Temporal association learning (TAL) allows for the linkage of distinct, nonsynchronous events across a period of time. This function is driven by neural interactions in the entorhinal cortical–hippocampal network, especially the neural input from the pyramidal cells in layer III of medial entorhinal cortex (MECIII) to hippocampal CA1 is crucial for TAL. Successful TAL depends on the strength of event stimuli and the duration of the temporal gap between events. Whereas it has been demonstrated that the neural input from pyramidal cells in layer II of MEC, referred to as Island cells, to inhibitory neurons in dorsal hippocampal CA1 controls TAL when the strength of event stimuli is weak, it remains unknown whether Island cells regulate TAL with long trace periods as well. To understand the role of Island cells in regulating the duration of the learnable trace period in TAL, we used Pavlovian trace fear conditioning (TFC) with a 60-sec long trace period (long trace fear conditioning [L-TFC]) coupled with optogenetic and chemogenetic neural activity manipulations as well as cell type-specific neural ablation. We found that ablation of Island cells in MECII partially increases L-TFC performance. Chemogenetic manipulation of Island cells causes differential effectiveness in Island cell activity and leads to a circuit imbalance that disrupts L-TFC. However, optogenetic terminal inhibition of Island cell input to dorsal hippocampal CA1 during the temporal association period allows for long trace intervals to be learned in TFC. These results demonstrate that Island cells have a critical role in regulating the duration of time bridgeable between associated events in TAL.
or increased temporal distance between any two events would signal that the events are less likely to be causally associated, therefore less relevant, and less likely to be stored and recalled. In fact, successful TFC depends on the strength of event stimuli and duration of temporal gap between events (Stiedl and Spiess 1997; Misane et al. 2005; Kitamura et al. 2014; Kitamura 2017). However, the underlying regulatory mechanism for TAL remains hidden. Previously we demonstrated that feedforward inhibition by the Island cells acts as a gating controller for the MECII inputs to the distal dendrites of HPC CA1 pyramidal cells in stratum moleculare (SM) (Kitamura et al. 2014) to control TFC when weaker (in this case diminished footshock intensity) unconditioned stimuli were delivered for TFC, indicating that Island cell activity controls the temporal association when the strength of two discontinuous events are relatively weaker. However, the way in which the EC-HPC network regulates TFC with a longer trace period still remains unknown. Because the activation of Island cells would result in a net inhibitory effect on the local network in CA1, imposing a tight and specific regulation on associations of events across the temporal gap in TAL (Crestani et al. 2002; Moore et al. 2010; Kitamura et al. 2014, 2015b), we hypothesized that the length of the temporal gap between events would also be modulated by this mechanism. In this study, we examined the role of the regulatory input to this circuit arising specifically from the Island cells in the MECII using apoptotic elimination of Island cells, chemogenetic neural inhibition, and optogenetic terminal inhibition methods within an L-TFC protocol to give a thorough and complete assessment of the circuit involvement while considering each technique’s unique features.

Results

Trace fear conditioning with long CS-US contingencies in mice

In the conditioning session of TFC on day 1, we subjected two groups of C57BL/6J mice to two different TFC protocols in which after a 240-sec acclimation, a 20-sec conditioned stimulus (CS); tone, 5 kHz, 80 dB) is followed by a 2-sec footshock as the unconditioned stimulus (US); shock, 1.0 mA, 2 sec) three times with either 20 sec as a naturally associative TFC or 60-sec trace periods separating the CS and US as a long TFC (L-TFC) that is harder to associate (Fig. 2A,B). Both groups exhibited the freezing response during the tone period compared with those in the baseline period (paired t-test, base vs. tone in 20-sec group, \( t_{11} = 7.39, P < 0.0001 \); base vs. tone in 60-sec group, \( t_{12} = 3.62, P < 0.004 \); mice conditioned with the 60-sec L-TFC protocol showed significantly lower freezing responses during both the tone period (unpaired t-test, \( t_{23} = 3.44, P < 0.002 \)) and the first minute post-tone period (unpaired t-test, \( t_{23} = 2.66, P < 0.01 \)) (Fig. 2D) compared with the performance of the mice conditioned with the 20-sec
Ablation of Island cells in MEC partially enhances L-TFC
To test the effect of the ablation of MECII Island cells on L-TFC, we bilaterally injected AAV8-EF1α-Rex-taCasp3-TEVp into the MEC of either Wfs1-Cre mice (Casp− group) or wild-type (WT) littermates (Casp+ group) as a control to induce Cre-dependent apoptosis in Island cells of the MEC. The genetically engineered procaspase 3 (pro-taCasp3) triggers cell-autonomous apoptosis by activation of the heterologous enzyme tobacco etch virus protease (TEVp) (Yang et al. 2013). At 4 wk after the injection of AAV8-EF1α-Rex-taCasp3-TEVp into MECII, we found virtually no Wfs1+ (a marker for Island cells) cells in the MECII of the Casp+ group, while Wfs1-Cre (a marker for Island cells) cells in the Casp− group (unpaired t-test, base: \(P<0.02\) ) (Fig. 3C,D). Together, these results suggest that the elimination of Island cells partially enhances L-TFC.

Chemogenetic inhibition of Island cell activity in MEC reduces L-TFC
To investigate the role of Island cell activity on L-TFC, we next examined chemogenetic neural activity inhibition using Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) (Armbruster et al. 2007; Zhu et al. 2014; Roth 2016). First, we examined chemogenetic neural activity inhibition using Designer Drugs for neuronal inhibition at the tested dosage (10 µM) (Fig. 4B), indicating a net reduction in neuronal excitability compared to the baseline levels without the presence of the DREADD agonist CNO in the bath solution. Interestingly, while the resting membrane potential (RMP) was quickly and significantly reduced from baseline RMP (3 min from CNO application, one sample t-test, \(t_8=4.228, P<0.01\); 5 min from CNO application, one sample t-test, \(t_8=2.540, P<0.05\)), we observed a gradual rebound of the reduced RMP in a subset of Island cells (10 min from CNO application, one sample t-test, \(t_8=2.132, P=0.065\) (Fig. 4C), contrary to what has been seen using chemogenetic techniques for neuronal inhibition at the tested dosage (10 µM) of CNO in the hippocampus and hypothalamus (Krashe et al. 2011; Zhu et al. 2014), suggesting a robust mechanism may exist for the maintenance and balancing of the RMP in a subpopulation of Island cells.

Behavioral observation of the DREADDs’ effects on L-TFC were performed using two groups of Wfs1-Cre mice; the hM4Di-mCherry-expressing (hM4Di-mCherry group) or mCherry-expressing (mCherry group), which had been injected with AAV8-hSynL-DIO-hM4Di-mCherry or AAV8-hSynL-DIO-mCherry, respectively, into the MEC. We administered the hM4Di-mCherry and mCherry groups CNO via intraperitoneal injection (4 mg/kg) 30 min before L-TFC. In the conditioning session on day 1, the hM4Di-mCherry group showed a similar freezing response to the control mCherry group (two-way repeated measure [RM] ANOVA, effect of condition: \(F_{1,18}=1.13, P>0.30\); effect of time: \(F_{1,578}=75.6, P<0.001\); interaction: \(F_{1,578}=0.98, P>0.51\) ) (Fig. 3B). In the testing session on day 2, however, while there was no difference in the freezing responses during either the baseline period or tone period between the Casp− and Casp+ groups (unpaired t-test, base: \(t_{8}=0.44, P>0.67\); tone: \(t_{8}=0.56, P>0.58\) ) (Fig. 3C,D), the Casp+ group showed a significantly higher freezing response during the first minute post-tone period compared with the Casp− group (unpaired t-test, \(t_{8}=2.73, P<0.01\) ) (Fig. 3D). Together, these results suggest that the elimination of Island cells in MEC partially enhances L-TFC.

Figure 3. Effects of ablation of Island cells in the medial entorhinal cortex (MEC) on long trace fear conditioning (L-TFC). (A) Representative images in the MEC of Wfs1-Cre mice (bottom panels) and Wfs1-Cre− littersmates (top panels) at 4 wk after AAV8-EF1α-Rex-taCasp3-TEVp injection. Scale bar, 200 µm. (B) Time course of freezing responses in apoptosis-inducible Wfs1-Cre mice (Casp+ group, \(n=8\)) and Wfs1-Cre− littersmates (Casp− group, \(n=12\)) during conditioning with 60-sec trace period on day 1 at 4 wk after the AAV injection. Gray and blue bars represent tone and shock, respectively. (C) Time course of freezing responses in both Casp− and Casp+ groups during the test. Gray bars each indicate 60-sec tone period. (D) The averaged freezing responses over the three bins during each tone period and that of the first 1-min, second, and third post-tone periods. Means ± SEM. Two-way repeated measure (RM) analysis of variance (ANOVA) for B and C. (*) \(P<0.05\) by unpaired t-test for D.
Optogenetic terminal inhibition of MECII Island cell synapses at dorsal hippocampal CA1 enhances L-TFC

To examine the role of Island cell inputs into dorsal HPC CA1 during the CS–US association period on L-TFC, we bilaterally injected either AAV2-EF1α-DIO-eArchT3.0-enhanced yellow fluorescent protein (eYFP) (Mattis et al. 2011) or AAV2-EF1α-DIO-eYFP as a control into the MEC of Wfs1-Cre mice, bilaterally implanted optic fibers into dorsal HPC CA1, and then subjected them to L-TFC with green light illumination (561 nm) only during the CS–US association periods (total 82 sec; 20 sec [tone] + 60 sec [trace] + 2 sec [shock]) (Fig. 5A). We have previously confirmed that the optogenetic terminal inhibition of Island cells at dorsal HPC CA1 increases the spiking activity in CA1 pyramidal cells (Kitamura et al. 2014), indicating that the optogenetic terminal inhibition suppresses the feed-forward inhibition of HPC CA1 pyramidal cells driven by Island cell inputs. In the conditioning session on day 1, eArchT3.0-eYFP-expressing Wfs1-Cre mice (ArchT-eYFP group) exhibited higher freezing responses compared with the control eYFP-expressing ones (eYFP group, two-way RM ANOVA, effect of condition: $F_{(1,16)} = 8.16, P < 0.01$; effect of time: $F_{(41,656)} = 41.1, P < 0.001$; interaction: $F_{(41,656)} = 2.28, P < 0.001$) (Fig. 5A). In the testing session on the conditioning session on day 1, eArchT3.0-eYFP-expressing Wfs1-Cre mice (ArchT-eYFP group) exhibited
day 2, the ArchT group showed higher freezing responses during the tone period and post-tone for the first 1-min period compared with those in the eYFP group (unpaired $t$-test, tone: $t_{8} = 2.74, P < 0.02$; 1 min: $t_{8} = 2.46, P < 0.03$) (Fig. 5B,C). Previous work demonstrates that Island cell inhibition with a short trace period (20 sec) has a similar boosting effect on freezing levels to what we see here with a 60-sec trace period, but that Island cell activity has no effect on delay fear conditioning, which does not have a temporal gap between CS and US (Kitamura et al. 2014). These results, combined with previous reports, suggest that the optogenetic terminal inhibition of Island cells in the MEC at dorsal HPC CA1 during the CS–US association periods enhances L-TFC.

**Discussion**

Previously, our group has established that Island cells are involved in the modulation of TFC with a 20-sec trace period, but not delay fear conditioning (Kitamura et al. 2014) or contextual fear conditioning (Kitamura et al. 2015b), indicating a specificity of involvement in TAL, rather than fear conditioning in general. In this study, by using an L-TFC protocol with a 60-sec trace period (Fig. 2), we examined the regulatory role of Island cells on TFC with a long temporal gap between events. We found that the elimination of Island cells in the MEC partially enhanced L-TFC (Fig. 3), chemogenetic manipulation of Island cells causes differential levels of efficacy and leads to a circuit imbalance that disrupts L-TFC (Fig. 4), and, finally, that optogenetic terminal inactivation of Island cells at dorsal HPC CA1 during CS-US association periods enhanced L-TFC (Fig. 5). Of particular interest was the comparison between local synaptic inactivation (Fig. 5) and gross circuit inhibition, respectively (Figs. 3 and 4). These experiments suggest that Island cells in MECII regulate L-TFC and the temporal inhibition of Island cell inputs into HPC CA1 only during CS-US association would be required for sufficient artificial facilitation of L-TFC.

Ablation of Island cells in the MEC using an inducible caspase construct (Yang et al. 2013) led to a significant increase of the freezing response in L-TFC (Fig. 3D), similar to the result of the optogenetic terminal inhibition of Island cells at HPC CA1 (Fig. 5C,D). Despite the similarity in the enhanced freezing response during the 1-min post-tone period, we observed no difference during the tone period in the Casp+ group compared with the Casp− group (Fig. 3D). While it may be possible that the lack of response during the tone period overall may be due to insufficient acquisition due to the prolonged absence of Island cells in the MEC, we also speculate that the Casp+ group might remember the timing of the footshock delivery. Given that the animals have had 4 wk between injection of the caspase virus and experimentation, the elimination of Island cells could cause compensation in the EC networks, which could lead to a partial restoration of function that is less amenable to modulation (Jinde et al. 2012; Couey et al. 2013; Zelikowsky et al. 2013; Ohara et al. 2019). Since the lateral entorhinal cortex (LEC), in particular, has a role in trace eyelink conditioning (Takehara-Nishiuchi et al. 2011; Morrissey et al. 2012; Pilkiv et al. 2017; Marks et al. 2020) and other forms of temporal learning (Chao et al. 2016), we suspect that the function may be partially picked up by the Ws’ Islands of the LECII, which were unaffected by our targeted ablation, and also project to the SL of CA1 (Kitamura et al. 2014). With the overlap in anatomical connectivity and local cytoarchitecture between LEC and MEC, it seems reasonable that there could be compensation in one segment of ECII when the other is compromised, and that the functional separation between MEC and LEC is not as discrete as assumed (Save and Sargolini 2017).

Previously it was determined that reducing neural input strength from Island cells or increasing MECII input enhanced TFC with weaker external stimuli in the 20-sec TFC paradigm (Kitamura et al. 2014). In this study, we showed that optogenetic terminal inhibition of Island cells in the MEC at dorsal HPC CA1 resulted in the greater freezing response in L-TFC, compared with the eYFP control group (Fig. 5B,C). Our experiment disinhibited the processed input from MECIII (Kitamura et al. 2014), leaving the rest of the HPC processing apparatus largely intact for fine-tuned processing of an artificially boosted temporal signal (McEchron et al. 2003; Pastalkova et al. 2008; MacDonald et al. 2011; Pelkey et al. 2017; Marks et al. 2019; Zhang et al. 2019; Zhou et al. 2020). Therefore, this experiment suggests that the Island cells in the MEC exert control over the allowed length of time between events that can become associated across time. Considering the extension of duration gained by reduced synaptic activity onto the inhibitory interneurons in SL of the dorsal hippocampus, it is reasonable to suppose that under normal conditions the strength or number of activated Island cells could vary, exerting a stronger or weaker control over the temporal signal and, therefore, which events become associated. Our study suggests that Island cells control L-TFC as well as TAL with low impact/salience events (e.g., reduced footshock intensity) for optimal adaptive memory formation by regulating the MECIII inputs into the HPC CA1, which would be driven by tone-induced persistent activity (Yoshida et al. 2008; Kitamura et al. 2014).

We observed that the hM4Di-mCherry-expressing mice showed a reduction of the freezing response in L-TFC compared with the mCherry control group (Fig. 4) following exposure to CNO. This result would appear to be indicative of the overall inhibition of Island cells, but yet appears contrary to our observations of increased freezing following optogenetic terminal inhibition of Island cells in the hippocampus (Fig. 5), and the extensive ablation of Island cells (Fig. 3); however, we believe this result points to a more dynamic process underlying the activity of Island cells in TAL. Our confirmatory process of patch clamping Island cells to verify function of the hM4Di construct yielded a surprising observation (Fig. 4B,C) that some Island cells showed a tendency to rebound after an initial hyperpolarization in response to CNO (Fig. 4C). In addition, we observed a wide spread of CNO effects on excitability (Fig. 4C), although the overall effect was clearly inhibitory. It is possible the population of Island cells may be more heterogeneous than previously understood (Berggaard et al. 2018; Grosser et al. 2021), perhaps with differential expression of voltage-gated ion channels. Within the population of Island cells in the MEC, differential expression of GABA receptor α3 has been observed and hypothesized to generate differential activity patterns (Puch et al. 2016; Berggaard et al. 2018; Grosser et al. 2021), so it is not unlikely that other differences may exist, and distinct functional subtypes of Island cells have yet to be defined. Furthermore, although the overall effect of the DREADDs is inhibitory, it does not result in a complete abolition of firing (as with optogenetic inhibition), merely a reduction. An unbalanced reduction, or differential levels of GABA activity across the total population would result in the scrambling of a normally coherent signal. This suggests that our result is not simply dependent on the overall inhibitory effect we observed, but also that the pattern of Island cell activity may be important, and that even a subtle unbalancing or shifting of the standard activity patterns of a subset of Island cells can have a drastic effect on the capability of TAL (Kitamura et al. 2014), essentially taking a well-coordinated signal that would normally result in TAL and scrambling it into a nonsense signal, resulting in the observed decrease of freezing rather than an increase as observed in the optogenetic terminal inhibition experiment and Island cell ablation experiment. Future studies focused on the diversity of single-cell responses of Island cells during TFC, the existence of functional subsets within the broad class of Island cells, and the generation of a coherent signal/decoding of the information...
in the Island cell signal might be called for in light of these findings.

In this study, we have used multiple techniques to identify the role of Island cells on L-TFC. As a methodological discussion, we note the advantage/disadvantages of each approach based on our experimental results. Optogenetic approaches allow for the temporally precise terminal inhibition, but are better suited to small subregions of the brain rather than whole-system inhibition. In our case, we successfully used targeted light illumination to silence Island cell axons, since Island cells specifically project to SL in HPC CA1 (Fig. 1). Chemogenetic neural manipulation allows whole-system inhibition of a cell type and all its projections, but causes a partial, rather than total inhibition of neural firing by biasing cells toward a hyperpolarized state. However, depending on neural cell type, it may cause artificial generation of an aberrant/nonsense signal, as we observed in Figure 4. Finally, cell type-specific ablation allows for complete elimination of the target population, but leaves time for systemic compensation to attempt to restore a homeostatic balance in the brain prior to behavioral testing. Although each of these techniques is powerful, the choice of which technique to use depends on the experimental question. Here, using these techniques on the same circuit system yielded differing results that on the surface appear to be contradictory; however, each provides a unique insight into the system that is not achievable with the others.

We have demonstrated through multiple complimentary techniques that the Island cells of MECII regulate the linking of temporally contiguous events in terms of weaker impact of events or longer duration of temporal gap between events. The identity of the driving factors that regulate Island cell activity for this linking function by MECII is still unclear. It has been suggested that the individual Island clusters may have unique processing capabilities, but to what degree remains unclear (Ray et al. 2014; Fuchs et al. 2016). A recent report has suggested a gradient of parvalbumin* interneuron activity along the dorsal ventral axis of the Island cell distribution, implying differential processing along this axis (Grosser et al. 2021). It is also not clear yet what the specific inputs to the Island cells are and what information is initiating their temporal-gating activity. One possibility is that the medial prefrontal cortex (mPFC), which has been shown to play a role in the retention of the conditioned response in trace eyeblink conditioning (Volle et al. 2016; Jarovi et al. 2018) as well as temporal order memory (Naya et al. 2017), may be involved in this gating, sending information about the salience of the objects involved in individual events, determining what degree of threat or benefit is involved, and therefore the relevance between any two events due to its involvement in stimulus and mnemonic discrimination, as well as working memory (Dollemanc-Van Der Weel and Witter 1996; Weible et al. 2000; Chiba et al. 2001; Knight et al. 2004; Gilmartin and McChern 2005; Guimaraes et al. 2011; Johnson et al. 2021). The involvement of amygdala seems likely given the importance of emotional valence in memory and threat discrimination, and the fact that the amygdala can facilitate integration of information transmitted by mPFC inputs to perihilar cortex into MEC layer II/III (Phillips and LeDoux 1992; Kajiwara et al. 2003; Paz et al. 2006; Wahlstrom et al. 2018). Understanding what the driving factors underlying this process are will give greater insight into TAL.

In this study, we have demonstrated that the Island cells in the MEC regulate the duration of the temporal gap between events in MECII by reducing and modulating the input from Island cells to SL-INS, we were able to artificially associate or separate two temporally contiguous events during L-TFC. These findings open new avenues for investigation into the driving factors of the external control of temporal association and the formation of larger episodic memories.

Materials and Methods

Animals

WT male C57BL/6j mice purchased from Jackson laboratory (between 12 and 20 wk old) and Wfs-1 Cre male and female mice (RBRC03751; between 4 wk and 20 wk old) were group housed with littermates (two to five mice per cage) in a 12-h (6 a.m.–6 p.m.) light–dark cycle, with food and water available ad libitum. All experiments were conducted during the light cycle. Mice were randomly assigned to experimental conditions. All animal procedures were conformed to National Institutes of Health (NIH) and institutional guidelines and approved by the University of Texas (UT) Southwestern Institutional Animal Care and Use Committee (IACUC).

Stereotaxic surgery and virus microinjection

All animal aseptic surgeries were conducted with a stereotaxic frame (David Kopf Instruments) and followed NIH and UT Southwestern IACUC guidelines. Mice were anesthetized with 4% isoflurane for induction and with 1%–2% isoflurane for maintenance of anesthesia during surgery. A small amount of 2% lidocaine was placed on and under the skin as a topical analgesic and a small hole was drilled above each injection site. Microinjections were completed with a 10-µL Hamilton microsyringe and a glass micropipette filled with mineral oil and attached to a microsyringe pump (World Precision Instruments) with viral injections performed at a rate of 2 nL/sec. Coordinates are given relative to Bregma (in millimeters). The EC was targeted at AP: −4.85, ML: ±3.45, DV: −3.30. The micropipette was allowed to remain in place for 5 min following each injection to allow backflow of the viral solution. For optogenetic experiments, after injection, a Doric patchcord optical fiber (200-µm core diameter) was placed above the SL-layer in the CA1 region (Bregma: AP: −2.20, ML: ±1.5, DV: −1.30) as described previously (Kitamura et al. 2014). At the end of surgery, mice were given 0.9% sterile saline and meloxicam (2 mg/kg) as an analgesic, placed on a heating pad until fully recovered from the anesthesia, and were allowed to recover for a minimum of 3 d before returning to group housing with cage mates. After finishing all behavioral procedures, we performed histology to verify the target sites and expression of AAV injection.

Trace fear conditioning

TFC was performed on WT or Wfs-1-Cre male mice aged between 12 and 20 wk during the light cycle with minor modifications of the method as described previously (Kitamura et al. 2014). All animals were handled 5 min a day for five consecutive days prior to experimentation. The protocol of fear conditioning in WT mice with 20- or 60-sec trace periods was performed in a soundproof fear conditioning chamber (Med Associates). On day 1, mice were placed in context A (dim white light, white plastic semicircular board inserted into the chamber scented with 1% acetic acid) and allowed to explore for 240 sec, at which point a 20-sec tone (85 dB, 5 kHz) was played as conditioned stimulus (CS) followed by either a 20- or 60-sec trace, and then a 2-sec, 1.0-mA footshock as unconditioned stimulus (US). This was repeated two more times, starting at 402 and 564 sec trace, and then a 2-sec, 1.0-mA footshock as unconditioned stimulus (US). This was repeated two more times, starting at 402 and 564 sec trace, and then a 2-sec, 1.0-mA footshock as unconditioned stimulus (US). This was repeated two more times, starting at 402 and 564 sec trace, and then a 2-sec, 1.0-mA footshock as unconditioned stimulus (US). This was repeated two more times, starting at 402 and 564 sec trace, and then a 2-sec, 1.0-mA footshock as unconditioned stimulus (US). This was repeated two more times, starting at 402 and 564 sec trace, and then a 2-sec, 1.0-mA footshock as unconditioned stimulus (US). This was repeated two more times, starting at 402 and 564 sec trace, and then a 2-sec, 1.0-mA footshock as unconditioned stimulus (US). This was repeated two more times, starting at 402 and 564 sec trace, and then a 2-sec, 1.0-mA footshock as unconditioned stimulus (US). This was repeated two more times, starting at 402 and 564 sec trace, and then a 2-sec, 1.0-mA footshock as unconditioned stimulus (US). This was repeated two more times, starting at 402 and 564 sec trace, and then a 2-sec, 1.0-mA footshock as unconditioned stimulus (US). This was repeated two more times, starting at 402 and 564 sec trace, and then a 2-sec, 1.0-mA footshock as unconditioned stimulus (US). This was repeated two more times, starting at 402 and 564 sec trace, and then a 2-sec, 1.0-mA footshock as unconditioned stimulus (US). This was repeated two more times, starting at 402 and 564 sec trace, and then a 2-sec, 1.0-mA footshock as unconditioned stimulus (US).
by a function generator. Mice were then placed in context A and allowed to explore for 180 sec, at which point a 20-sec tone (75 dB, 2000 Hz) was played, followed by a 60-sec trace, and then a 2-sec, 0.75-mA footshock. This was repeated two more times, starting at 442 and 604 sec. During the CS–US pairing periods (82 sec) (Fig. 5A), mice received green light stimulation (15 mW, both hemispheres). Mice remained in the conditioning chamber for a total of 826 sec. On day 2, mice were placed in context B and allowed to explore for 180 sec, at which point the same 60-sec tone was played, followed by 180 sec of post-tone period. This was repeated two more times and mice were then returned after 880 or 960 sec in the chamber, respectively.

In the chemogenetic experiments, Wts1-L-Cre mice were bilaterally inoculated with AAV-Hsyn-DIO-hM4Di-mCherry (2.9 × 10^{13} gc/mL; Addgene) or AAV-Hsyn-DIO-mCherry (2.3 × 10^{13} gc/mL; Addgene) at AP: −4.85, ML: ±3.45, DV: −3.30 at a volume of 200 nl/side. Mice were allowed to recover for 2 wk before returning to group housing with cagemates. One week after group rehousing, mice were subjected to behavioral experiments or electrophysiological experiments. We performed the same behavioral procedure followed as the long trace (60-sec) TFC protocol described above. At 30 min prior to the conditioning session (Fig. 4C), these Wts1-Cre mice were intraperitoneally injected with CNO at dose of 4 mg/kg in sterile saline (Enzo).

In the induced-apoptosis experiment, Wts1-L-Cre mice and WT littermates were bilaterally inoculated with AAV-EF1a-Flex-taCas3-TEVp (4.0 × 10^{12} gc/mL; Addgene) at AP: −4.85, ML: ±3.45, DV: −3.30 at a volume of 300 nl/side. Mice were allowed to recover for 2 wk before returning to group housing with cagemates. Two weeks after the group rehousing, we performed the same L-TFC protocol described above at 4 wk after the surgery. All behavioral experiments were performed by a researcher blind to experimental conditions.

Immunohistochemistry
Mice were deeply anesthetized with a ketamine (75 mg/kg)/dexamethasone (1 mg/kg) cocktail by intraperitoneal injection and perfused transcardially with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Brains were removed and post-fixed in 4% PFA in PBS for 24 h at 4°C and then sliced sagittally using a 2-sec, 0.75-mA footshock. This was repeated two more times, and mice were then returned after 500 pA over 800 msec. After baseline recordings were obtained, ongoing passive recording of the membrane potential was engaged while 10 µM CNO (HelloBio) was added into the extracellular media. A timestamp was made in the recording when CNO was added to the tissue chamber, 3 min after recording began. This 3-min period is used as the baseline measurement. The recording was ended 10 min after the timestamp, and the ramp and current step protocols were repeated. Recordings were acquired with a lowpass filter at 4 kHz, and a sampling rate of 10 kHz at 1x gain.

All electrophysiological data was filtered using the Chebyshev method at 1200Hz prior to analysis. Rheobase was determined using the current ramp protocol to identify the current level at which cells began firing. Change in membrane potential was measured as the difference in membrane potential between the timestamp and the -3, -5, and -10 min mark. Baseline drift was corrected from the slope of the pre-CNO application period.

Statistics
Calculated statistics of all data are presented as means ± SEM. Experimenters were blinded to conditions of experiments during data acquisition and analysis. The experimental designs were counterbalanced. Statistical analyses were performed using GraphPad Prism 8 software. Physiological assessments were analyzed using paired t-tests for within subject measurements of rheobase and firing frequency. Assessments of RMP reduction were analyzed using one sample t-tests relative a nonchanging theoretical baseline. For behavioral experiments, comparisons of data between two groups were analyzed with two-tailed unpaired t-test, and multiple-group comparisons were assessed using two-way RM ANOVA when applicable, followed by Bonferroni’s post hoc test. Using by paired t-tests for within subject measurements of averaged freezing percent between baseline and tone. All statistical tests assumed an α level of 0.05. For all figures, * p < 0.05.

Acknowledgments
This work was supported by grants from Endowed Scholar Program to T.K., Brain Research Foundation to T.K. (BRFSG-2018-04),
Faculty Science and Technology Acquisition and Retention Program to T.K., the Brain & Behavior Research Foundation to T.K. (26391) and J.Y. (28801), the Whitetail Foundation to T.K. (2019-05-38), National Institute of Mental Health to T.K. (R01MH121034), and W.D.M. (F32MH122082), Daiichi Sankyo Foundation of Life Science to J.Y., the Uehara Memorial Foundation to J.Y., Japan Society for the Promotion of Science, N.Y. (201860573) and Human Frontier Science Program to T.K. (RGY0072/2018).

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Received March 15, 2021; accepted in revised form July 8, 2021.