USA) was implanted a week later. 2) AAV2-Cre was injected
141 alone and, one week later, after applying AAV-flex-GCaMP6f to the surface of the GRIN lens using
142 silk fibroin, the lens was implanted in the PFC. After craniotomy, cortical tissue was aspirated and
143 the lens lowered to 3.5 mm, then cemented. Rats received dexamethasone (1 mg/kg)
144 postoperatively.

145 *Behavior*

146 Contexts for behavior tests: Fear conditioning was performed in Context A, a rat conditioning
147 cage with a speaker and a grid floor for footshock (Coulbourn Instruments, USA). Tone tests used
148 Context B for behavior tests after TPP⁺ or PBS infusion, and Context B', inside a sound-attenuating
149 box, for in vivo recording and optogenetic inactivation experiments. Behavior was recorded by
150 CCD camera and freezing quantified with ezTrack¹⁹.

151 Weak and strong fear conditioning: For freezing behaviors (Figure 1a and Figure S6a), rats
152 received five weak pairings of tones (2.5 kHz, 20 s, 75 dB) and footshock (0.5 mA, 1 s), with a 20
153 s trace interval for trace conditioning (Figure 1a) or co-termination for delayed conditioning
154 (Figure S6a). For prelimbic recording on Day 2 (Figure 2-6 and Figure S7), a strong protocol of
155 eight trace pairings (0.7 mA) was used, with twenty tones in context B. The strong protocol also
156 served the optogenetic study (Figure 1g-h) and drug infusion on Day 2 (Figure S5), with retrieval
157 tested on Day 3.

158 Drug infusion into the prelimbic cortex: Fear conditioning began two weeks after cannula
159 implantation. TPP, Ru360 (both Sigma, USA) or PBS was infused into the PL 30 min before
160 behavior test. In all experiments except those of Figure S5, infusion preceded conditioning; for
161 drug infusion prior to tone test (Figure S5), it preceded the tone test on Day 2.

162 Optogenetic inactivation: For cell type specific inactivation (Figure 1g), trace fear conditioning
163 began two weeks after fiber implantation. Fibers were connected to a 593.5 nm laser
164 (Lasercap, China) at 9-10 mW. Under the strong protocol, the prelimbic area was illuminated
165 during trace and post-CS period of conditioning (Day 1) and tone test (Day 2), respectively, to
166 investigate the role of persistent activity of CPn and COM neurons. On Day 3, rat received ten
167 tones without illumination to test retrieval of extinction memory.

168 Operant delayed match to position (DMTP): Working memory was evaluated with an operant
169 DMTP task in which a nose poke matching the sample location was rewarded with Kool-Aid (0.4
170 g/ml). Animals progressed through habituation, pretraining, match, 1 s and 4 s DMTP stages at 30
171 trials/h with 70% success, then performed 4 s DMTP for two days, received PBS or TPP 30 min
172 before the next session, and continued for two more days.

173 *Unit recordings and data processing*

174 One week after implantation, rats acclimated for 15 min before trace fear conditioning. Prelimbic
175 activity was recorded through a 32-channel RHD headstage (Intan, USA) with Open Ephys
176 software at 30 kHz and a 500 to 5000 Hz bandpass filter. Behavior was recorded by CCD camera
177 and synchronized with tones (Bonsai software). After recording, 30 μ A was applied for 8 s to
178 locate the bundle tip; brains were post-fixed in 4% paraformaldehyde (PFA), sectioned at 300 μ m
179 and imaged with a fluorescence microscope.

180 For spike sorting, we modified a Matlab package on github
181 (<https://github.com/nghorbani/NeuralDataAnalysis>). Recordings from -20 s to +60 s of all trials
182 were concatenated with tone onset as zero and spikes detected by amplitude thresholding at 5
183 σ , where σ was the median absolute deviation of the filtered signal divided by 0.6754²⁰. Principal
184 component features were fitted with a Gaussian mixture model using the split-and-merge

185 expectation-maximization algorithm²¹, followed by manual sorting. Spikes wider than 200 μ s with
186 firing rates below 15 Hz were regarded as putative pyramidal neurons and used for further
187 analysis (Figure S2b-e).

188 To select cells with high activity during CS and post-CS period (20 - 40 s and 40 - 60 s in Figure 2c),
189 we calculated z scores for firing frequencies and interspike intervals (ISIs), z_f and z_{isi} , relative to
190 baseline (pre-CS). z_{isi} was obtained from a test for comparing two Poisson means (Krishnamoorthy
191 and Thomson 2004):

192
$$z_{isi} = (\lambda_1 - \lambda_2) / \sqrt{(\lambda_1 / N_1 + \lambda_2 / N_2)}$$

193 , where λ_1 and λ_2 are the mean ISIs during baseline and during CS or Post-CS, and N the sample
194 size. Cells satisfying one of two following criteria during CS or post-CS period were classified as CS
195 and PA cells, respectively: (1) $z_f > 1.96$ ($p < 0.05$); (2) $z_{isi} > 1.96$ and $z_f > 1.28$ ($p < 0.1$).

196 For CS-responsive cells in Figures 3-4, we selected putative pyramidal cells which satisfy at least
197 one criterion among following three conditions: (1) epoch z_f $p < 0.05$; (2) epoch isi $p < 0.05$ and
198 epoch z_f $p < 0.1$; (3) z_f $p < 0.001$ (or $z_f > 3.09$) in any of 1 s bins during the CS and post-CS periods.
199 Peak timing of time-encoding cells was found from Gaussian-filtered z score ($\sigma = 4$ s), and firing
200 sequence consistency was tested by comparing summed cosine similarity of population vectors
201 with 1000 shuffles of cell indices (Figures S8c and S9c).

202 Whole-cell patch recording

203 Brain slices were prepared from Sprague Dawley rats (21-28 days). Brains were chilled in ice-cold
204 low-calcium artificial CSF (aCSF) containing (in mM): 110 choline chloride, 10 glucose, 25 NaHCO_3 ,
205 2.5 KCl, 1.25 NaH_2PO_4 , 2 Na-pyruvate, 3 ascorbate, 0.5 CaCl_2 and 7 MgCl_2 , pH 7.4 saturated with
206 carbogen (95% O_2 , 5% CO_2). Coronal prefrontal and visual cortex slices (300 μ m) were cut with a
207 vibratome (Leica, Germany), recovered at 36 $^\circ\text{C}$ for 30 min and kept in standard aCSF, identical
208 except for 125 NaCl in place of choline chloride, 1.3 CaCl_2 and 1 MgCl_2 , also perfused during
209 recording.

210 Whole-cell current clamp and voltage clamp recordings were performed at 32 $^\circ\text{C}$ using an EPC-10
211 (HEKA, Germany) or Integrated Patch Clamp amplifier (SUTTER instrument, USA) at 10 kHz.

212 Recording pipettes (3-5 M Ω) were filled with (in mM): 130 K-gluconate, 8 KCl, 20 HEPES, 4 MgATP,
213 0.3 NaGTP, 1 MgCl₂ and 0.1 EGTA, pH 7.3 with KOH. EPSCs were recorded from the PL at -65 mV;
214 data in which series resistance exceeded 20 M Ω or changed (>20%) were discarded. Monophasic
215 pulses (2-15 mV, 100 μ s/pulse) were delivered through an aCSF-filled pipette placed in L2/3 within
216 300 μ m from the soma of a postsynaptic L5 cell.

217 For PTP induction, baseline EPSCs of around 100 pA were evoked by paired pulses (Inter-spike
218 Intervals, 50 ms) every 5 s 20 times. PTP was induced by stimulation mimicking the firing patterns
219 of an exemplary CS responsive L2/3 pyramidal neuron during CS period (Figure 5m), after which
220 paired pulse stimulations were resumed for 50 times.

221 In vivo Ca²⁺ imaging and data processing

222 Three weeks after virus injection, rats were subject to trace fear conditioning, and the next day
223 the Miniscope (V4; Open Ephys, Portugal) was docked to the baseplate. Signals were recorded
224 with Miniscope DAQ software (Aharoni Lab, UCLA) at 25 frames per second and 600 $\times$ 600 pixels.
225 To reduce photobleaching, signal was recorded separately per trial, from 30 s before cue onset
226 for 110 s. To confirm GCaMP6f expression and lens location, coronal prefrontal sections (300 μ m)
227 were imaged with a fluorescence microscope.

228 Videos were down-sampled to 300 $\times$ 300 pixels in ImageJ (NIH, USA), concatenated over five tone
229 trials, and motion-corrected using NoRMCorre²² in MATLAB. Data were then processed with a
230 constrained non-negative matrix factorization algorithm optimized for microendoscopic data
231 (CNMF-E)²³, in which ROIs were merged or excluded by spatial overlap, centroid distance, peak-
232 to-noise ratio and spike size, and spiking activity inferred by foopsi deconvolution. Neurons were
233 visually inspected and those with non-neuronal morphology removed. After binarizing non-zero
234 values, the first five tone test trials were averaged to reduce trial-to-trial variability.

235 Statistics

236 Data was analyzed with Igor Pro 7, SPSS, Prism, MATLAB and R; sample sizes and tests are given
237 in the figure legends. Freezing was analyzed by repeated measures two-way ANOVA with Tukey
238 and Bonferroni post hoc tests. Mean z-scores of PBS and TPP groups during CS1, CS2 and Trace

239 (or post-CS) were compared with a linear mixed effects model (time and drug as fixed effects;
240 animals and neurons as random effects)²⁴, fitted with “nlme”²⁵ and compared with “emmeans”²⁶.
241 Wilcoxon matched-pairs signed rank tests were used for paired groups and chi-square tests for
242 proportions. Significance: n.s., $p > 0.05$; * $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Data
243 are mean $\pm$ s.e.m.

244

245 Results

246 *Inhibiting prelimbic mitoPTP accelerates trace fear memory extinction*

247 We investigated trace fear conditioning and its extinction in rats, a form of Pavlovian associative
248 learning in which animals should associate conditioned stimulus (CS, tone) and unconditioned
249 stimulus (US, electric shock), traced by a 20 s interval. Prelimbic persistent activity occurs during
250 trace fear conditioning, and optogenetic silencing of the prelimbic cortex during the trace interval
251 impairs trace fear memory acquisition^{16,27}. To determine whether prelimbic mitoPTP contributes
252 to persistent activity and associative learning, we examined the effects of TPP⁺, an inhibitor of
253 NCLX (mitochondrial Na⁺/Ca²⁺ exchanger), on prelimbic local network activity and trace fear
254 conditioning¹.

255 To test if there is any adverse effect of TPP⁺ in the prelimbic cortex¹, we conducted patch clamp
256 experiments in rat brain slices and examined whether TPP⁺ affects the intrinsic electrical
257 properties of prelimbic neurons. The bath application of 2 μ M TPP⁺ did not exert significant
258 impacts on input resistance, action potential (AP) threshold, and resting membrane potential of
259 pyramidal neurons in layer 2/3, and 5, and of fast-spiking interneurons in layer 5 (Figure S3).
260 Furthermore, we made slices in rats infused with 1 μ L of 20 μ M TPP⁺ into the prelimbic cortex.
261 This in vivo treatment of TPP⁺ abolished PTP ex vivo at L2/3-CPh synapses within 300 μ m, but not
262 farther away, from the infusion site (Figure S4).

263 We next examined the effect of TPP⁺ on trace fear conditioning in rats. TPP⁺ (20 μ M) or its vehicle
264 (phosphate-buffered solution, PBS, 1 μ L each) was injected into the prelimbic area 30 minutes
265 before trace fear conditioning on Day 1. Rats underwent five trials of CS-US pairing in context A

266 (Figure 1a). Both groups displayed similar freezing levels during conditioning (Figure 1b). On the
267 following day, rats were presented ten times with the CS alone (called tone test) in a distinct
268 context (context B) to measure the formation and extinction of trace fear memory (Figure 1a).
269 The TPP⁺-treated group exhibited an accelerated extinction of conditioned fear. The freezing of
270 the TPP group began to decrease earlier compared to that of the PBS group (Figure 1b, c). It
271 should be noted, however, that the freezing behavior of the TPP group was similar to that of the
272 PBS group for the first three trials (Figure 1b, c), suggesting that the acquisition and retrieval of
273 trace fear memory were not affected, but its extinction was accelerated by TPP infusion.

274 Due to negative mitochondrial potential, positive-charged TPP is effectively sequestered within
275 the mitochondria with minimal washout over time, prompting us to hypothesize that accelerated
276 extinction on Day 2 results from persisting effects of TPP⁺. To test this, we examined the effects
277 of TPP⁺ injected on Day 2. Separate groups of rats underwent trace fear conditioning consisting
278 of eight CS-US pairings. The following day, rats received prelimbic injection of either TPP or PBS
279 30 minutes prior to tone test (Figure S5a). Similar to the effects of Day 1 injection (Figure 1b),
280 freezing levels in the TPP group began to decline earlier than those in the PBS group. Notably,
281 both groups exhibited comparable freezing levels during the first few trials on Day 2 (Figure S5b).
282 These findings suggest that TPP injected on Day 1 persists in mitochondria, exerting an inhibitory
283 effect on mitoPTP through Day 2. Furthermore, we assessed the retrieval of extinction memory
284 on Day 3 by observing fear responses to the tone in Context B. The TPP group displayed
285 significantly lower freezing levels than the control group, indicating that lower freezing on Day 2
286 results from facilitation in extinction memory formation rather than a deficit in fear expression²⁸
287 (Figure S5c).

288 While both TPP⁺ and Ru360 block mitoPTP⁴, they operate through distinct mechanisms: TPP
289 inhibits mitochondrial Na⁺/Ca²⁺ exchanger, a primary pathway for post-tetanic Ca²⁺ release,
290 whereas Ru360 blocks mitochondrial Ca²⁺ uptake via MCU during tetanic stimulations^{1, 29, 30}. To
291 confirm that inhibition of mitoPTP is responsible for the behavioral impacts of TPP⁺, either Ru360
292 (100 nM) or PBS (1 μL each) was infused into the prelimbic cortex of trace fear-conditioned rats
293 30 minutes prior to the tone test on Day 2. Ru360-treated rats exhibited significantly accelerated
294 extinction of conditioned fear responses compared to controls without affecting initial fear

memory retrieval (Figure S5d). Moreover, lower freezing levels on Day 3 support that prelimbic infusion of Ru360 accelerated fear extinction learning (Figure S5e). As expected, in vitro study revealed that bath-applied 10 nM Ru360 suppressed mitoPTP at L2/3-CPh synapses (Figure S5g, h) without affecting intrinsic properties (Figure S5i-k). Collectively, these findings demonstrate that two mechanistically distinct pharmacological agents targeting mitoPTP resulted in the same behavioral outcome, suggesting that mitoPTP acts as a constraint on extinction learning.

TPP⁺ has little effect on delayed fear conditioning and a working memory task performance

To further test if TPP⁺ treatment has any effect on fear expression per se, we performed delayed fear conditioning, in which CS and US are temporally contiguous (Figure S6a). The prelimbic cortex is required for fear expression in this task, while it is dispensable for conditioning²⁸. The TPP group showed no significant difference from the PBS group in acquisition of delayed fear memory and fear extinction (Figure S6b-c).

The prelimbic area is thought to hold task-relevant information during a trace period not only in trace fear conditioning but also in working memory tasks^{14, 17, 31}. Given that TPP⁺ has little effect on CS-US association during trace fear conditioning, we performed an operant delayed-match-to-position working memory task to test whether TPP⁺ has any effects on the ability to hold task-relevant information during a trace period (Figure S6d). Rats treated with either TPP⁺ or PBS similarly performed in the working memory task (Figure S6e-g), indicating that TPP⁺ has little effect on maintenance of working memory. Overall, these findings suggest that blocking prelimbic mitoPTP at L2/3-CPh synapses specifically accelerates extinction learning after trace, but not delayed, fear conditioning with minimal effects on acquisition of trace or delayed fear memory and the performance of a working memory task.

320 *Silencing prelimbic corticopontine neurons accelerates fear memory extinction*

321 Given that TPP⁺ treatment impairs mitoPTP specifically at L2/3-CPh synapses¹¹, we hypothesized
322 that the TPP⁺ effects on extinction learning are mediated by preferential inhibition of CPh
323 neurons. To test this, we performed cell type-specific optical silencing experiments. We injected
324 AAV-DIO-ArchT-GFP or AAV-DIO-GFP into the prelimbic cortex, and retro-AAV-Cre into the pons
325 or the contralateral prelimbic cortex to express the inhibitory opsin ArchT specifically in CPh and
326 COM neurons, respectively (Figure 1d, e). To express ArchT in COM neurons on the contralateral
327 prelimbic cortex, we used a pair of fDIO-ArchT-GFP and retro-AAV-flipase (Figure 1e). For the
328 control group, AAV-DIO-GFP and retroAAV-Cre were injected into the prelimbic cortex and pons,
329 respectively. As expected, CPh neurons were labeled only in L5, and COM neurons were observed
330 both in layers 3 and 5 of the prelimbic cortex (Figure 1d, e). We confirmed that ArchT-mediated
331 hyperpolarizing photocurrents successfully inhibited the firing of the labeled neurons (Figure 1f).

332 Rats were habituated to the conditioning chamber and presented with eight CS-US pairing trials
333 for trace fear conditioning. We optogenetically silenced ArchT-expressing CPh or COM neurons
334 during the trace interval of trace fear conditioning (Day 1) and the post-CS period of the tone test.
335 For the tone test, tone alone was presented 20 times in a distinct context (Day 2) (Figure 1g).
336 Optogenetic silencing of either cell type did not affect freezing behaviors during trace fear
337 conditioning and the first five trial block of the tone test, compared with the control group (Figure
338 1h-i). On the other hand, silencing CPh cells, not COM cells, accelerated the extinction of
339 conditioned fear, an effect similar to the TPP treatment (Figure 1h-i). The day after extinction
340 training (day 3), the CPh-silenced group showed significantly lower freezing during the initial two
341 trials compared with the other groups, further confirming that silencing CPh neurons facilitates
342 extinction learning (Figure 1h-i). Overall, these findings imply that TPP treatment accelerates
343 extinction learning by inhibiting CPh neurons as silencing CPh neurons phenocopied TPP
344 treatment.

345

346 *TPP⁺ inhibits persistent activity and accelerates extinction of prelimbic CS responses*
347 *during tone tests*

348 To directly measure the effect of TPP⁺ on prelimbic neuronal activity during extinction learning,
349 we performed in vivo electrophysiological recordings (Figure 2a). Behavioral procedures were the
350 same as those for optogenetic experiments (Figure 1g). TPP⁺ or PBS was injected into the
351 prelimbic cortex 30 min before trace fear conditioning on Day 1. On the next day (Day 2), we
352 examined the activity of prelimbic neurons during the tone test (Figure 2a). The CS responses of
353 prelimbic neurons dissipated over trials, and the trial-dependent changes were accelerated by
354 TPP⁺ infusion (Figure 2b), reflecting earlier decline of freezing behavior during the tone test in the
355 TPP-treated and CPn-silenced animals (Figure 1b and 1h). Based on z scores for firing frequencies
356 (z_f) and interspike intervals (z_{si}) relative to their baseline values, neurons displaying distinctly high
357 activity during 20 s tone presentation (CS period) of the first block (five trials/block) of the tone
358 test were categorized into CS cells (see Methods). The time course of mean CS cell activity over
359 each of 20 trials of the tone test is shown in Figure 2c. When CS cell activity during the first half
360 of CS period (CS-early phase) was analyzed, the activity of the TPP group was similar for the first
361 five trials but decayed earlier than that of the PBS group (Figure 2d-e). To examine the effects of
362 TPP⁺ on the time course of CS cell activity before their CS responses decline, we compared their
363 activity time courses averaged over the first block (trials 1-5) between two groups (Figure 2f).
364 Whereas two groups exhibited similar initial response to the tone presentation, CS cell activity in
365 the TPP group decayed faster resulting in significantly lower activity during the second half of CS
366 period (CS-late phase) and post-CS period (Figure 2g). Accordingly, when we define neurons
367 exhibiting distinctly high activity during the post-CS period as persistently active (PA) cells, the
368 proportion of PA cells among CS cells was lower in the TPP group (PBS, 26/60; TPP, 4/32; $p =$
369 0.0056), whereas the proportion of CS cells in the total number of cells was not different between
370 two groups (PBS, 60/186; TPP, 31/124; $p = 0.2122$, Figure 2h). Notably, similar effects were also
371 observed when TPP⁺ was delivered 30 minutes before the extinction learning task (Figure S7).
372 Collectively, these results indicate that TPP⁺ treatment selectively reduces CS-triggered persistent
373 activity of prelimbic neurons without affecting early CS response. Together with the finding that
374 TPP⁺ treatment accelerates extinction learning on Day 2 (Figure 1), these results suggest that

375 prelimbic persistent activity during the tone test may play a role in maintaining CS-US associative
376 memory, impeding extinction learning.

377

378 *Early CS-responsive cells showing persistent activity belong to CPn cells*

379 To characterize the timing of peak activity of TPP⁺-sensitive CS-responsive cells, we ordered
380 putative pyramidal neurons displaying distinctly high activity during CS (20 s) and post-CS period
381 (40 s) according to the timing of their peak activity. For this analysis, we included cells displaying
382 $z_i > 3.09$ (or $p < 0.001$) at any 1 second bins of CS and post-CS periods as well as CS and PA cells
383 (collectively called CS-responsive cells, see Methods). The firing sequence was well preserved
384 across first five trials of the tone test (Figure S8). CS-responsive cells were classified into CS-early,
385 CS-late and Delay cells, which have a timing of peak activity during early and late 10 s of CS
386 presentation and 40 s post-CS period of the tone test, respectively (Figure 3a-c). The proportions
387 of these three cell types were not altered by TPP⁺ treatment (Figure 3c). The time course of mean
388 neuronal activity in each cell type, however, revealed that TPP⁺ selectively reduced the persistent
389 activity of CS-early cells ($p = 0.017$, LME model, Figure 3d), while it did not affect the activity of
390 other cell types (Figure 3e-f).

391 Next, we examined cell type-specific Ca²⁺ spike activity during the tone test. The Ca²⁺ sensor
392 GCaMP6f was specifically expressed in CPn and COM neurons using the same viral approaches
393 described in Figure 1d-e (Figure 4a-b). The Ca²⁺ spike activity was recorded during the tone test
394 on Day 2 following trace fear conditioning, and analyzed using CNMF-E (Figure 4c-d)²³. The time
395 course of CS-responsive cell activity averaged over first five trials was not different between CPn
396 and COM cells (Figure 4e-f). We selected CS-responsive cells, and sorted according to their peak
397 activity timing. The patterns of firing sequence were largely consistent over first five trials (Figure
398 S9). The cells participating in the firing sequence were classified according to their peak activity
399 timing into CS-early, CS-late and Delay cells (Figure 4g-h). COM cells encompassed a significantly
400 larger number of CS-early cells compared to CPn cells ($p=0.001$) (Figure 4g-i). Time courses of
401 activity averaged over each cell type are compared between COM and CPn cells in Figure 4j-l. CS-
402 early cells exhibited strong activity immediately following the CS onset both in COM and CPn cells,

but the tone response of COM cells was rather transient compared to CPh cells (Figure 4j). Notably, post-CS persistent activity of CS-early cells was observed specifically in CPh cells but not in COM cells ($P = 0.0175$, LME model, Figure 4j), whereas post-CS activity in other cell types was not significantly different between COM and CPh cells (Figure 4k-l). Because TPP⁺ specifically reduced persistent activity of CS-early cells (Figure 3a-b), these findings suggest that the effects of TPP⁺ on the CS-early cells are specific to CPh cells.

The firing pattern of L2/3 pyramidal neurons induces mitoPTP in L5 CPh neurons

We previously showed that TPP⁺ inhibits post-tetanic potentiation evoked by 25 Hz stimulation at prelimbic L2/3-CPh synapses in vitro¹¹, but it remains to be tested whether CS-evoked L2/3 pyramidal neuronal activity in vivo can induce PTP in CPh neurons. We recorded L2/3 neuronal activity patterns during first five trials of the tone test in trace fear-conditioned rats (Figure 5a). The activity time course and proportions of CS and PA cells in L2/3 neurons were not different from those of layer-nonspecific recordings (Figure 5b-d). The mean firing rate of CS cells in L2/3 increased from 1.5 Hz to 2.7 ± 0.3 Hz in response to CS (Figure 5e-f). The distribution of inter-spike intervals (ISIs) averaged over CS cells revealed that ISIs shorter than 120 ms significantly increased in the CS period compared to the baseline (Figure 5g), indicating that burst firings are responsible for the CS-evoked increased activity. We used the firing pattern of an exemplar L2/3 CS cell during tone presentation as a stimulation template (Figure 5h-l), and tested whether this patterned stimulation of L2/3 induces PTP in CPh cells in vitro (Figure 5h-m). To mimic in vivo conditions, recordings were done at 32°C and 1.3 mM [Ca²⁺] in the bath. Under these conditions, PTP was readily induced at L2/3-CPh synapses (Figure 5n-p), and significantly reduced by bath-applying TPP⁺ (Figure 5n-p), suggesting that CS-evoked L2/3 prelimbic activity can induce mitoPTP at L2/3-CPh synapses of the prelimbic cortex.

TPP⁺ also reduces persistent activity during trace fear conditioning

Because TPP⁺ inhibited persistent activity during the tone test, we further examined whether TPP⁺ has similar effects during trace fear conditioning on Day 1 (Figure 6a). Rats were habituated to the conditioning chamber and presented with three trials of CS alone (trial 1-3). Then, the rats underwent subsequent eight CS-US pairing trials (trial 4-11) for trace fear conditioning. The CS-responsive activity emerged in the trial 5, whereas CS presentation alone (trial 1-3) did not alter prelimbic activity relative to baseline (Figure 6b-d). The effects of TPP⁺ on the network activity during trace fear conditioning were similar to those during the tone test. TPP⁺ treatment reduced the persistent activity of prelimbic neurons without affecting early CS responses (Figure 6e-f). The proportion of PA cells among CS cells were lowered by TPP⁺ (Figure 6g), whereas that of CS cells was not altered by TPP⁺ (59% vs. 55%), indicating that TPP⁺ specifically reduces persistent activity of prelimbic neurons in trace fear conditioning as well as in subsequent tone test.

Discussion

Post-tetanic potentiation at the prelimbic L2/3-CPh synapse is induced by post-tetanic mitochondrial Ca²⁺ release via mNCX, which is blocked by TPP⁺^{1, 3, 11}. In the present study we examined the network and behavioral consequences of inhibition of mitoPTP at these synapses using TPP⁺. TPP⁺ not only accelerated extinction learning but also selectively reduced persistent activity of CS-encoding CPh neurons during retrieval of trace fear memory. This notion is supported by in vivo electrophysiological recordings and Ca²⁺ imaging, which revealed that persistent activity of early CS-responsive cells (CS-early cells) was observed among CPh cells but not among COM cells, and it was selectively reduced by TPP⁺. Moreover, optogenetic silencing of CPh cells during post-CS period phenocopied the behavioral effects of TPP⁺. Finally, CS-evoked characteristic firing patterns of prelimbic L2/3 pyramidal neurons successfully induced mitoPTP at L2/3-CPh synapses. Together with our previous study¹¹, these results suggest that mitoPTP at prelimbic L2/3-CPh synapses maintains persistent activity of prelimbic CPh neurons during retrieval of trace fear memory, which may impede extinction learning.

455 The present study critically relies on the pharmacological agent TPP⁺, which blocks mNCX, the
456 primary pathway of post-tetanic mitochondrial Ca²⁺ release^{1, 4, 32}. Several lines of evidence,
457 however, validate our interpretation of TPP⁺ effects in the present study. First, previous studies
458 indicate that TPP⁺ specifically inhibits mitoPTP with little adverse effects. Low concentration TPP⁺
459 did not affect presynaptic AP shapes, Ca²⁺ current, mitochondrial respiration and short-term
460 plasticity at < 50 Hz in vitro ^{1,32}. In line with these findings, TPP⁺ had little effect on the intrinsic
461 properties including after-depolarization, firing frequency-current injection relationship, Input
462 resistance, resting membrane potential and AP threshold in L2/3 and L5 pyramidal cells and fast-
463 spiking interneurons (Figure S0). Second, TPP⁺ did not affect the early network response to CS
464 (Figure 2f, 3d, 6e), but it specifically reduced the delayed response, arguing against a possibility
465 that TPP⁺ has non-specific adverse effects. Third, our behavioral tests showed that the TPP⁺ effect
466 was specific to extinction learning with little effects on trace fear conditioning and the working
467 memory task, supporting little adverse effects of TPP⁺. Lastly, the behavioral phenotype induced
468 by TPP⁺ was recapitulated by Ru360, which inhibits mitoPTP through a mechanism distinct from
469 TPP⁺, inhibition of MCU ^{29, 4}. These findings suggest that the effects of TPP⁺, acceleration of
470 extinction learning are reduced persistent activity of CS cells, are mediated by mitochondrial
471 calcium signaling rather than off-target effects. Nevertheless, we cannot completely rule out the
472 adverse effects of TPP⁺ treatment, and further examinations should follow.

473 Activity and plastic changes in the prelimbic area are required for new memory formation of CS-
474 US association during TFC^{16, 33, 34}, but not for retrieval of trace fear memory in already conditioned
475 animals¹⁵. Nevertheless, extinction of trace fear memory was accelerated by inhibition of the
476 prelimbic cortex¹⁵. More generally, silencing of the prelimbic cortex promoted reversal learning
477 in an operant conditioning task of cue-reward pairing while it did not affect recall of already
478 learned rule¹⁵. Our results elaborated these previous findings by elucidating prelimbic cell type-
479 specific activity that impedes extinction learning. We showed that acceleration of extinction
480 learning closely correlates with TPP-induced inhibition of prelimbic persistent activity during the
481 post-CS period of the tone test. As aforementioned, we presented evidence supporting that TPP-
482 sensitive persistent activity occurs in CPn cells. Therefore, we conclude that post-CS persistent
483 activity of prelimbic CPn cells interferes with extinction learning. It remains to be elucidated

484 whether prelimbic persistent activity also contributes for an animal to adhere to the previously
485 learned rule even if the animal is repeatedly exposed to an alternative rule.

486 Inactivation of the prelimbic cortex during trace intervals impaired trace fear memory formation,
487 suggesting that prelimbic activity during the trace interval contributes to temporal associative
488 learning^{16, 35}. In our study, however, TPP⁺ did not affect fear memory formation even though it
489 significantly reduced persistent activity during the trace interval. This finding was supported by
490 little effect of TPP⁺ on the working memory task (Figure S6d), suggesting that TPP⁺ does not impair
491 network activity that links temporally separated CS and US. It is possible that TPP⁺-insensitive
492 persistent activity may convey CS information, given that TPP⁺ has little effects on persistent
493 activity of CS-late and delay cells (Figure 3). Alternatively, a change in the population activity of
494 the prelimbic network rather than persistent activity itself may carry the CS information during
495 trace intervals.

496 Our data indicate that CS presentation induces burst firing of prelimbic L2/3 pyramidal neurons,
497 which can trigger PTP in CPn neurons thereby interfering with rapid extinction learning. The
498 mediodorsal thalamus may drive this bursting activity because it comprises the major long-range
499 inputs and burst firings of thalamic afferents provide net excitatory inputs to prelimbic L2/3
500 pyramidal neurons^{36, 37}. Indeed, enhanced burst firing in the mediodorsal thalamus impairs
501 extinction learning³⁸. Investigating direct interactions between the prelimbic cortex and
502 mediodorsal thalamus during extinction learning will confirm this notion.

503 Post-tetanic mitochondrial Ca²⁺ release is inhibited by mild mitochondrial depolarization, which
504 is observed in pre-symptomatic stage of Alzheimer disease⁶. Consistently we have shown that
505 young adult Alzheimer disease model mice phenocopied the effects of TPP⁺: mild mitochondrial
506 depolarization in L2/3 pyramidal neurons and the lack of post-tetanic potentiation at L2/3-CPn
507 synapses, phenocopying the effect of TPP⁺ treatment¹¹. Therefore, TPP⁺-induced network and
508 behavioral changes may provide mechanistic insights to preclinical signs of brain disorders
509 involving mitochondrial dysfunction.

510

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516

517 **Conflict of Interest**

518 Authors declare that there is no competing financial interests in relation to this study.

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606

607

Figure Legends

Fig. 1

Figure 1. Both TPP⁺ infusion into the prelimbic area and optogenetic silencing of corticopontine neurons accelerated extinction of trace fear memory. a-c: Time course of freezing behavior after PBS or TPP injection into the prelimbic cortex (PL) 30 minutes before the trace fear conditioning. a, Schematic of experiment procedure. PBS or TPP was infused into the prelimbic cortex 30 minutes before the trace fear conditioning. b, Time course of freezing ratio of PBS or TPP-treated rats during trace fear conditioning (left; day 1) and tone test (right; day 2) (PBS, n = 6; TPP, n = 12; Conditioning on D1, $F_{1,18} = 0.120$, $P = 0.733$; Tone test on D2, $F_{1,16} = 5.930$, $P = 0.027$; Repeated measure two-way ANOVA (RM-ANOVA) for main effect of groups). c, Mean freezing ratios during tone test in PBS and TPP groups, presented as the average of 2 trials during tone test ($F_{1,16} = 4.756$, $P = 0.044$, RM-ANOVA for main effect of groups). d-e: Schematic of the corticopontine (CPn) and commissural (COM) neuron-specific expression of ArchT. d, Upper, to inactivate CPn activity, AAV2-retro-EF1a-Cre and AAV5-CAG-DIO-ArchT-tdTomato were injected into the pons and PFC, respectively. Lower, representative images of DIO-ArchT-tdTomato virus expression in CPn neurons and the optic fiber placement in the PL. Scale bar, 500 μm . e, Upper, to inactivate commissural (COM) neuron activity, the mixture of AAV2-retro-EF1a-Cre and AAV5-CAG-fDIO-ArchT-GFP and the mixture of AAV2-retro-EF1a-Flpo and AAV5-CAG-DIO-ArchT-tdTomato were injected into the contralateral and ipsilateral PFC, respectively. Lower, representative images of DIO-ArchT-tdTomato and fDIO-ArchT-GFP virus expression and optic fiber placement in the PL. Scale bar, 500 μm . f, Left, representative image of the ArchT-tdTomato-expressing PL neuron. Scale bar, 10 μm . Right, illumination of 593 nm light inhibited action potentials induced by step current injections (colored trace, at 80 and 90 pA) in the same cell. Scale bar, 500 ms, 20 mV. g, Schematic of neuron type-specific optical silencing experiment procedure. Illumination at 593 nm was applied during the trace interval of fear conditioning and post-CS period of the tone test to inactivate CPn and COM neurons. h, Time courses of freezing ratio during trace fear conditioning (left), tone test (middle), and extinction retrieval test (right) in control (GFP) and COM- or CPn-

636 silenced animals (black, GFP; green, CCM; purple, CPh) (For tone test, $F_{2,27} = 9.960$, $P = 0.001$, RM-
 637 ANOVA for main effect of group; For GFP vs CPh, $P = 0.001$; For GFP vs CCM, $P = 0.823$, Tukey's
 638 post hoc; For extinction retrieval test, $F_{2,24} = 4.453$, $P = 0.023$, RM-ANOVA for main effect of group;
 639 For GFP vs CPh, $P = 0.018$; For GFP vs CCM, $P = 0.283$, Tukey's post hoc). i, Mean freezing ratio
 640 presented as the average of 5 trials during the tone test (left, RM-ANOVA for main effect of group,
 641 $F_{2,27} = 9.876$, $P = 0.001$; Tukey's post hoc for GFP vs CPh, $P = 0.001$; for GFP vs CCM, $P = 0.710$),
 642 and as the average of 2 trials during the extinction retrieval tests (RM-ANOVA for main effect of
 643 group, $F_{2,24} = 4.118$, $P = 0.029$; Tukey's post hoc for GFP vs CPh $P = 0.023$; for GFP vs CCM, $P =$
 644 0.333). Post hoc comparisons with Bonferroni correction were conducted to investigate
 645 differences between groups at each time point. All data are mean $\pm$ S.E.M., * $P < 0.05$, ** $P < 0.01$,
 646 *** $P < 0.001$.

647

648 *Fig. 2*

649 Figure 2. TPP⁺ reduces persistent activity and accelerates extinction of prelimbic CS responses
 650 during tone test. a, Schematic of single unit recording during the tone test on Day 2 in rats which
 651 underwent PBS and TPP⁺ infusion into the prelimbic (PL) area 30 min prior to trace fear
 652 conditioning on Day 1. b, Heatmap of z-scores of individual cells averaged over CS period of each
 653 trial (PBS, n=186 cells from 10 rats; TPP, n=124 cells from 7 rats). Color bar, z-score. c, Heatmap
 654 of mean z-scores averaged over all CS cells of PBS (upper, n=60) and TPP groups (lower, n = 32)
 655 during the tone test. CS cells are defined as putative pyramidal neurons displaying distinctly high
 656 activity during CS period compared to baseline (see Methods for detail). Vertical white lines
 657 indicate the CS period (20-40 s) and expected UStiming (60 s). d, Activity changes of CS cells over
 658 trials in PBS (blue) and TPP (red) animals during the early 10 s of CS presentation (CS-early period).
 659 e, Mean z-scores of CS cells during CS-early period (20-30 s) across five trial blocks (5 trials/block).
 660 Linear mixed-effects (LME) models (TPP vs. PBS): for main effect of group, $F_{1,13} = 10.587$, $P = 0.006$;
 661 for trial blocks 2/3/4 $P = 0.001$, $P = 0.062$, $P = 0.0436$, respectively; Tukey's post hoc test. f, Time-
 662 dependent changes of mean z-score of CS cells averaged over first five trials in PBS (blue) and TPP
 663 (red) groups. Gray box, CS period (20-40 s). Vertical dashed line, expected UStiming. g, Mean z-

664 scores of CS cells averaged over three epochs shown in f (CS-early, CS-late, and post-CS periods).
 665 LME models for 1 s-binned activities between TPP vs. PBS groups: for CS-late period (30-40s), $F_{1,13} = 4.678$, $P = 0.0497$; for post-CS period (40-60s), $F_{1,13} = 6.601$, $P = 0.0233$. h, Proportions of CS
 666 and PA cells observed in the tone test. PA cells are defined as neurons exhibiting distinctly high
 667 activity during the post-CS period. The numbers in the parentheses on the pie chart indicate cell
 668 counts. Chi-squared test: for CS_{PBS} (60/186) vs. CS_{TPP} (32/124), $P = 0.275$; for PA_{PBS} (29/186) vs. PA_{TPP}
 669 (8/124), $P = 0.0243$. For LME models, time and drug treatment were set as fixed effects, while
 670 individual animals and neurons were set as random effects to account for repeated measures and
 671 clustering by animals. All data are mean $\pm$ S.E.M., Shaded areas represent S.E.M., * $P < 0.05$, *** P
 672 < 0.001 .
 673

674

675 *Fig. 3*

676 Figure 3. Effects of TPP⁺ on the activities of cell types classified by peak activity timing. a-b,
 677 Sequential activity of CS-responsive cells in PBS and TPP injected groups. CS-responsive cells are
 678 defined as CS, PA cells and neurons displaying $z_i > 3.09$ (or $p < 0.001$) at any 1 second bins of CS
 679 (20-40 s) and post-CS (40-60 s) periods. Data from experiments of Day 1 and Day 2 TPP⁺ injections
 680 (shown in Fig. 2 and Fig. S7, respectively) were merged for these plots to collect sufficient number
 681 of cells responsive to tone tests. Upper, Heat maps displaying activity time courses of individual
 682 cells (z-scores) averaged over first five trials of the tone test for PBS(a) and TPP⁺ (b) treated group.
 683 For each group, CS-responsive cells were selected from electrophysiological recordings as shown
 684 in Fig. 2 and Fig. S7 from animals received prelimbic infusion of PBS(a, n = 63 out of 186 total cells
 685 from 10 rats) and TPP⁺ (b, n = 64 out of 209 total cells from 12 rats) and sorted according to their
 686 timing of the peak z score. The peak timing in each cell was determined from a gaussian-filtered
 687 time sequence of z scores ($\sigma = 4$ s). Lower, Histograms for counts of cells displaying peak activity
 688 in each of 1 s bin. Vertical dashed lines demarcate the beginning of tone, first 10 s of CS period
 689 (CS-early), second 10 s of CS period (CS-late), and 10 s after expected US timing. c, Pie charts for
 690 the proportions of CS-early, CS-late and Delay cells from PBS (upper, n = 63) and TPP (lower, n =
 691 64) groups. CS-responsive cells were categorized into three types based on their peak activity

692 timing. The numbers in the parentheses on the pie chart indicate cell counts. Chi-square test: for
 693 CS-early cell, $P = 0.5387$; for CS-late cell, $P = 0.6874$; for Delay cell, $P = 0.3609$. d-f, Mean time-
 694 dependent activities of CS-early cells (d), CS-late cells (e), and Delay cells (f) as shown in a-b. The
 695 cell numbers for the three cell types are shown in c. Right, Mean z-scores averaged over each of
 696 three epochs (CS-early, CS-late and post-CS period). LME models of 1 s-binned CS-early cell
 697 activities (PBSvs TPP): for CS-late period (30-40 s), $P = 0.0709$; for post-CSperiod (40-60 s), $F_{1,16} =$
 698 7.076, $P = 0.0171$. LME models of Delay cell activities (PBSvs TPP⁺): for CS-early period, $P = 0.698$;
 699 for CS-late period, $P = 0.241$; for post-CSperiod, $P = 0.6901$. Time and drug treatment were set as
 700 fixed effects of LME models, while individual animals and neurons as random effects. All data are
 701 mean $\pm$ S.E.M., Shaded areas represent S.E.M., * $P < 0.05$.

702

703 *Fig. 4*

704 Figure 4. Cell-type specific activity during tone tests. a-b, Schematic of the methods for CPh (a)
 705 and OOM (b) cell-specific virus expression. a, Upper, to record CPh activity, AAV2-retro-EF1a-Cre
 706 and AAV1-hSyn-flex-GCaMP6f were injected into the pons and the prelimbic area (PL), respectively.
 707 A GRIN lens was implanted into the PL. Lower, representative image of GCaMP6f expression in
 708 the PL. b, Upper, to record OOM activity, AAV2-retro-EF1a-Cre and AAV1-hSyn-flex-GCaMP6f were
 709 injected into the contralateral and ipsilateral PL, respectively. A GRIN lens was implanted into the
 710 ipsilateral PL. Lower, representative image of GCaMP6f expression in the PL. Scale bar, 500 μ m. c,
 711 Representative Miniscope image of OOM cells obtained using a 1 mm GRIN lens. d, Representative
 712 raw and intermediate data used for CNMF-E analyses of Miniscope images. Upper, raw calcium
 713 trace from an exemplary neuron. Lower, simulated calcium trace (red) calculated from
 714 convolution of inferred spike (blue) and a unitary calcium transient, which were estimated from
 715 CNMF-E. Scale bar, 1 min. e, Mean z-score of all CS-responsive OOM cells (green; $n = 192$ out of
 716 618 total cells from 13 rats) and CPh (purple; $n = 186$ out of 682 total cells from 13 rats) neurons.
 717 For each cell, z scores were averaged over first five trials of tone test. f, Mean z-scores of CS cells
 718 averaged over each of three epochs (CS-early, CS-late, and post-CS periods). g-h, Upper, heat map
 719 was plotted similar to Fig. 3a-b for OOM (g) and CPh (h) cells (OOM cells: $n = 229$ out of 618 total

cells. CPh cells: $n = 232$ out of 618 total cells). For each cell, time series of 1 s binned z scores were averaged over first five trials of the tone test. Lower, histogram for counts of cells displaying peak activity in each 1 s bin. Vertical dashed lines demarcate the beginning of tone, first 10 s of CS period (CS-early), second 10 s of CS period (CS-late), and 10 s after expected US timing. i, CS-responsive cells shown in g and h are classified into CS-early, CS-late and Delay cells according to peak activity timing, and their proportions in OOM (upper) and CPh (lower) cells are displayed as pie charts. The numbers in the parentheses indicate cell counts. Chi-square test (OOM vs CPh): for CS-early cell, $P = 0.001$; for CS-late cell, $P = 0.0781$; for Delay cell, $P = 0.0945$. j-l, Left, mean time-dependent activities (z scores) of CS-early cells (j), CS-late cells (k) and Delay cells (l) of OOM (green) and CPh (purple) cells, shown in g and h. Right, mean z-scores averaged over each of three epochs (CS-early, CS-late and post-CS period) for CS-early (j), CS-late (k) and delay (l) cells. Green, OOM cells. Purple, CPh cells. LME models for 1 s-binned CS-early cell activities between OOM vs. CPh: CS-early period (30-40 s), $F_{1,24} = 5.5889$, $P = 0.0265$; post-CS period (50-70 s), $F_{1,24} = 6.5147$, $P = 0.0175$. LME model of CS-late cell activities during CS-early period (OOM vs CPh), $F_{1,22} = 4.4251$, $P = 0.0471$; post-CS period (50-70 s), $F_{1,22} = 4.101$, $P = 0.0551$. Time and cell types (OOM vs CPh) were set as fixed effects of LME model, while individual animals and cells were set as random effects. All data are mean $\pm$ S.E.M., Shaded areas represent S.E.M., * $P < 0.05$.

Fig. 5

Figure 5. In vivo activities of prelimbic L2/3 pyramidal neurons can induce TPP⁺-sensitive post-tetanic potentiation in L5 CPh neurons. a, Representative image for the location of stereotrode bundles to record prelimbic L2/3 neuron activity. Scale bar, 500 μ m. b, Heatmap of z-scores averaged over all L2/3 CS and PA cells ($n = 36/121$ cells from 4 rats) during tone tests. Vertical white lines, CS period and expected US timing. c, Time-dependent changes of mean z-score of L2/3 CS and PA cells averaged over first five trials of the tone test. For comparison, the z-score trace from layer-nonspecific recordings (denoted as PBS) is reproduced from Fig. 2f. Gray box, CS period. Vertical dashed line, expected US timing. d, Percentages of CS and PA cells in prelimbic L2/3 during tone test (CS_{L2/3}, 36/121; PA_{L2/3}, 16/121). The numbers on the pie chart represent

748 proportions in percentages. Numbers in the parentheses are cell counts. e, Time-dependent
 749 changes of mean spike frequency of all CS and PA cells in L2/3 ($n = 36/121$). Horizontal bar, CS
 750 period. f, Spike raster plots (upper) and corresponding post-event time histograms (PETHs) of spike
 751 counts (lower) of two representative L2/3 CS cells for first 5 trials. Bin width of PETH, 1 s. g, Mean
 752 spike counts of L2/3 cells as a function of inter-spike intervals (ISIs) during the baseline (black) and
 753 CS periods (orange). Bin width, 0.01 s. For each of CS and PA cells, spike counts were summed
 754 over 20 s of baseline or a CS period over first five trials. For visibility, spike counts with $ISI > 1$ s
 755 were truncated. Comparing ISIs shorter than 120 ms of baseline vs. CS period revealed that $F_{1,6965}$
 756 $= 21.631$, $P < 0.05$ (LME model). Gray vertical dashed line indicates ISI at 120 ms. h, Spike activity
 757 of the cell #97 shown in f (left) during baseline and CS period in the 1st trial. i-j, Histogram of ISIs
 758 of cell #97 in the baseline and the CS period of 1-5 trials. Note that most ISIs were shorter than
 759 200 ms for both periods. k-l, Plots of instantaneous frequencies ($1/ISIs$) of cell #97 during baseline
 760 and the CS period of the 1st trial. m, EPSCs trace at L2/3-CPn synapses evoked by patterned
 761 stimulation mimicking the firing pattern of h during CS period. n, Representative paired-pulse
 762 EPSCs before (left; black) and after TPP^+ (right; red) applications. Dotted line indicates the peak
 763 EPSC amplitude after patterned stimulation under the TPP conditions. o, Time courses of mean
 764 baseline-normalized EPSC amplitudes at L2/3-CPn synapses before and after patterned
 765 stimulation applied at $t = 0$. Experiments were done in the in vivo-mimicking conditions (1.3 mM
 766 $[Ca^{2+}]_o$, 32 °C). Left, control ($n = 8$). Right, after 2 μM TPP^+ application ($n = 8$). p, Post-tetanic
 767 potentiation at L2/3-CPn synapses before and after applying TPP^+ . For Post-tetanic potentiation in
 768 control vs TPP conditions, $P = 0.0078$, Wilcoxon matched pairs signed rank test. All data are mean
 769 $\pm$ S.E.M., Shaded areas represent S.E.M., ** $P < 0.01$.

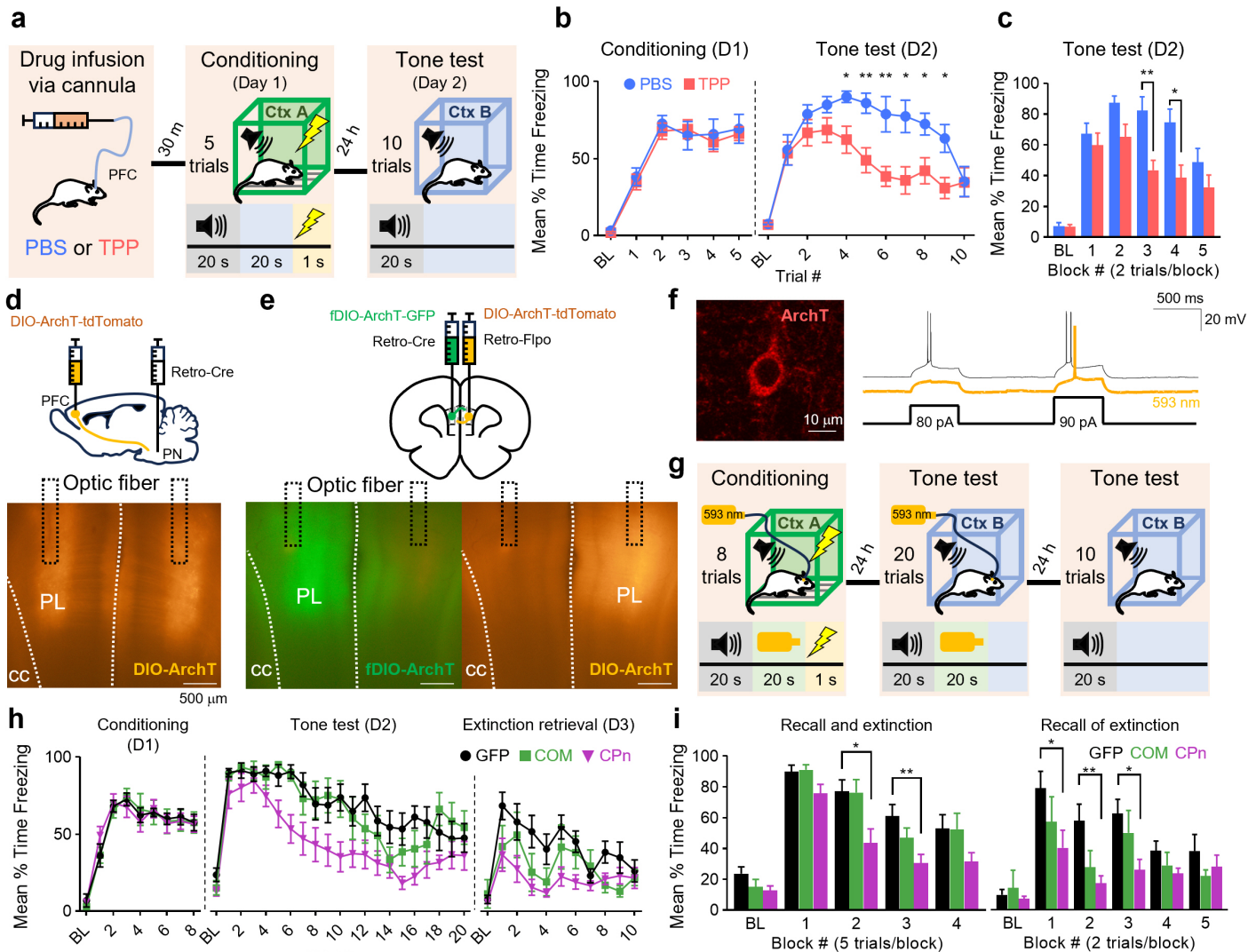
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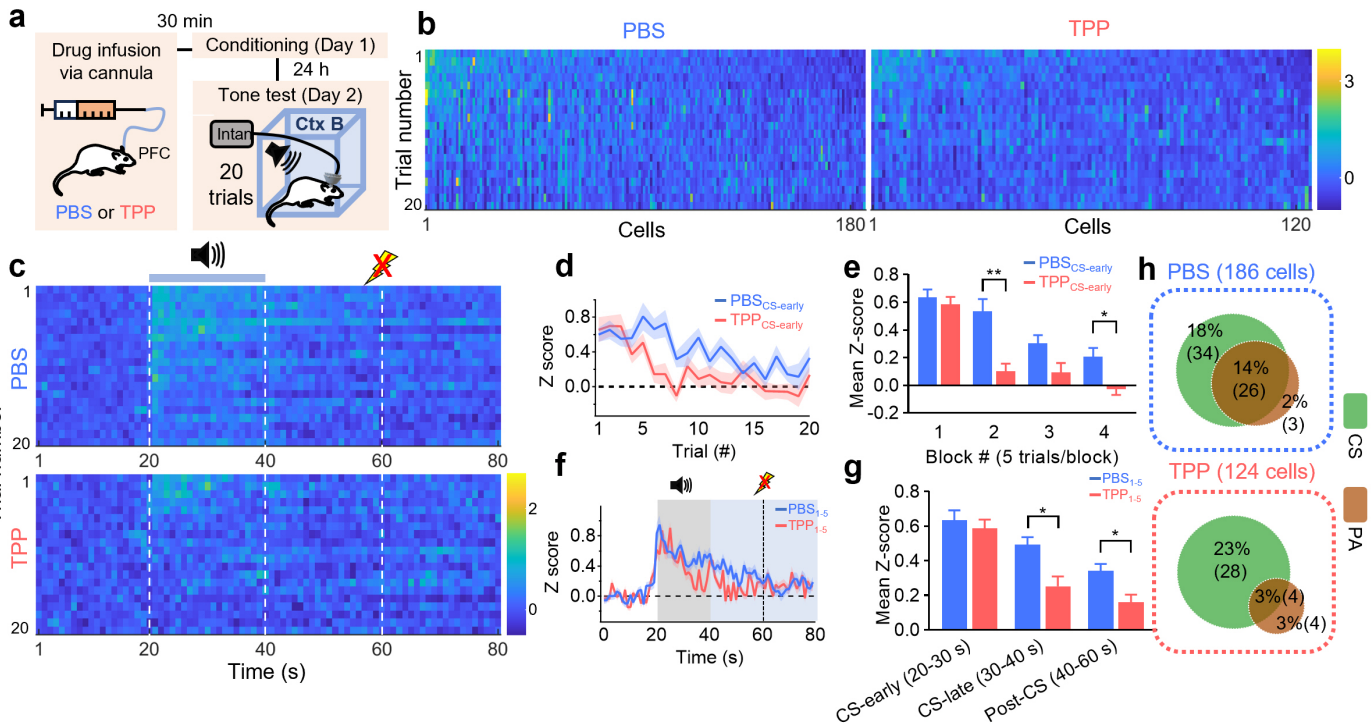
771 *Fig. 6*

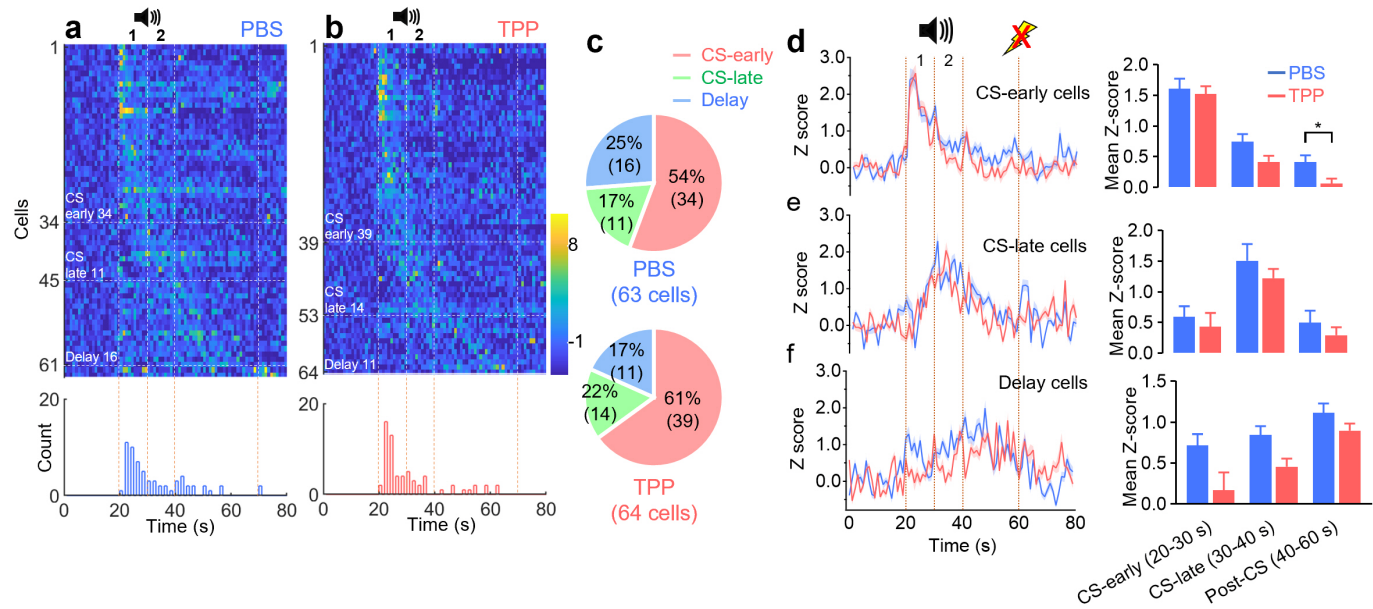
772 Figure 6. TPP^+ infusion into the PL reduces the persistent activity of PL neurons during TFC a,
 773 Schematic of single unit recording during trace fear conditioning in rats which received PBS or
 774 TPP^+ infusion into the PL 30 min prior to conditioning. Rats were habituated to the conditioning
 775 chamber and presented with three trials of CS alone (trial 1-3) and then underwent subsequent

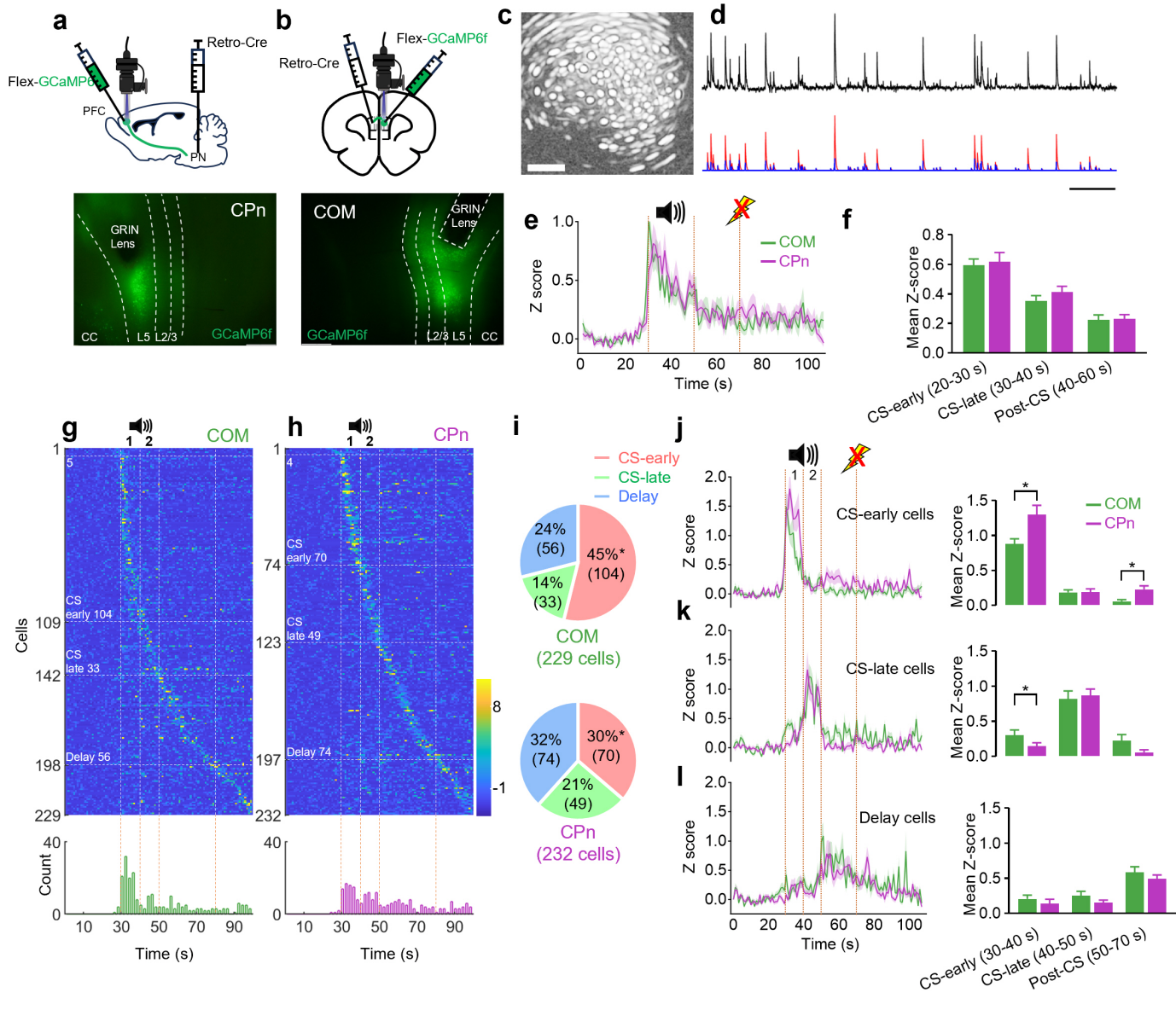
776 eight CS-US pairing trials (trial 4-11) for trace fear conditioning. b, Heatmap of z-scores of
777 individual cells activity averaged over the CS period of each trial (left, PBS, n=134 cells from 5 rats;
778 right, TPP, n=104 cells from 5 rats). Color bar, z-score. Cells were sorted based on their activities.
779 c, Heatmap of z-scores averaged over all CS cells of PBS (upper, n = 79) and TPP groups (lower, n =
780 57) as a function of time. CS cells are defined as the same as in Fig. 2. CS-only and CS+US trials are
781 demarcated by the horizontal dashed line. The vertical white lines divide pre-CS, CS and trace
782 periods. Color bar, z-score. d, Mean activity changes of CS cells over trials in PBS (blue) and TPP
783 (red) animals during CS-early periods (first 10 s of CS). e, Mean z-scores of CS cell activity as a
784 function of time. Z-scores shown in c were averaged over trials 5 to 11 for the PBS and TPP groups.
785 The CS period is indicated by a gray box, the trace interval by a blue box, and the US presentation
786 by a yellow box. f, Summary for the mean z-scores averaged over three epochs shown in e (CS-
787 early, CS-late, and trace periods). For 1 s-binned activities during the trace period of TPP vs PBS
788 group, $F_{1,8} = 5.846$, $P = 0.041$, LME model with time and drug treatment set as fixed effects, and
789 individual animals and neurons as a random effect to account for repeated measures and
790 clustering by animals. g, Percentages of CS and PA cells observed during conditioning. PA cells are
791 defined as the same as in Fig. 2. The proportion of PA cells among CS cells were significantly lower
792 in the TPP group compared to the PBS group (29/79 vs. 7/57, $p = 0.0028$), whereas that of CS cells
793 was not altered by TPP⁺ (59% vs. 55%). Chi-squared test: for CS_{PBS} (79/134) vs. CS_{TPP} (57/104), $P =$
794 0.191; for PA_{PBS} (33/134) vs. PA_{TPP} (13/104), $P = 0.0120$. All data are mean $\pm$ S.E.M., Shaded areas
795 represent S.E.M. *, $P < 0.05$.

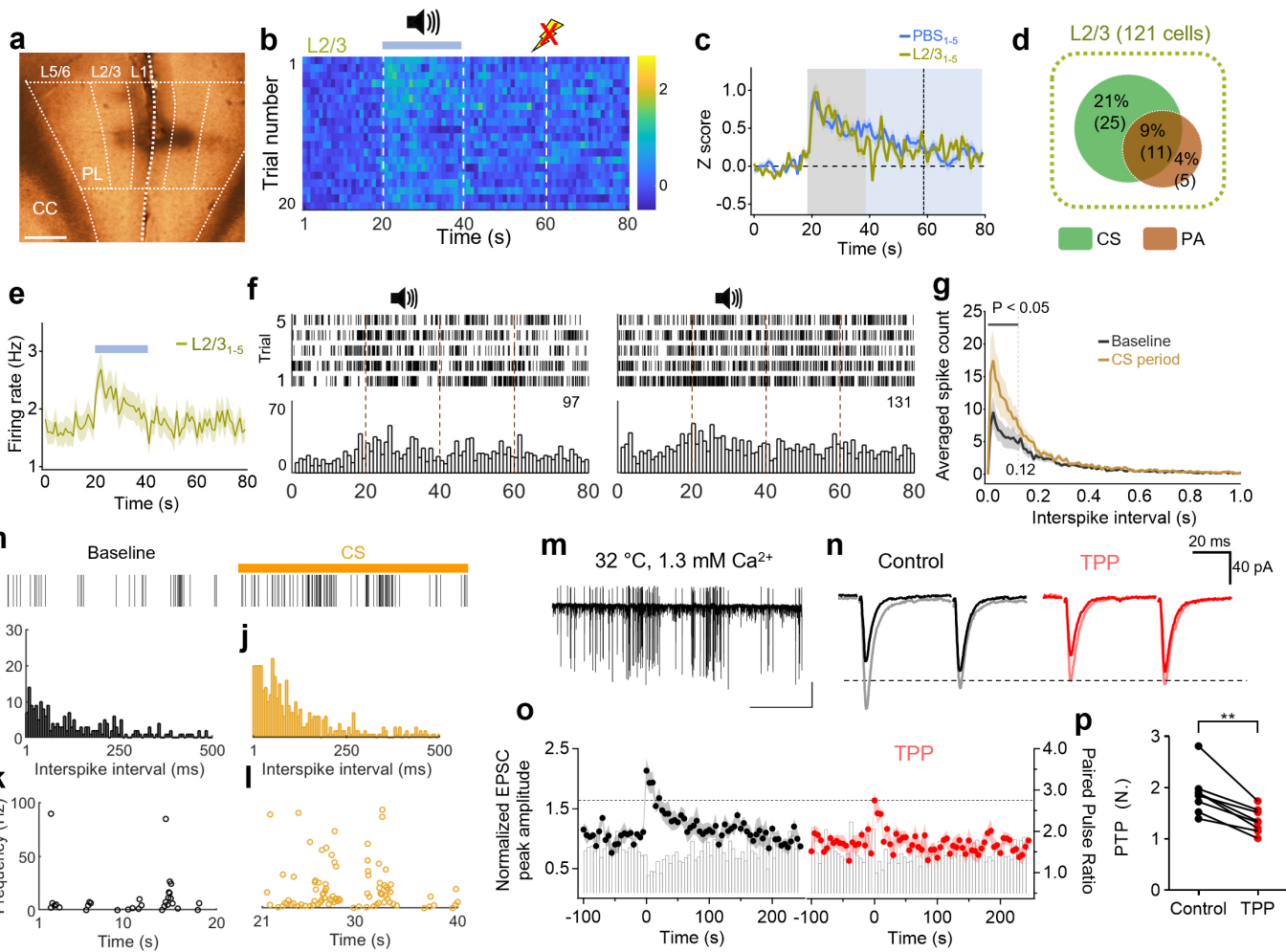
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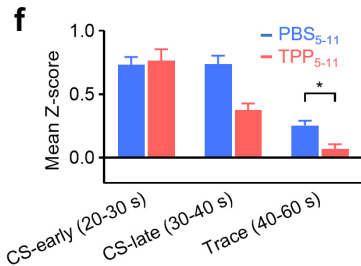
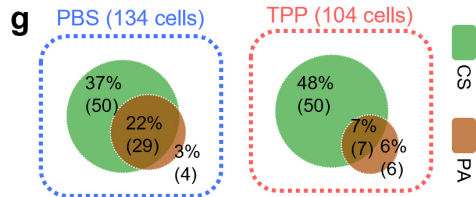
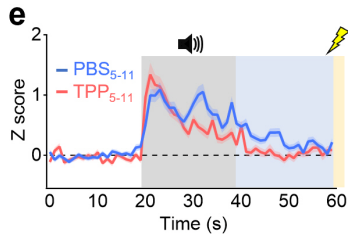
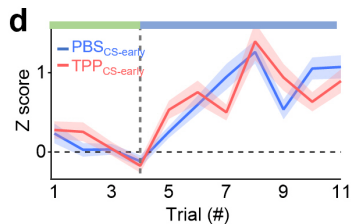
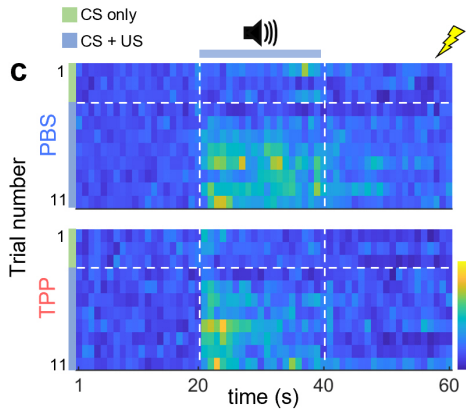
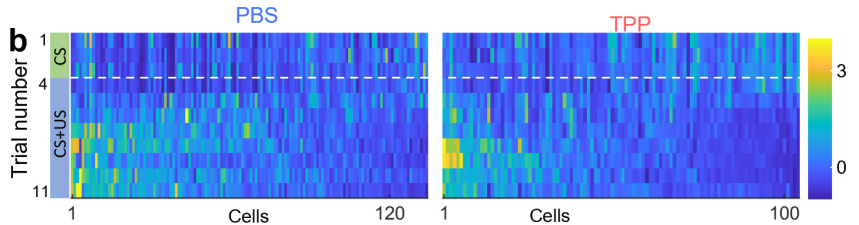
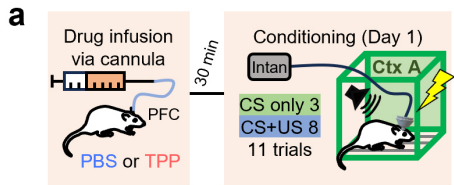












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