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Distinct hippocampal engrams control extinction and relapse of fear memory

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Learned fear often relapses after extinction, suggesting that extinction training generates a new memory that coexists with the original fear memory; however, the mechanisms governing the expression of competing fear and extinction memories remain unclear. We used activity-dependent neural tagging to investigate representations of fear and extinction memories in the dentate gyrus. We demonstrate that extinction training suppresses reactivation of contextual fear engram cells while activating a second ensemble, a putative extinction engram. Optogenetic inhibition of neurons that were active during extinction training increased fear after extinction training, whereas silencing neurons that were active during fear training reduced spontaneous recovery of fear. Optogenetic stimulation of fear acquisition neurons increased fear, while stimulation of extinction neurons suppressed fear and prevented spontaneous recovery. Our results indicate that the hippocampus generates a fear extinction representation and that interactions between hippocampal fear and extinction representations govern the suppression and relapse of fear after extinction.

Maladaptive learned fear is commonly treated using therapies based on extinction, i.e., repeated exposure to a fear-evoking stimulus in the absence of threat[1,2]. Extinction does not permanently eliminate the fear memory. Fear can, for instance, relapse with the passage of time, a phenomenon known as spontaneous recovery[3,4]. The transience of extinction suggests that extinction training does not erase learned fear but, instead, establishes a new memory that suppresses the fear memory or competes with it for expression[5,6]. The acquisition of an extinction memory depends critically on prefrontal cortex (PFC)-to-amygdala projections[7,8], but the mechanisms governing the expression of extinction are not well understood. Based on evidence that inhibiting hippocampal activity interferes with extinction learning[9] and the context-dependency of extinction retrieval[10–12], we investigated how the activity of hippocampal ensembles regulates the expression of an extinction memory.

The hippocampal dentate gyrus plays a critical role in the acquisition of contextual fear memory—fear of a place where an aversive experience occurred. Contextual fear acquisition activates a sparse ensemble of dentate gyrus granule cells, sometimes termed 'fear engram cells', whose reactivation is necessary[13] and sufficient[14–17] for the expression of contextual fear. Fear engram cells retain their ability to evoke fear when optogenetically stimulated at remote time points[18] and after amnestic treatments[16,19], suggesting that they constitute a stable neural representation of contextual fear memory. However, it is unknown how extinction training affects the activity of fear engram cells. We recently discovered that activity in the dentate gyrus is necessary for the extinction of contextual fear[9], which led us to hypothesize that extinction training might suppress fear by altering the activity of fear engram cells in the dentate gyrus.

We used an activity-dependent neuronal tagging transgenic mouse line[13] to indelibly label and manipulate dentate gyrus granule cells active during either contextual fear acquisition or extinction. We report that extinction training suppresses the reactivation of neurons that were active during fear training (fear acquisition neurons), while causing a different population—putative extinction neurons—to become active. Silencing neurons tagged during fear acquisition decreased fear, whereas silencing neurons tagged during extinction training increased fear after extinction. Conversely, optogenetic stimulation of neurons tagged during fear acquisition increased fear, whereas stimulation of neurons tagged during extinction decreased fear. Our data lead us to hypothesize that fear acquisition and fear extinction are represented by unique ensembles of dentate gyrus granule cells and that suppression and relapse of fear after extinction are controlled by the activity of these ensembles.

Results

Extinction suppresses reactivation of fear acquisition neurons. We used the ArcCreERT2 transgenic mouse line to tag and manipulate neurons active during either contextual fear conditioning (CFC) acquisition or fear extinction. In these mice, activity of the immediate early gene (IEG) *Arc* drives the expression of tamoxifen-dependent CreERT2 recombinase. An injection of 4-hydroxytamoxifen (4-OHT) transiently activates recombinase activity, thereby permanently tagging Arc-expressing neurons with a reporter (Fig. 1a). Previous work demonstrates that: (1) ArcCreERT2 reporter expression closely mirrors that of endogenous Arc protein; (2) the system detects unique hippocampal ensembles active in different contexts; (3) CFC retrieval is associated with reactivation of neurons tagged during fear acquisition; and (4) silencing neurons tagged during fear acquisition impairs the behavioral expression of fear[13,20,21].

We first sought to determine if extinction training affects the reactivation of dentate gyrus fear acquisition neurons. ArcCreERT2::channelrhodopsin (ChR2)-enhanced yellow fluorescent protein (eYFP)^{flx} mice were injected with 4-OHT before CFC training to tag active neurons with eYFP. Five days later, one group

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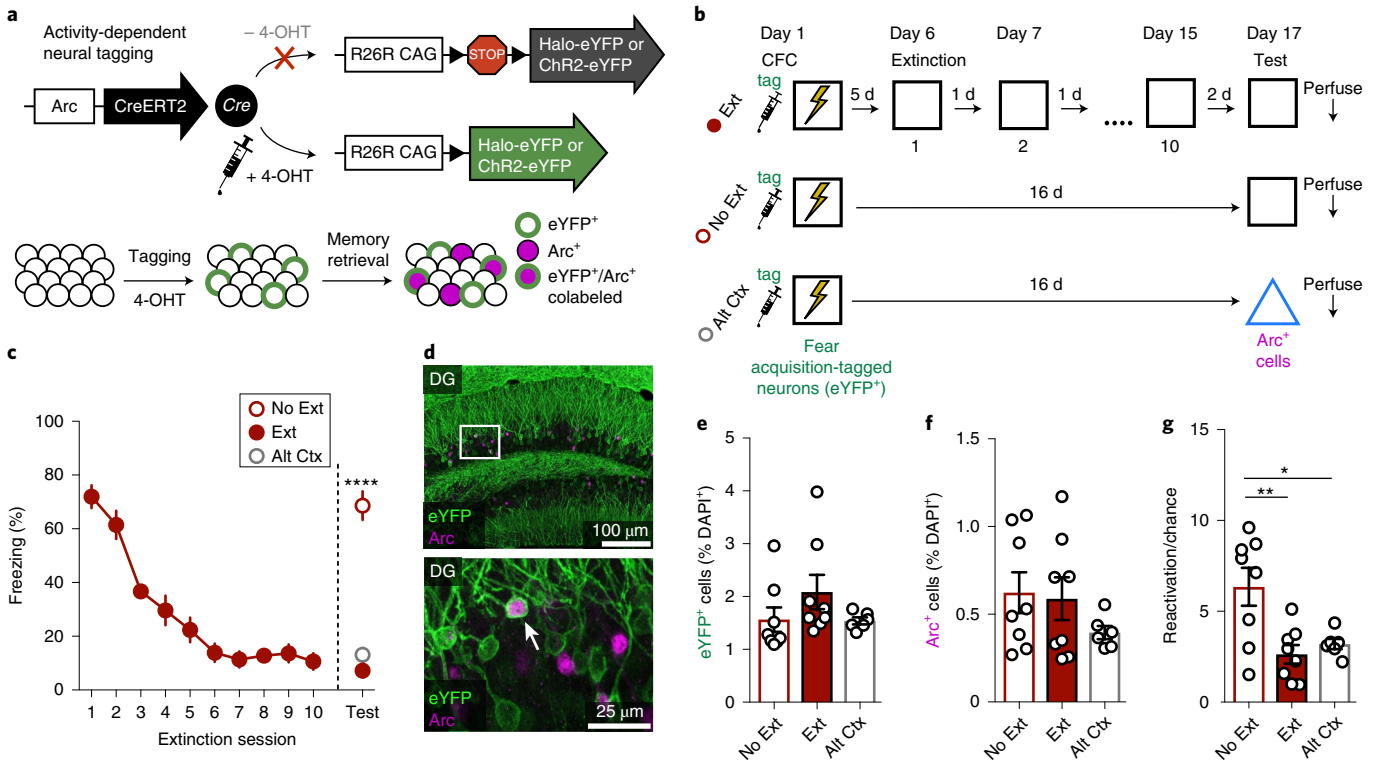

Fig. 1 | Extinction suppresses reactivation of fear acquisition-tagged neurons. a, Genetic design. Administration of 4-OHT to ArcCreERT2 mice activates permanent expression of a reporter in neurons active around the time of the injection. **b**, Experimental design. **c**, Freezing behavior declined across the 3-min extinction sessions. The No Ext group had significantly more freezing than the Ext and Alt Ctx groups during a retrieval test (one-way ANOVA with Sidak's post hoc test, $F_{(2,19)}=78.99$, $P<0.0001$). **d**, Representative image of eYFP$^+$ and Arc$^+$ immunofluorescence in the dentate gyrus (DG). The white box indicates the area of magnification (bottom). The white arrow denotes a colabeled eYFP$^+$/Arc$^+$ cell. **e**, The Ext, No Ext, and Alt Ctx groups displayed a similar percentage of eYFP$^+$ cells among DAPI$^+$ cells. **f**, Fear retrieval, extinction retrieval, and alternate context exposure activated a similar percentage of Arc$^+$ cells among DAPI$^+$ cells. **g**, The number of eYFP$^+$/Arc$^+$ reactivated cells was significantly higher in the No Ext group compared to the Ext and Alt Ctx groups. Reactivation was calculated as the percentage of eYFP$^+$/Arc$^+$ cells divided by the chance percentage (one-way ANOVA with Sidak's post hoc test, $F_{(2,19)}=7.55$, $P=0.004$). No Ext: $n=8$ mice; Ext: $n=8$ mice; Alt Ctx: $n=6$ mice. Data are presented as mean ± s.e.m. *$P<0.05$, **$P<0.01$, ****$P<0.0001$.

of mice (the Ext group, $n=8$) began extinction training, which consisted of ten 3-min exposures to the conditioned context without shock. The other groups remained in the home cage during this time. Mice were then exposed to the conditioned context (No Ext, $n=8$) or an alternate context (Alt Ctx, $n=6$) to test for conditioned fear (Fig. 1b). Freezing to the conditioned context decreased in the Ext group across the ten extinction sessions (Fig. 1c; repeated-measures one-way analysis of variance (ANOVA), $F_{(9,63)}=45.52$, $P<0.0001$). During the test session 2 d after the final day of extinction (and 16 d after training), the freezing of the No Ext group exceeded that of the Ext and Alt Ctx groups ($F_{(2,19)}=78.99$, $P<0.0001$). Mice were perfused 90 min after the retrieval session. Immunohistochemistry against Arc and eYFP was used to assess reactivation of the eYFP$^+$ neurons that had been tagged during fear acquisition (Fig. 1d). The Ext, No Ext, and Alt Ctx groups displayed similar numbers of eYFP$^+$ (Fig. 1e; $F_{(2,19)}=1.53$, $P=0.243$) and Arc$^+$ (Fig. 1f; $F_{(2,19)}=1.15$, $P=0.339$) cells as a percentage of 4,6-diamidino-2-phenylindole (DAPI)$^+$ cells. Reactivation of eYFP$^+$ cells was assessed by dividing the percentage of eYFP$^+$/Arc$^+$ colabeled cells among DAPI$^+$ cells by the chance percentage ((eYFP$^+$/DAPI$^+$)×(Arc$^+$/DAPI$^+$)×100)[22–24]. Reactivation was reduced in the Ext and Alt Ctx groups compared to the No Ext group (Fig. 1g and Supplementary Fig. 1; Supplementary Table 1; $F_{(2,19)}=7.55$, $P=0.004$), demonstrating that extinction suppresses the reactivation of dentate gyrus fear acquisition neurons to a similar extent as exposure to a neutral, alternate context. Furthermore, because the number of Arc$^+$ neurons did not differ among the groups, we infer that retrieval of fear and retrieval of extinction similarly engage the dentate gyrus but activate distinct neuronal ensembles.

Fear retrieval and extinction retrieval reactivate distinct ensembles. To test the hypothesis that retrieval of fear and retrieval of extinction recruit unique dentate gyrus ensembles, we performed an experiment where neurons active during fear training or fear extinction were tagged in separate cohorts of mice. ArcCreERT2::halorhodopsin (Halo)-eYFP^{flx} mice were subjected to CFC and extinction. 4-OHT was injected either immediately after fear acquisition (Acq-Tag; $n=16$) or immediately after the tenth day of extinction (Ext-Tag; $n=15$). 4-OHT injected immediately after CFC effectively tags fear engram neurons[20] and was performed to prevent the injection from becoming a conditioned stimulus or retrieval cue. Mice were returned to the context either 5 d later for an extinction retrieval test or 28 d later for a spontaneous recovery test (Fig. 2a). Freezing to the conditioned context decreased across the extinction sessions in both groups (Fig. 2b; repeated-measures two-way ANOVA, effect of session ($F_{(9,261)}=69.4$, $P<0.0001$), no effect of group ($F<1$) or group X session interaction ($F<1$)). Freezing remained low during the extinction retrieval test but was elevated during the spontaneous recovery test (two-way ANOVA, effect of test ($F_{(1,27)}=10.04$, $P=0.004$)). All mice were perfused 90 min after their test session to evaluate the reactivation of fear- and extinction-tagged neurons during low fear (extinction retrieval) and high

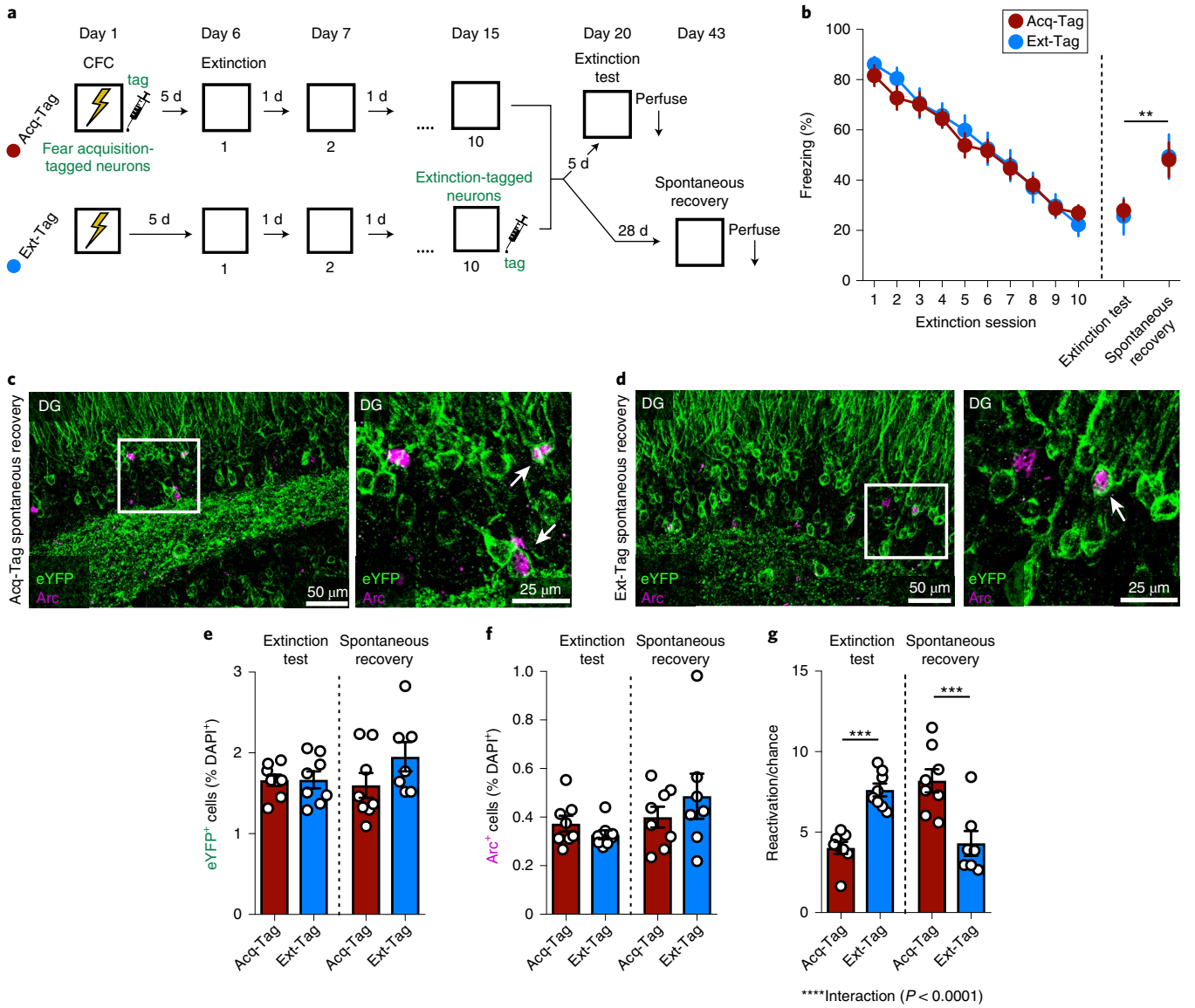

Fig. 2 | Fear retrieval and extinction retrieval reactivate distinct neural ensembles in the dentate gyrus. a, Experimental design. **b**, Freezing behavior declined across the 5-min extinction sessions. Freezing remained low in mice tested 5 d after extinction (extinction retrieval) but was increased in mice tested 28 d after extinction (spontaneous recovery) (two-way ANOVA, effect of test, $F_{(1,27)} = 10.04$, $P = 0.004$). **c**,**d**, Representative images of eYFP$^+$ and Arc$^+$ immunofluorescence in the dentate gyrus of Acq-Tag (**c**) and Ext-Tag (**d**) mice during a spontaneous recovery test. The white box indicates the area of magnification (right). The white arrow denotes colabeled eYFP$^+$/Arc$^+$ cells. **e**, The Acq-Tag and Ext-Tag groups displayed similar percentages of eYFP$^+$ cells among DAPI$^+$ cells. **f**, Extinction retrieval and spontaneous recovery activated similar percentages of Arc$^+$ cells among DAPI$^+$ cells. **g**, Ext-Tag mice displayed a significantly greater percentage of reactivated eYFP$^+$/Arc$^+$ cells (relative to chance) than Acq-Tag mice during an extinction retrieval test. The pattern was reversed during the spontaneous recovery test (two-way ANOVA with Sidak's post hoc test, significant test × group interaction, $F_{(1,27)} = 41.47$, $P < 0.0001$). Acq-Tag extinction test: $n = 8$ mice; Ext-Tag extinction test: $n = 8$ mice; Acq-Tag spontaneous recovery: $n = 8$ mice; Ext-Tag spontaneous recovery: $n = 7$ mice. Data are presented as mean ± s.e.m. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

fear (spontaneous recovery) states (Fig. 2c,d). We predicted that extinction-tagged neurons would be reactivated during the 5-d test, whereas fear acquisition-tagged neurons would be reactivated during the 28-d test.

The Acq-Tag and Ext-Tag groups displayed similar numbers of eYFP$^+$ cells (Fig. 2e; no effect of test ($F < 1$), group ($F_{(1,27)} = 1.87$, $P = 0.183$), or test × group interaction ($F_{(1,27)} = 1.76$, $P = 0.196$)), and Arc$^+$ cells (Fig. 2f; no effect of test ($F_{(1,27)} = 3.32$, $P = 0.080$), group ($F < 1$), or test × group interaction ($F_{(1,27)} = 1.68$, $P = 0.206$)). During the 5-d test, Ext-Tag mice displayed an increased amount of reactivated eYFP$^+$/Arc$^+$ neurons compared to Acq-Tag mice (Fig. 2g and Supplementary Fig. 1; significant test × group interaction ($F_{(1,27)} = 41.47$, $P < 0.0001$)). The pattern reversed during the 28-d test, with Acq-Tag mice displaying a higher number of reactivated eYFP$^+$/Arc$^+$ neurons than Ext-Tag mice. These data demonstrate that fear acquisition and extinction activate different populations of dentate gyrus neurons. Expression of extinction is associated with reactivation of neurons that were active during extinction training, whereas expression of fear during spontaneous recovery is associated with the reactivation of neurons that were active during fear acquisition.

Silencing extinction-tagged neurons impairs extinction retrieval. Next, we evaluated whether the reactivation of extinction-tagged

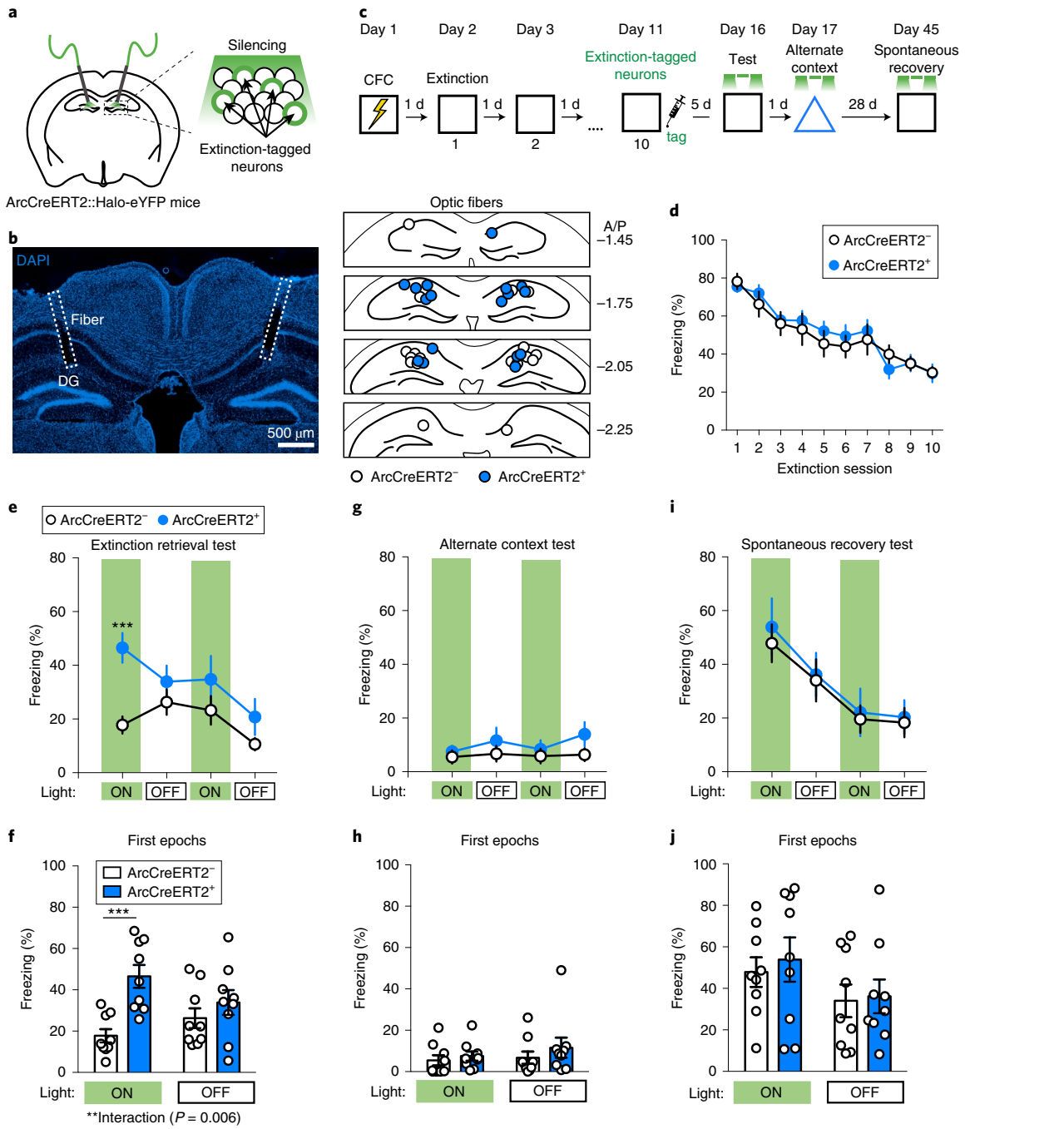

Fig. 3 | Silencing extinction-tagged neurons impairs extinction retrieval. a, Schematic for silencing extinction-tagged neurons in the dentate gyrus of ArcCreERT2::Halo-eYFP^{flx} mice. **b**, Left: example of optic fiber placement. Right: coronal figures showing the fiber implantation sites in the dorsal dentate gyrus. **c**, Experimental design. Light was delivered during context tests at minutes 0–3 and 6–9. **d**, Freezing declined across the 5-min extinction sessions and did not differ between ArcCreERT2$^+$ and ArcCreERT2$^-$ mice. **e**, Freezing behavior during the extinction retrieval test (three-way repeated-measures ANOVA, significant genotype × epoch × light interaction, $F_{(1,14)} = 5.40$, $P = 0.036$). **f**, In the extinction retrieval test, silencing extinction-tagged neurons increased freezing in ArcCreERT2$^+$ mice during the first light ON epoch compared to ArcCreERT2$^-$ mice (two-way repeated-measures ANOVA with Sidak's post hoc test, significant genotype × light interaction, $F_{(1,16)} = 10.24$, $P = 0.006$). **g**, Freezing behavior during the alternate context test. **h**, In the alternate context test, silencing extinction-tagged neurons had no effect on freezing behavior during the first light ON and OFF epochs. **i**, Freezing behavior during the spontaneous recovery test. **j**, In the spontaneous recovery test, silencing extinction-tagged neurons had no effect on freezing behavior during the first light ON and OFF epochs. ArcCreERT2$^-$: $n = 9$ mice; ArcCreERT2$^+$: $n = 9$ mice. Data are presented as mean ± s.e.m. $^{**}P < 0.01$, $^{***}P < 0.001$.

neurons is necessary for the expression of extinction. ArcCreERT2$^+$::Halo-eYFP^{flx} and their ArcCreERT2$^-$::Halo-eYFP^{flx} littermates ($n = 9$ per group) were implanted bilaterally with optical fibers targeting the dorsal dentate gyrus (Fig. 3a,b); 2–4 weeks later, all mice received CFC followed by extinction training. 4-OHT was injected immediately after the tenth day of extinction, thereby expressing Halo in the Arc$^+$ cells activated during extinction (Fig. 3c).

Freezing declined across the extinction sessions and did not differ between ArcCreERT2$^+$ and ArcCreERT2$^-$ mice (Fig. 3d; effect of session ($F_{(9,135)} = 35.43$, $P < 0.0001$), no effect of genotype ($F < 1$)

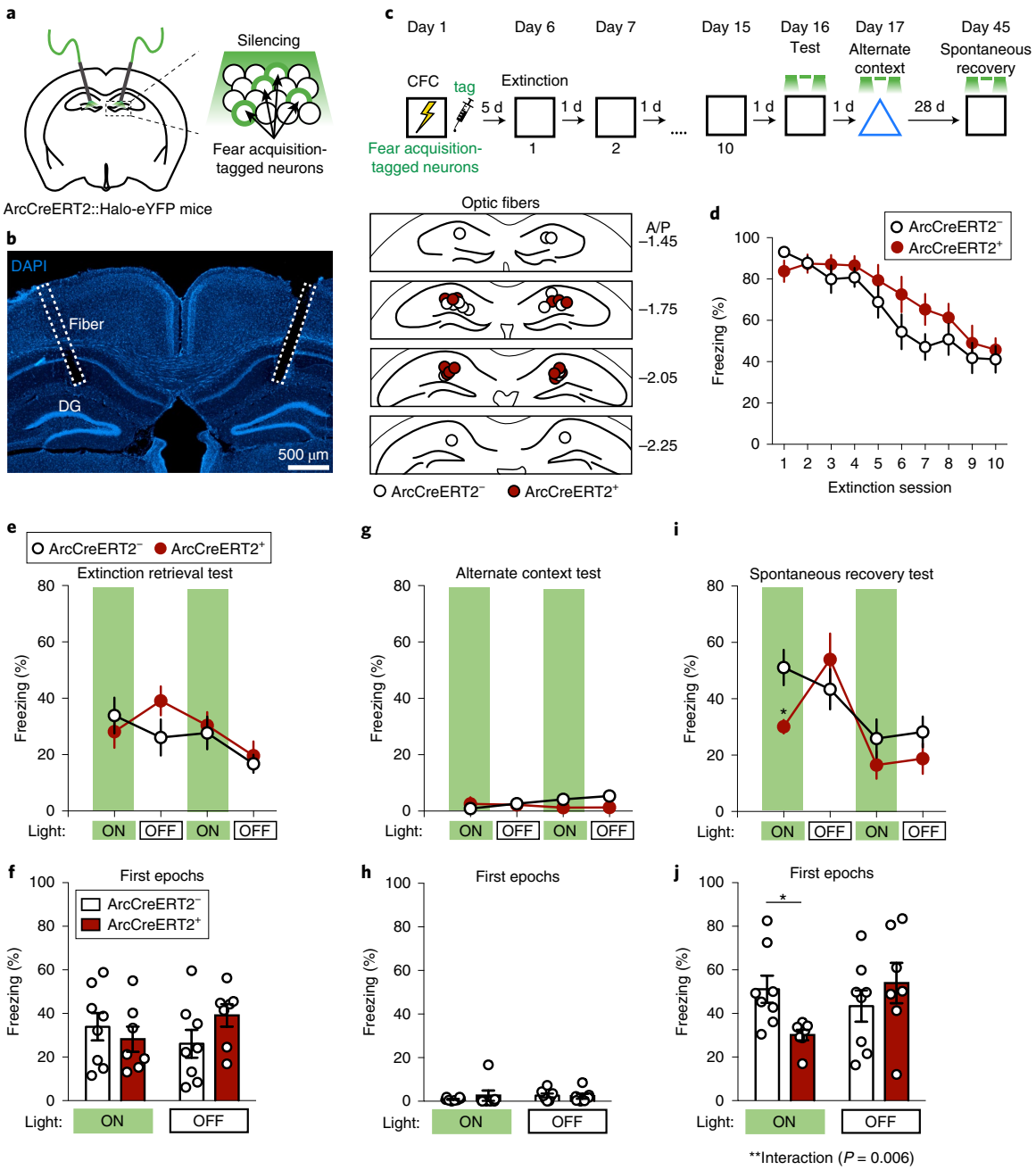

Fig. 4 | Silencing fear acquisition-tagged neurons reduces spontaneous recovery of fear. a, Schematic for silencing fear acquisition-tagged neurons in the dentate gyrus of ArcCreERT2::Halo-eYFP^{flx} mice. **b**, Left: example of optic fiber placement. Right: coronal figures showing the fiber implantation sites in the dorsal dentate gyrus. **c**, Experimental design. Light was delivered during context tests at minutes 0–3 and 6–9. **d**, Freezing declined across the 5-min extinction sessions and did not differ between ArcCreERT2$^+$ and ArcCreERT2$^-$ mice. **e**, Freezing behavior during the extinction retrieval test. **f**, In the extinction retrieval test, silencing fear acquisition-tagged neurons had no effect on freezing behavior during the first light ON and OFF epochs. **g**, Freezing behavior during the alternate context test. **h**, In the alternate context test, silencing fear acquisition-tagged neurons had no effect on freezing behavior during the first light ON and OFF epochs. **i**, Freezing behavior during the spontaneous recovery test (three-way repeated-measures ANOVA, significant genotype × epoch × light interaction, $F_{(1,11)} = 10.01$, $P = 0.009$). **j**, In the spontaneous recovery test, silencing fear acquisition-tagged neurons reduced freezing behavior in ArcCreERT2$^+$ mice during the first light ON epoch compared to ArcCreERT2$^-$ mice (two-way repeated-measures ANOVA with Sidak's post hoc test, $F_{(1,13)} = 10.68$, $P = 0.006$). ArcCreERT2$^-$: $n = 8$ mice; ArcCreERT2$^+$: $n = 7$ mice. Data are presented as mean ± s.e.m. $^*P < 0.05$, $^{**}P < 0.01$.

or genotype × session interaction ($F < 1$)). Five days later, mice were returned to the context for a 12-min extinction retrieval test. Extinction-tagged neurons were silenced bilaterally with green light (9–12 mW, continuous) during minutes 0–3 and 6–9 of the test. Silencing extinction-tagged neurons increased freezing during the first light ON epoch; freezing returned to control levels during the light OFF epoch (Fig. 3e,f; significant genotype × epoch × light interaction ($F_{(1,14)} = 5.40$, $P = 0.036$)). Interestingly, inhibiting extinction-tagged neurons had no effect on freezing during the second light ON epoch (Fig. 3e and Supplementary Fig. 2), suggesting that although these cells contribute to the initiation of extinction retrieval, they are not necessary for maintaining retrieval after it has occurred. Silencing extinction-tagged neurons had no effect on freezing in a neutral, alternate context (Fig. 3g,h; no effect of

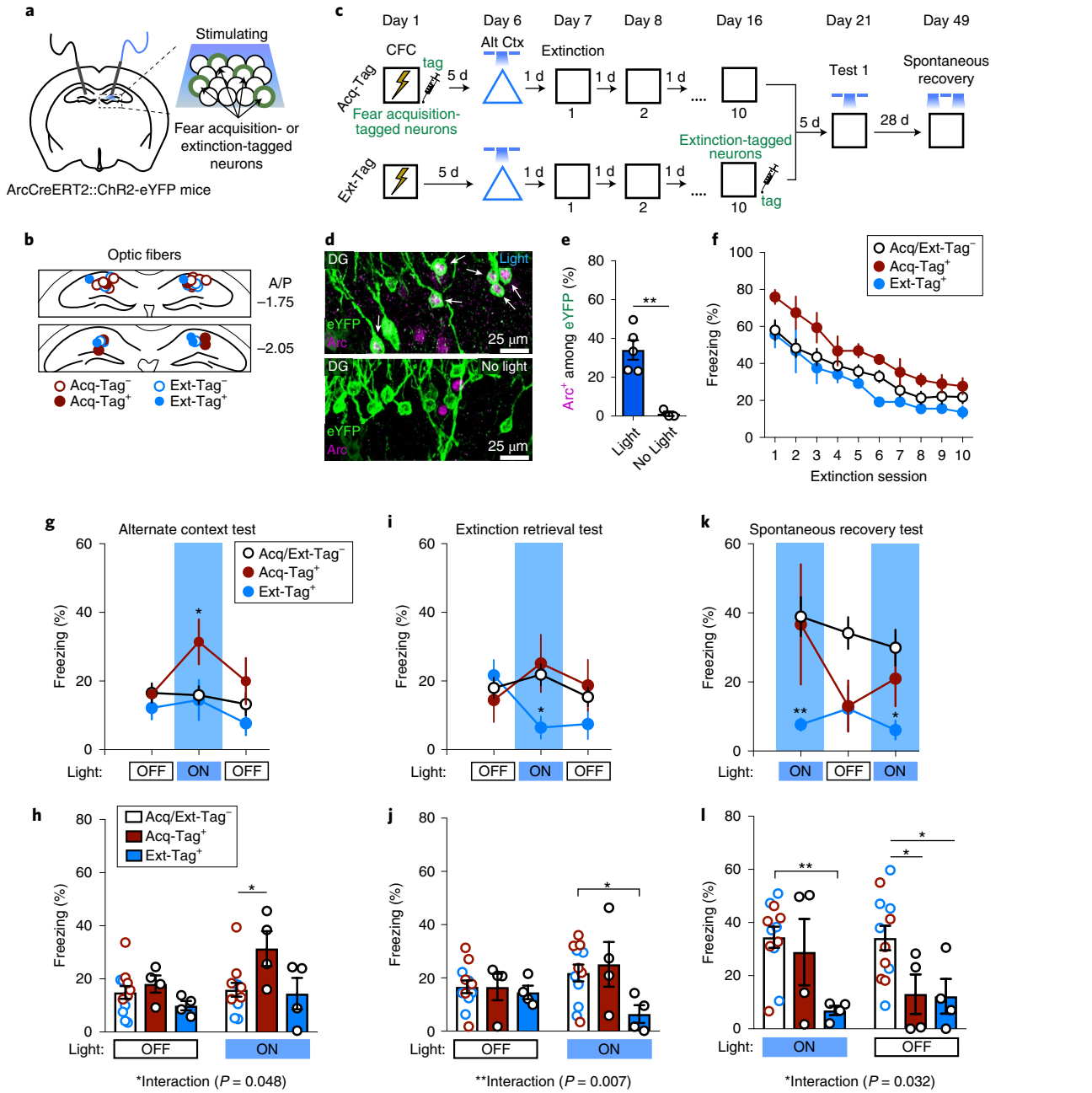

Fig. 5 | Stimulating fear acquisition-tagged neurons potentiates fear, whereas stimulating extinction-tagged neurons suppresses fear. a, Schematic for stimulating fear acquisition-tagged or extinction-tagged neurons in the dentate gyrus of ArcCreERT2::ChR2-eYFP^{flx} mice. **b**, Coronal figures showing fiber implantation sites in the dorsal dentate gyrus. **c**, Experimental design. Light was delivered during alternate context and extinction tests during minutes 3–6. For the spontaneous recovery test, light was delivered during minutes 0–3 and 6–9. **d**, Representative images of eYFP$^+$ and Arc$^+$ immunofluorescence in the dentate gyrus of Acq-Tag$^+$ mice presented with light (top) or without light (bottom). The white arrows denote colabeled eYFP$^+$/Arc$^+$ cells. **e**, Light increased the percentages of Arc$^+$ cells among eYFP$^+$ cells in Acq-Tag$^+$ and Ext-Tag$^+$ mice (two-sided *t*-test, $t_{(6)} = 4.86$, $P = 0.003$). The white arrows denote colabeled eYFP$^+$/Arc$^+$ cells. Light: $n = 5$ mice; No light: $n = 3$ mice. **f**, Freezing declined across the 5-min extinction sessions and did not differ between groups. **g**, Freezing behavior during the alternate context test (two-way repeated-measures ANOVA with Sidak's post hoc test, effect of light, $F_{(2,34)} = 3.31$, $P = 0.048$). **h**, In the alternate context test, stimulating fear acquisition-tagged neurons increased freezing during the light ON epoch in Acq-Tag$^+$ mice compared to Acq/Ext-Tag$^-$ mice. Light OFF epochs were averaged (two-way repeated-measures ANOVA with Sidak's post hoc test, significant group × light interaction, $F_{(2,17)} = 3.65$, $P = 0.048$). **i**, Freezing behavior during the extinction retrieval test (two-way repeated-measures ANOVA with Sidak's post hoc test, significant group × light interaction, $F_{(4,34)} = 3.09$, $P = 0.029$). **j**, In the extinction retrieval test, stimulating extinction-tagged neurons decreased freezing during the light ON epoch in Ext-Tag$^+$ mice compared to Acq/Ext-Tag$^-$ mice. The light OFF epochs averaged (two-way repeated-measures ANOVA with Sidak's post hoc test, significant group × light interaction, $F_{(2,17)} = 6.78$, $P = 0.007$). **k**, Freezing behavior during the spontaneous recovery test (two-way repeated-measures ANOVA with Sidak's post hoc test, effect of group, $F_{(2,17)} = 4.92$, $P = 0.021$). **l**, In the spontaneous recovery test, stimulating extinction-tagged neurons reduced freezing during the light ON epochs in Ext-Tag$^+$ mice compared to Acq/Ext-Tag$^-$ mice. The light ON epochs were averaged (two-way repeated-measures ANOVA with Sidak's post hoc test, significant group × light interaction, $F_{(2,17)} = 4.24$, $P = 0.032$). Acq-Tag$^-$: $n = 6$ mice; Acq-Tag$^+$: $n = 4$ mice; Ext-Tag$^-$: $n = 6$ mice; Ext-Tag$^+$: $n = 4$ mice. Data are presented as mean ± s.e.m. $^*P < 0.05$, $^{**}P < 0.01$.

genotype ($F_{(1,14)}=2.82$, $P=0.12$) or interactions ($P>0.20$)). To rule out an order effect, 1 d after the alternate context test, mice were given a second exposure to the conditioning context with light presentations. Silencing extinction-tagged neurons again increased freezing during the first light ON epoch but not during the second epoch (Supplementary Fig. 3).

When mice were returned to the conditioned context 28 d after the alternate context test, silencing extinction-tagged neurons had no effect on spontaneous recovery (Fig. 3i,j; no effect of genotype ($F<1$) or interactions ($F<1$)). These results demonstrate that dentate gyrus neurons active during fear extinction are necessary for the expression of extinction memory. The activity of extinction-tagged neurons is not necessary during spontaneous recovery of fear, possibly because spontaneous recovery reflects the failure to reactivate the extinction memory.

Silencing fear acquisition-tagged neurons reduces spontaneous recovery of fear. Reactivation of hippocampal fear acquisition neurons is necessary for the expression of contextual fear[13,25]. It is unknown if the activity of these neurons is also required for the expression of fear after extinction, such as during spontaneous recovery. To address this question, ArcCreERT2^{+}::Halo-eYFP^{flx} ($n=7$) and ArcCreERT2^{-}::Halo-eYFP^{flx} littermates ($n=8$) were implanted bilaterally with optical fibers targeting the dorsal dentate gyrus (Fig. 4a,b); 2–4 weeks later, mice received CFC training followed immediately by an injection of 4-OHT, thereby expressing Halo in fear acquisition neurons, and then a course of extinction training (Fig. 4c).

Contextual fear decreased across the 10 d of extinction; freezing did not differ between genotypes (Fig. 4d; effect of session ($F_{(9,117)}=27.07$, $P<0.0001$), no effect of genotype ($F_{(1,13)}=1.25$, $P=0.284$) or genotype × session interaction ($F_{(9,117)}=1.48$, $P=0.165$)). Five days after the final extinction session, mice were returned to the context for an extinction retrieval test with green light delivered during minutes 0–3 and 6–9. Silencing fear acquisition-tagged neurons had no effect on freezing during the light ON epochs of this test (Fig. 4e,f). Although the genotype × epoch × light interaction reached significance ($F_{(1,11)}=5.42$, $P=0.040$), the pairwise between-group tests were not significant. The next day, light was delivered during exposure to an alternate context. Silencing fear acquisition-tagged neurons had no effect on freezing behavior (Fig. 4g,h; no effect of genotype ($F_{(1,11)}=1.60$, $P=0.232$) or interactions ($P>0.07$)). In contrast, when mice were returned to the conditioning context for a spontaneous recovery test 28 d later, silencing fear acquisition-tagged neurons reduced contextual fear (Fig. 4i,j; significant genotype × epoch × light interaction ($F_{(1,11)}=10.01$, $P=0.009$)). Freezing in ArcCreERT2^{+} mice increased during the first light OFF epoch but was reduced again during the second light ON epoch. These results suggest that the neurons active during fear acquisition are not required for extinction retrieval but are necessary for the spontaneous recovery of fear.

Stimulating fear acquisition-tagged neurons potentiates fear whereas stimulating extinction-tagged neurons suppresses fear. Our findings suggest that reactivation of neurons active during extinction training suppresses fear and the failure of these neurons to reactivate contributes to fear relapse after extinction. To determine whether the activity of extinction neurons is sufficient to suppress fear and attenuate fear relapse, we generated mice expressing the optogenetic neural activator ChR2 in fear acquisition-tagged or extinction-tagged neurons. ArcCreERT2^{+}::ChR2-eYFP^{flx} and their ArcCreERT2^{-}::ChR2-eYFP^{flx} littermates were implanted bilaterally with optical fibers targeting the dorsal dentate gyrus (Fig. 5a,b); 2–4 weeks later, all mice received CFC and extinction training (Fig. 5c). 4-OHT was injected either immediately after fear acquisition (Acq-Tag^{+}: $n=4$; Acq-Tag^{-}: $n=6$) or immediately after the tenth day of extinction (Ext-Tag^{+}: $n=4$; Ext-Tag^{-}: $n=6$), thereby expressing ChR2 in fear acquisition-tagged or extinction-tagged neurons. We confirmed that 3 min of unilateral blue light (10 Hz, 20 ms pulses, 0.75–0.9 mW) delivered in an alternate context increased the percentage of Arc^{+} cells among eYFP^{+} cells in Acq-Tag^{+} and Ext-Tag^{+} mice (Fig. 5d,e and Supplementary Fig. 4; $t_{(6)}=4.86$, $P=0.003$). Groups not expressing ChR2 (Acq-Tag^{-} and Ext-Tag^{-}) performed similarly in all test sessions and were combined into a single control group.

Five days after fear acquisition and 1 d before extinction training, mice were tested for 9 min in an alternate context with light delivered during minutes 3–6. This test was performed to confirm that stimulation of fear acquisition-tagged neurons potentiates fear expression. Indeed, stimulating fear acquisition-tagged neurons increased freezing during the light ON epoch (Fig. 5g,h; significant group × light interaction ($F_{(2,17)}=3.65$, $P=0.048$)). Because this test occurred before 4-OHT administration in Ext-Tag^{+} mice, light stimulation failed to alter behavior in these mice.

Contextual fear decreased across the 10 d of extinction; freezing did not differ between groups (Fig. 5f; effect of session ($F_{(9,153)}=41.55$, $P<0.0001$), no effect of group ($F_{(2,17)}=3.34$, $P=0.060$) or genotype × session interaction ($F<1$)). Five days after the final extinction session, mice were returned to the context for an extinction retrieval test with light delivered during minutes 3–6. Stimulating extinction-tagged neurons reduced freezing. Stimulating fear acquisition-tagged neurons had no effect on freezing, (Fig. 5i,j; significant group × light interaction ($F_{(2,17)}=6.78$, $P=0.007$)). In an open field test, stimulation of fear acquisition-tagged or extinction-tagged neurons failed to affect center time, center distance, or total distance traveled (Supplementary Fig. 5).

The mice were returned to the context 28 d later for a spontaneous recovery test, with light delivered during minutes 0–3 and 6–9. In contrast to previous test sessions, light stimulation was delivered at the beginning of the session to determine if stimulation of extinction-tagged neurons could prevent spontaneous recovery of fear, which is typically strongest at the start of the test session. Stimulating extinction-tagged neurons blocked spontaneous recovery, an effect that persisted even after light termination (Fig. 5k,l; significant group × light interaction ($F_{(2,17)}=4.24$, $P=0.032$)). For consistency with the previous light stimulation sessions, mice were given a second exposure to the context on the following day, with light delivered in the middle of the session (Supplementary Fig. 6). Stimulating extinction-tagged neurons again reduced freezing and there was no effect of stimulating fear acquisition-tagged neurons. These results demonstrate that neurons active during fear acquisition and fear extinction have opposing effects on fear behavior. Whereas activity of fear acquisition neurons is sufficient to induce fear, activity of extinction neurons is sufficient to suppress fear.

Discussion

We used activity-dependent neuronal tagging to investigate how extinction training influences contextual fear representations in the dentate gyrus. Previous research demonstrates that recall of contextual fear requires reactivation of the dentate gyrus neurons that were active during fear acquisition[13]. Our results demonstrate that extinction training suppresses reactivation of the fear acquisition ensemble and recruits a different ensemble of dentate gyrus neurons putatively representing the extinction memory. These fear extinction neurons were more active than fear acquisition neurons during a test session 5 d after extinction, when fear expression was low. In contrast, during a spontaneous recovery test 28 d after extinction, fear acquisition neurons were more active than extinction neurons. Optogenetic manipulations revealed that reactivation of fear extinction neurons is necessary and sufficient for the expression of extinction, whereas reactivation of fear acquisition neurons is necessary for spontaneous recovery. Our data suggest that fear acquisition and

extinction training have unique ensemble representations in the dentate gyrus, and competition between these ensembles controls expression of fear after extinction.

The role of the hippocampus in CFC is thought to involve generating context representations that acquire the ability to activate the expression of fear through hippocampal interactions with the amygdala[26–28]. Behavioral evidence suggests that changes in context valence are associative in nature, in that they reflect changes in the ability of a stable context representation to evoke fear, rather than the generation of new context representations[29]. This view is supported by evidence that rodents can be fear-conditioned to a remembered context representation[30] and by evidence that pairing artificial reactivation of a context representation with shock is sufficient to produce fear of a previously neutral context[15,16]. However, other evidence suggests that changes in context valence might alter context representations in the hippocampus. In the hippocampal CA1 region, the place fields of some neurons remap during fear conditioning and/or extinction[31]. Fear acquisition and extinction also induce IEG expression in different ensembles of CA1 pyramidal cells[32]. Our results demonstrate that unique ensemble representations of fear and extinction are present upstream in the dentate gyrus and that the activity of these dentate gyrus ensembles plays a causal role in expression of fear and extinction. The maintenance of separate fear and extinction representations in the hippocampus appears to provide a mechanism for fear relapse after extinction.

Whereas lesions and non-specific silencing of the dentate gyrus fail to impair expression of context fear and other learned responses[33–35], paradoxically, we found that silencing sparse populations of fear acquisition-tagged or extinction-tagged neurons alters fear expression. A recent study using computational simulations of hippocampal function provides a potential resolution to this paradox[9]. The simulations suggest that when only engram cells are silenced, the remaining activity in the dentate gyrus is contextually inappropriate and interferes with recall. Large-scale dentate gyrus silencing minimizes this inappropriate activity, allowing accurate recall through cortical inputs to the CA3 region (bypassing the dentate gyrus), which are sufficient for correct recall under some circumstances. This model leads us to speculate that silencing extinction cells could lead to increased activity of fear acquisition neurons, while silencing fear acquisition neurons could lead to increased activity of extinction neurons. Testing this hypothesis requires new methods that enable tagging of multiple ensembles in the same mice.

Although our conclusions are different, our results are not inconsistent with a recent study showing that reactivation of the dentate gyrus neurons active during recall of a remote fear memory contributes to fear extinction[36]. In that study, neurons were tagged during a shock-free test session, which presumably evoked both fear recall and extinction. Our findings suggest that such a procedure will yield a heterogeneous tagged population. It is also possible that the hippocampal mechanisms of extinction vary depending on whether the memory being extinguished is recent (our study) or remote[36,37].

The existence of distinct populations of fear acquisition and extinction neurons in the hippocampus parallels what has been observed in the PFC and amygdala. The basolateral amygdala (BLA) contains unique populations of excitatory neurons that respond to extinguished or non-extinguished fear cues and project to different targets[38,39]. In the PFC, neurons in the prelimbic subdivision preferentially respond to fear-associated cues, while those in the infralimbic subdivision respond to extinguished cues[40–42]. Whereas the opposing cell types in the BLA and PFC can be distinguished based on molecular profiles and connectivity[43–45], it remains to be determined whether the populations in the dentate gyrus are similarly differentiated. The fear-tagged and extinction-tagged neurons we observed in the dentate gyrus were intermingled and could not be distinguished from each other based on morphology or location. Nevertheless, we speculate that the activity of dentate gyrus fear acquisition neurons induces fear expression through interactions with ventral CA1 projections to the BLA[46]. The activity of extinction neurons might reduce fear by interfering with recall of hippocampal fear representations or by activating fear-suppressive circuitry in the amygdala, PFC, or another region through polysynaptic mechanisms[8,47,48]. It is also possible that fear and extinction ensembles are associated with different oscillation frequencies, similar to what has been shown in PFC-amygdala circuits[49].

In conclusion, we demonstrated that fear and extinction memories have distinct ensemble representations in the dentate gyrus. We hypothesize that competition between these ensemble representations in the hippocampus determines whether fear is expressed or suppressed after extinction training. Because fear relapse represents a significant challenge for the treatment of anxiety and fear disorders[50], interventions that potentiate the activity of the hippocampal extinction ensemble or suppress reactivation of the fear acquisition ensemble may be of therapeutic value.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41593-019-0361-z.

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Author contributions

A.F.L., C.A.D., and M.R.D. conceived the project and designed the experiments. A.F.L., S.C.L., and S.L.S. performed the cell reactivation experiments. A.F.L. performed the optogenetic behavioral experiments. A.F.L., E.T.B., C.R.C., F.S., M.J.M., K.P.S., S.C.L., and S.L.S. quantified the cells and analyzed the data. C.A.D. generated the ArcCreERT2 mice. A.F.L. and M.R.D. wrote the manuscript. M.R.D. supervised the project.

Competing interests

The authors declare no competing interests.

Additional information

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Methods

Animals. Adult male and female ArcCreERT2::Halo-eYFP^{flx} and ArcCreERT2::ChR2-eYFP^{flx} mice aged 2–7 months were used for all experiments (Supplementary Table 2). ArcCreERT2::Halo-eYFP^{flx} and ArcCreERT2::ChR2-eYFP^{flx} mice were generated by breeding heterozygous (+/−) ArcCreERT2 mice[13] with homozygous (+/+) Rosa26-CAG-stop^{flx}-eNpHR3.0-eYFP (Ai39) or Rosa26-CAG-stop^{flx}-ChR2(H134R)-eYFP (Ai32) mice[51], originally purchased from The Jackson Laboratory. These crosses generated ArcCreERT2^{+} or ArcCreERT2^{-} mice heterozygous for either the Halo-eYFP^{flx} or ChR2-eYFP^{flx} allele. Mice were housed with their littermates in groups of 1–5 in plastic cages with woodchip bedding and maintained on a 12 h light–dark cycle (7:00–19:00) in a temperature- and humidity-controlled vivarium. Food and water were provided ad libitum. Experiments were conducted during the light phase. Mice were randomly assigned to groups before the start of each experiment. As sex × genotype statistical interactions were not present, male and female data were aggregated. A cohort of six mice shipped from Columbia and potentially exposed to extreme heat did not extinguish and was removed from further analysis. Five mice were found to have no eYFP expression suggestive of a genotyping error and were removed from further analysis. No statistical methods were used to predetermine sample size, but sample sizes were based on those in previously published studies[13,15]. All procedures were approved by the University of Texas at Austin Institutional Animal Care and Use Committee.

Surgery. For the optogenetic experiments, mice received stereotaxic surgery 2–4 weeks before behavioral experimentation. Mice were anesthetized with 3% isoflurane (1.5 l min^{-1}) vaporized in pure oxygen, head-fixed in a stereotaxic surgical frame, and maintained under anesthesia with 1.5% isoflurane (0.75 l min^{-1}). Ophthalmic ointment was applied to prevent the eyes from drying. A skin incision was made to expose the skull. The skull was scoured with an acidic gel etchant and a layer of OptiBond epoxy (Kerr Corporation) was applied. Fiber-optic cannulas were bilaterally implanted at a 20° angle targeting the dorsal dentate gyrus (from bregma: anterior/posterior −2.0 mm; medial/lateral ± 1.3 mm; dorsal/ventral −1.5 mm). Optic fiber implants were constructed by following previously published methods[52], using 1.25 mm ceramic ferrules (Kientec Systems) and 200 µm core, 0.39 numerical aperture multimodal fiber (Thorlabs). The fibers were secured in place with a layer of dental cement (Bosworth). After the surgery, mice were injected subcutaneously with ketoprofen (5 mg kg^{-1}) in sterile saline (0.9%) to provide analgesia.

Neuronal tagging. Recombination was induced with 4-OHT (Sigma-Aldrich). 4-OHT was dissolved by sonication in 10% ethyl alcohol/90% sunflower seed oil at 10 mg ml^{-1}. Mice were transported from the vivarium to an adjacent holding room at least 3 h before the 4-OHT injections to minimize transportation-induced IEG activity. Activity-dependent neuronal tagging was induced by a single intraperitoneal injection of 4-OHT (55 mg kg^{-1}) administered either before (Fig. 1) or immediately after the CFC session for the fear acquisition-tagged mice, or immediately after the tenth extinction session for the extinction-tagged mice. After the 4-OHT injection, mice were moved to an isolated room and housed in the dark for 3 d, which helped minimize non-specific labeling[13]. Mice were then returned to the vivarium with a regular 12 h light–dark cycle for the remainder of the experiment.

Optogenetics. Cranial implants were bilaterally connected via fiber-optic patch cables to a light source interfaced with a FC/PC rotary joint (Doric Lenses). Photoinhibition was performed bilaterally with a 140 mW, 532 nm laser (Shanghai Dream Lasers Technology) delivered continuously during the light ON epochs with an intensity of 9–12 mW at the end of the fiber-optic implant. ChR2 photostimulation was performed unilaterally with a 17.2 mW, 470 nm light-emitting diode (Thorlabs) delivered in 20 ms pulses at 10 Hz with an intensity of 0.75–0.9 mW at the end of the fiber-optic implant.

CFC. All mice were handled for 1–2 min per day for 4–5 d before behavioral testing. During the handling sessions, mice were habituated to fiber-optic cables by allowing them to explore a novel cage while attached to patch cables. Animals were transported from the vivarium to a holding room adjacent to the test room at least 1 h before experimentation. Mice were moved individually to and from the conditioning room in an opaque container. The transport containers were cleaned with a 70% ethyl alcohol solution between uses. Mice were connected to the fiber-optic patch cables for every behavioral session.

Behavioral testing occurred in 30.5 × 24 × 21 cm^3 conditioning chambers (Med Associates), with two aluminum sidewalls, a Plexiglas door and ceiling, and a white vinyl back wall. Chambers were contained within a larger, sound-attenuating chamber equipped with a fan to provide approximately 65 dB ambient noise. An overhead white light illuminated the chamber continuously throughout the procedures.

Two distinct contexts were used—a conditioning context and an alternate context. The conditioning context contained a straight stainless steel rod floor (36 rods spaced 8 mm from center to center), was cleaned with a 70% ethyl alcohol solution between uses and was scented with 1% acetic acid solution in the waste tray below the floor. The alternate context occurred in the same chambers and contained a flat plastic floor covered in woodchip bedding and a curved plastic insert along the back wall. The chamber was cleaned and scented with disinfecting wipes (Clorox).

For fear conditioning, three 2-s 0.75 mA scrambled foot shocks were delivered through the floor 180, 240, and 300 s after the mice were placed in the context. Mice were removed 30 s after the final footshock and returned to their home cage.

All behavioral sessions were video recorded at 30 frames per second using a near-infrared camera mounted to the interior door of the chamber. The videos were manually scored for freezing behavior by an investigator blind to the experimental conditions. Freezing was defined as the absence of movement, with the exception of those related to breathing.

Extinction. Extinction sessions consisted typically of 5 min unreinforced exposures to the conditioning context once per day for 10 d (exception: 3-min sessions for Fig. 1b–g).

Tests and optogenetic manipulation. For the test sessions in the cell reactivation experiments, mice were placed in the conditioning or alternate context for 5 min. The tests for the silencing experiments took place in either context for 12 min. The light ON epochs occurred during minutes 0–3 and 6–9. Tests for the ChR2 stimulation experiments took place in either context for 9 min. The light ON epoch occurred during minutes 3–6 for the extinction retrieval and alternate context tests. The light ON epochs occurred during minutes 0–3 and 6–9 for the spontaneous recovery test. Spontaneous recovery tests occurred 27–28 d after the last test session. The photostimulation-induced IEG experiment took place in the alternate context for 9 min. For mice that received photostimulation, light was delivered during minutes 3–6.

Open field. The open field was a 40 × 40 cm^2 arena with opaque plastic walls 35 cm high. Overhead lights provided 80 lux illumination measured in the center of the arena. Mice were connected to fiber-optic patch cables and placed into the arena for 15 min. Photostimulation consisted of a 5 min epoch from minutes 5 to 10. The light parameters were identical to those reported for the tests. The center was defined as an 18.5 × 18.5 cm^2 zone in the center of the arena. The behavior was recorded using a ceiling-mounted digital camera and analyzed with video-tracking software (ANY-maze).

Tissue preparation and immunohistochemistry. Ninety minutes after the CFC test, mice were deeply anesthetized with ketamine/xylazine (150/15 mg kg^{-1}) and transcardially perfused with 1× PBS, followed by 4% paraformaldehyde in 1× PBS. Brains were extracted and post-fixed overnight at 4 °C in 4% paraformaldehyde and then transferred to a 30% sucrose in 1× PBS medium at 4 °C for 2 d; 35-µm coronal sections were collected on a cryostat and stored in cryoprotectant at −20 °C.

For immunohistochemistry, sections were washed in 1× PBS and blocked at room temperature for 2 h in 10% normal donkey serum (NDS) in 1× PBS with 0.5% Triton X-100 (PBST). Sections were incubated with primary antibodies—1:2,000 rabbit anti-Arc (Synaptic Systems), 1:500 chicken anti-GFP (Abcam), and 1:1,000 rabbit anti-c-Fos (EMD Millipore)—diluted in 5% NDS in 1× PBST overnight at 4 °C. Sections were rinsed in 1× PBST and incubated in secondary antibodies (1:500 donkey anti-rabbit Cy3 and 1:500 biotinylated donkey anti-chicken; Jackson ImmunoResearch) in 1× PBST for 2 h at room temperature. Sections were rinsed in 1× PBST and incubated in tertiary antibody (1:250 avidin Cy-2; Jackson ImmunoResearch) and 1:1,000 DAPI for 1 h at room temperature. Sections were washed in 1× PBS, mounted onto slides, and coverslipped with ProLong Gold Antifade Mountant (Invitrogen). See the Life Sciences Reporting Summary for additional information.

Imaging and quantification. For Figs. 1 and 2, immunoreactive Arc^{+} cells in the granule cell layer of the dentate gyrus from every sixth section throughout the entire bilateral rostrocaudal axis of the hippocampus were counted exhaustively under fluorescent illumination (Zeiss Axio Imager M2) with a ×40 objective. Every Arc^{+} cell was evaluated for eYFP^{+} immunoreactivity to obtain eYFP^{+}/Arc^{+} colabeled cell counts. For eYFP^{+} cell estimates, fluorescent confocal images were obtained (Leica DM6000 confocal fixed stage system) with a ×40 objective from 4 locations sampled throughout the rostrocaudal axis of the dentate gyrus. Each image stack was acquired at 1 µm optical sections and eYFP^{+} cells were quantified from the z-stack images using ImageJ (NIH). To obtain DAPI^{+} estimates, the dentate gyrus volume from each mouse was measured using Stereo Investigator (v11). Next, a DAPI^{+} density estimate was obtained by counting DAPI^{+} cells in the dentate gyrus from 3 image stacks obtained with a ×63 objective of 4 ArcCreERT2::ChR2-eYFP^{flx} mice. Finally, the DAPI^{+} density estimate was multiplied by the dentate gyrus volume. For the photostimulation-induced IEG experiment, eYFP^{+} and Arc^{+} or c-Fos^{+} cells in the dorsal dentate gyrus were quantified from 3 image stacks obtained with a ×63 objective from a section near the site of the fiber-optic implant. For the CA3 counts, c-Fos^{+} and eYFP^{+} cells were counted exhaustively from every sixth section throughout the dorsal hippocampus. An investigator blind to treatment status performed all the cell counts. The amount of reactivation was normalized for chance overlap by dividing the percentage of colabeled^{+} among DAPI^{+} by chance ((eYFP^{+}/DAPI^{+}) × (Arc^{+}/DAPI^{+}) × 100)[22–24].

Statistical analysis. Data were analyzed using two-sided Student's *t*-tests, the Mann–Whitney *U*-test, or ANOVA, using repeated measures when appropriate. Significant ANOVAs were followed by a post hoc Sidak's test for multiple comparisons. Data distribution was assumed to be normal but this was not formally tested. Data analysis was performed on Prism version 6 (GraphPad Software) or JMP (v13, SAS Institute). The α value was set at 0.05 for all analyses. All data are presented as the mean ± 1 s.e.m.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All relevant data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Software and code

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Data collection	Fear conditioning videos recorded with Med Associates Video Freeze (2.7.0.106). Open field recorded with Stoeling ANY-Maze (4.99z). Confocal images collected with Leica LAS (2.7.3.9723)
Data analysis	Data analyzed with Image J (1.48v), Stopwatch+ (v1.5.1), Excel (v14.7.2), JMP (v14), and GraphPad Prism (v6)

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Life sciences

Study design

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Sample size	No statistical methods were used to predetermine sample size, but the sample sizes were based on those in previously published studies (Denny et al., 2014 & Ramirez et al., 2013).
Data exclusions	A cohort of 6 mice shipped to UT from Columbia and potentially exposed to extreme heat did not extinguish and was removed from further analysis. Five mice were found to have no eYFP expression suggestive of a genotyping error and were removed from further analysis.
Replication	All of the experiments, except the Halo-mediated silencing experiments, were replicated at least twice.
Randomization	Mice were randomly assigned to groups at the start of each experiment.
Blinding	Investigators performed experiments with knowledge of the group allocations. Investigators were blind to the group allocations during cell quantification and behavioral video scoring.

Materials & experimental systems

Policy information about availability of materials

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☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☐	☒ Research animals
☒	☐ Human research participants

Antibodies

Antibodies used	For Arc or cFos immunohistochemistry: Rabbit anti-Arc primary antibody (Synaptic Systems, 156 003), dilution 1:2000 Rabbit anti-cFos primary antibody (Millipore, ABE457), dilution 1:1000 Donkey anti-rabbit Cy3 secondary antibody (Jackson ImmunoResearch Labs, 711-165-152), dilution 1:500 For eYFP immunohistochemistry: Chicken anti-GFP (Abcam, ab13970), dilution 1:500 Biotin donkey anti-chicken secondary antibody (Jackson ImmunoResearch Labs, 703-065-155), dilution 1:500 Cy2-conjugated streptavidin (Jackson ImmunoResearch Labs, 016-220-084), dilution 1:250
Validation	Antibodies were first validated before use. Primary antibody was tested in different dilutions on sample mouse tissue to determine optimal primary antibody concentration. Similar immunohistochemistry reagents and protocols were used in previously published reports (Denny et al., 2014; Cazzulino et al., 2016).

Research animals

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Animals/animal-derived materials	Male and female ArcCreERT2 mice, Halo-eYFPflx mice (Ai39), and ChR2-eYFPflx mice (Ai32) aged two-seven months used in all experiments.

Method-specific reporting

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ Magnetic resonance imaging