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Social behavior in mice following chronic optogenetic stimulation of hippocampal engrams

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ABSTRACT

The hippocampus processes both spatial-temporal information and emotionally salient experiences. To test the functional properties of discrete sets of cells in the dorsal dentate gyrus (dDG), we examined whether chronic optogenetic reactivation of these ensembles was sufficient to modulate social behaviors in mice. We found that chronic reactivation of discrete dDG cell populations in male mice largely did not affect social behaviors in an experience-dependent manner. However, we found that social behavior in a female exposure task was increased following chronic optogenetic stimulation when compared to pre-stimulation levels, suggesting that the protocol led to increased social behavior, although alternative explanations are discussed. Furthermore, multi-region analysis of neural activity did not yield detectable differences in immediate-early gene expression or neurogenesis following chronic optogenetic stimulation. Together, these results suggest that the effects of chronic optogenetic stimulation in the dDG on social behaviors are independent of the contextual experience processed by each cellular ensemble.

1. Introduction

Social behaviors are dramatically impaired across many psychiatric disorders, though the underlying mechanisms sufficient to precipitate or alleviate such impairments remain largely unknown. Promisingly, previous studies have demonstrated that chronic optogenetic reactivation of both cell bodies and projection-specific elements can "reprogram" circuit-level and behavioral outputs in healthy and maladaptive states (Creed, Pascoli, & Lüscher, 2019; Tye, 2014). However, the behavioral effects of chronic optogenetic stimulation of memory ensembles are region-specific and experience-dependent. Specifically, reactivating dDG cells that were previously active during female exposure was sufficient to rescue depressive-like behavior in mice, while chronically reactivating dDG cells previously active during fear conditioning was sufficient to either suppress or enhance a context-specific memory (Ramirez et al., 2015; Chen et al., 2019). To elucidate the potential lasting effects of chronic circuit-level manipulations, we examined whether chronic optogenetic reactivation of ensembles in the dDG which are active during putative positive or negative experiences is sufficient to alter behavior and brain activity during three social tests. Our findings suggest that chronic optogenetic stimulation alters social behavior, and that these effects are independent of the contextual experience processed by the reactivated hippocampal ensemble.

2. Methods

2.1. Subjects

Wild-type C57BL/6J male mice (40–41 days; Charles River Laboratories) were housed with littermates in groups of 2–5 mice per cage. Mice were acclimated to the animal facility for 72 h upon delivery before experimental procedures began and kept on a 12:12-hour light cycle (lights on at 7:00). Food and water were available *ad libitum*. Animals were put on a diet containing 40 mg/kg doxycycline (dox) after the acclimation period and 24–48 h before receiving surgery between 6 and 7 weeks of age. Following surgery, mice were group-

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housed with littermates and left for 10 days to recover prior to experimentation. Animals were handled for 2–4 days (2 min daily per animal) at the end of the recovery period. They were also habituated to optogenetic stimulation conditions by plugging the patch cord into their headcaps and allowing them to walk around freely for 2 min per day for 2 days, prior to the start of the experimental period. All procedures related to mouse care and treatment were in accordance with Boston University and National Institutes of Health guidelines for the Care and Use of Laboratory animals.

2.2. Virus constructs and packaging; stereotaxic surgery

The pAAV$_9$-*c-Fos*-tTA and pAAV$_9$-TRE-ChR2-eYFP plasmids were constructed as described previously (Ramirez et al., 2013). Using these plasmids, AAV$_9$ viruses were generated at the Gene Therapy Center and Vector Core at the University of Massachusetts Medical School. Viral titres were 1×10^{13} genome copy per milliliter for AAV$_9$-TRE-ChR2-eYFP and 1.5×10^{13} genome copy per milliliter for AAV$_9$-*c-Fos*-tTA.

All surgeries were performed under stereotaxic guidance and the following coordinates are given relative to bregma. Anesthesia was induced using 3.5% isoflurane inhalation and maintained throughout surgery at 1.5–2.0%. Animals received bilateral craniotomies using a 0.6 mm diameter drill bit for dDG injections. The needle was slowly lowered to the target site of −2.2 mm AP, ± 1.3 mm ML, −2.0 mm DV (relative to Bregma). A cocktail consisting of 300 nL of AAV$_9$-c-Fos-tTa (300nL) + AAV$_9$-TRE-ChR2-eYFP (300nL) was infused into the dDG (100nL min^{-1}) using a 33-gauge needle attached to a mineral-oil filled 10 μL gastight syringe (Hamilton, #7653-01) (Fig. 1A). The needle remained at the target site for 2 min post-injection before being slowly withdrawn. A bilateral optical fiber implant (200 μm core diameter; Doric Lenses) was lowered above the dDG injection site at −1.6 mm DV. Two bone anchor screws were secured into the skull at the anterior edges of the surgical site to anchor the implant. Layers of adhesive cement (C&B Metabond) followed by dental cement (Stoelting) were spread over the surgical site to secure the optical fiber implant to the skull. Mice received 0.1 mL buprenorphine (0.03 mg/mL IP) and were transferred to recovery cages atop heating pads until recovery from anesthesia. Mice were given 10 days to recover before the start of the experiment. Injections were verified histologically. Only data from mice with bilateral opsin expression present in the dDG were used for analyses.

2.3. Neuronal tagging of behavioral epochs and counterbalanced behavior

In order to label the dDG cells active during a behavioral epoch, the dox diet was substituted with normal mouse chow; this occurred 48 h prior to the epoch to allow for complete clearance of dox and to open the window for activity-dependent neuronal tagging (Garner et al., 2012; Liu et al., 2012) (Fig. 1B-C). The mice were divided into 3 groups, and each group received a different "tagged" behavioral epoch to start. However, all mice received all behavioral epochs counterbalanced using a balanced Latin square design over a period of 3 days (negative-neutral-positive; positive-negative-neutral; neutral-negative-positive). 1) *Footshock (negative)*: animals were placed in a fear conditioning chamber and given a 4-shock protocol over a period of 500 s (1.5 mA, 2 s duration, 198 s, 278 s, 358 s, 438 s). 2) *Female exposure (positive)*: one female mouse (PD 30–40) was placed in a clean homecage with a clear, ventilated acrylic top. The experimental animal was then placed into the cage and allowed to freely interact with the female for 1 h. This session was recorded with a web-camera (Logitech HD). 3) Clean homecage (neutral): mice were individually placed in a clean homecage with a clear, ventilated acrylic cage top for 500 s. Immediately following the tagged behavioral epoch, mice were placed back into their homecage and again given access to dox to close the neuronal tagging window. Mice were weighed daily and monitored for health.

2.4. Pre-stimulation female exposure

The total amount of time that male mice interacted with a female mouse - defined as sniffing, chasing, mounting, or other contact initiated by the male - within the first 5 min of the 1 h, pre-optogenetic stimulation exposure was manually scored (termed "baseline" time point).

2.5. Chronic optogenetic stimulation protocol

Optical stimulation was administered twice daily during the light cycle at approximately 10:00 and again at 15:00 daily for 5 days, to animals at 8–9 weeks of age. Prior to the start of the session, laser output was tested to ensure that at least 10 mW of power was delivered at the end of the patch cord (Doric Lenses). Each stimulation session lasted for 10 min (450 nm, 20 Hz, 15 ms pulse width) and was conducted in an almond-scented custom-built acrylic rectangular chamber with striped walls under dim lighting. The first round of post-stimulation behavioral tests began one day after the cessation of this protocol.

2.6. Post-stimulation behavioral assays (resident intruder test, social interaction test, female exposure test)

Behavioral experiments were conducted 24 h after the final chronic optogenetic stimulation session. All behavioral assays were recorded using a web-camera (Logitech HD).

2.6.1. Resident intruder test

Experimental animals and their homecage enrichment were transferred from their homecage to a clean holding cage with their cagemates. The homecage with bedding was used as an experimental chamber with a clear, ventilated acrylic cage top. One experimental mouse from the cage was placed back into the homecage and allowed to acclimate for 1 min, after which a novel conspecific juvenile male (PD 24–28) was introduced into the experimental male's homecage for a 5-minute test session. Interaction was manually scored by the experimenter and was measured as experimental male-initiated behavior (defined as chasing, sniffing, or grooming the juvenile conspecific intruder).

2.6.2. Social interaction test

An open arena (24″ x 24″) with black walls was used for the social interaction test. Two inverted wire cups of diameter 4″ and height 4.25″ (Spectrum Diversified Galaxy Pencil Holder) were placed in the arena in opposite corners, each set 6″ away from the corner of the arena. Red lab tape was placed on the floor of the arena around the outside of the wire cup to demarcate a diameter 4 cm larger than that of the cup. A juvenile male conspecific (PD 24–28) was placed into one wire cup (herein referred to as conspecific cup), while the other cup was left empty (herein referred to as empty cup). The test animal was placed into the middle of the arena and was allowed to freely explore the arena for 10 min. Post hoc, experimenters scored the total amount of time that the experimental animal spent within each region outlined by tape, and computed the time spent with the conspecific cup, as well as the difference score (percent time spent with empty cup subtracted from percent time spent with conspecific cup).

2.6.3. Female exposure

One female mouse (PD 30–40) was placed into a clean homecage with a clear acrylic, ventilated cage top, which was used as the interaction chamber. The experimental male mouse was then placed into the chamber and was allowed to interact freely for 5 min. The amount of time the male mouse interacted with the female - defined as sniffing, chasing, mounting, or other contact initiated by the male - was manually scored post hoc.

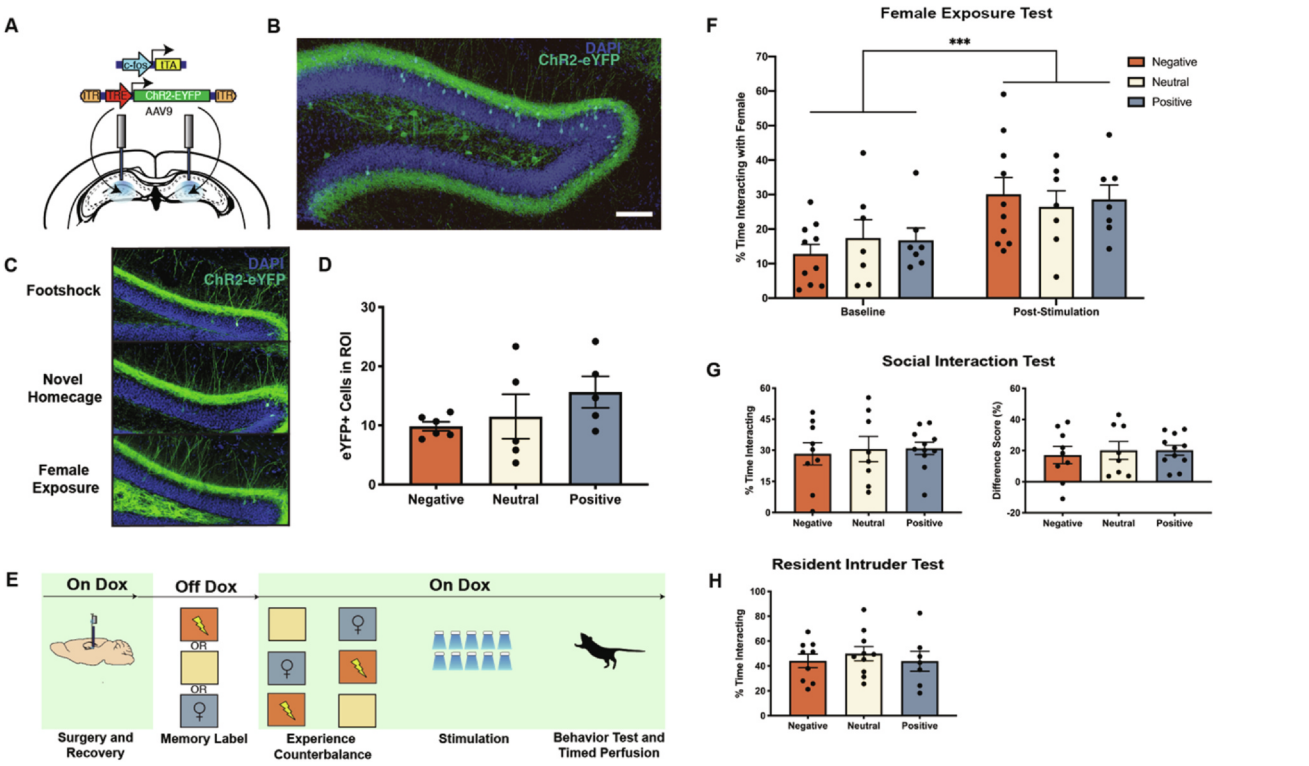


Fig. 1. Chronically stimulating dDG ensembles encoding a footshock, novel homecage, or female exposure experience does not differentially affect social behaviors. (**a**) Viral constructs for doxycycline (dox)-gated activity-dependent expression of ChR2 in the dDG. The immediate early gene *c-Fos* drives tetracycline transactivator (tTA), which binds to its response element (TRE) to in turn drive expression of ChR2 in a dox-regulated manner. (**b**) Representative image depicting expression of ChR2-eYFP (green) in the dDG. Scale bar represents 100 μm. (**c**) Representative images of ChR2-EYFP in DG for each group. (**d**) Ensemble sizes are not significantly different for different behavioral epochs (One-Way ANOVA, $F_{2,13} = 1.392$, $p = 0.2834$ (Negative n = 6, Neutral n = 5, Positive, n = 5) (**e**) Behavioral schedule and groups used. Green regions depict periods in which dox was present in the diet, and white regions depict regions where dox was removed to tag active cells ("memory label"). The orange box with a shock symbol represents a four-shock protocol, the tan-colored box represents a clean homecage exposure, and the gray box with a female symbol represents exposure to a female conspecific. (**f**) Chronic stimulation of negative, neutral or positive ensembles increases female interaction time (2 Way RM ANOVA, Main Effect of Time: $F_{1, 21} = 22.30$, ***$p = 0.0001$ (Negative n = 10, Neutral n = 7, Positive n = 7) (**g**) Chronically stimulating dDG ensembles encoding a negative, neutral, or positive experience does not differentially affect post-stimulation behavior in the social interaction test or (**h**) resident intruder test. Two separate cohorts of mice underwent the protocol in a) with either the social interaction test or resident intruder test on the final day. Difference score represents the difference in time spent interacting with the conspecific cup and the empty cup in the social interaction test. Social interaction test, One-Way ANOVA Time interacting: $F_{2,25} = 0.09415$, $p = 0.9105$; Difference Score: $F_{2,25} = 0.1382$, $p = 0.8716$ (Negative n = 9, Neutral n = 8, Positive n = 11), Resident intruder test: One-Way ANOVA $F(2,23) = 0.3150$, $p = 0.7329$ (Negative n = 9, Neutral n = 10, Positive n = 7). Data are presented as mean ± s.e.m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.7. Neurogenesis

In a separate cohort of animals, stereotaxic surgery was performed to infuse pAAV$_9$-*c-Fos*-tTA + pAAV$_9$-TRE-ChR2-eYFP into the dDG and mice were then left undisturbed to recover for 10 days. On day 11, animals were taken off dox for 48 h and left undisturbed in their homecages. Animals were then split into 3 groups: footshock (negative), novel homecage (neutral), or female exposure (positive) (refer to: *Experience tag)*. Cells active during these behavioral epochs were labelled. Mice were then subjected to the chronic optogenetic stimulation protocol (see *Chronic Stimulation Protocol*) and were then left undisturbed in their homecages for 7 days to allow for optimal doublecortin expression (Couillard-Despres et al., 2005). On the 8th day, animals were euthanized, and their brains were extracted for immunohistochemical staining (see *Immunohistochemistry)*. Doublecortin-positive cells in the upper and lower blade of the DG granule cell layer were manually counted by an experimenter (see *Cell Counting*).

2.8. Immunohistochemistry

Mice were overdosed with isoflurane and perfused transcardially first with 40 mL ice cold 1X phosphate-buffered saline (PBS) followed by 40 mL ice cold 4% paraformaldehyde in PBS. Brains were extracted and stored at 4 ˚C, first in 4% paraformaldehyde for 48 h, and subsequently in PBS. A vibratome was used to obtain 50-μm coronal slices, which were stored in 24-well plates in PBS at 4 ˚C. These slices were incubated with 1X PBS with 2% Triton (PBS-T) + 5% normal goat serum (NGS) for one hour at room temperature for blocking. Primary antibodies were diluted in PBS-T + 5% NGS as follows: guinea pig anti-c-Fos (1:1000, Synaptic Systems, #226 004), chicken anti-GFP (1:1000, Invitrogen, #A10262), and rabbit anti-doublecortin (1:500, Synaptic Systems, # 326 003). Slices were incubated in the primary solution at 4 ˚C for 24 h on an orbital shaker. This was followed by three 10 min washes in PBS-T, shaking at room temperature. Slices were then incubated with a secondary antibody solution for 2 h at room temperature, shaking. Secondary antibodies were diluted in PBS-T + 5% NGS as follows: Alexa 555 goat anti-guinea pig (1:200, Invitrogen, #A21435), Alexa 488 goat anti-chicken (1:200, Invitrogen, #A11039), and Alexa 555 goat anti-rabbit (1:200, Invitrogen, #A21429). Again, this was followed by three 10-minute washes in PBS-T at room temperature, shaking. Slices were then mounted onto microscope slides with VECTASHIELD® Hardset™ Antifade Mounting Medium with DAPI (Vector Labs, #H-1500).

2.9. c-Fos quantification

The total number of neurons immunoreactive for c-Fos were counted in several brain regions - prefrontal cortex (PFC), nucleus accumbens core (NAcc Core), nucleus accumbens shell (NAcc Shell), lateral septum (LS), dorsomedial hypothalamus (dmHyp), lateral hypothalamus (LatHyp), dorsal CA1 (dCA1), dorsal CA3 (dCA3), basolateral amygdala (BLA), and lateral habenula (LHb) - to measure neuronal activity in these areas during defined behavioral assays (female exposure test, resident intruder test, and social interaction test). Animals were euthanized 90 min following these tasks, to maximize the robustness of c-Fos expression. Following brain extraction, 3 coronal slices were selected from each of the following regions: approximately + 1.15 AP, −2.2 AP, and −2.78 AP. This allowed for visualization of the six brain regions of interest. Following c-Fos staining, the brain regions of interest were imaged using a confocal microscope (Zeiss LSM-800). Images were then processed using FIJI software. The Despeckle tool was used to reduce background noise, and the Subtract Background tool was used to create greater contrast between cells and background. Each brain region z-stack was set to include a 320 μm × 320 μm region of interest (ROI), then processed using the 3D Iterative Thresholding of the 3D ImageJ Suite (Ollion et al., 2013). The settings for thresholding were held constant for each brain region of each cohort, with a minimum threshold and preliminary size filter, to maintain consistency in image processing and cell counting parameters between animals. The thresholded images were then z-projected to create flat images of the thresholded objects. In order to isolate cell objects from artifacts such as blood vessels or noise, the images were then run through a pipeline created in Cell Profiler 3.1.8 software that identified objects of a more stringent size and shape. The number of c-Fos-positive cells was recorded for each ROI and averaged within each animal.

2.10. Doublecortin quantification

Following chronic reactivation of dDG cells encoding a negative, neutral, or positive experience, animals were left undisturbed in the homecage for 7 days to allow for optimal expression of doublecortin, a marker of neurogenesis. The number of neurons in dDG and vDG immunoreactive for doublecortin (DCX) was examined to determine levels of neurogenesis. In FIJI software, DCX-positive cells were selected in each layer in the z-stack with the Oval tool and added to the ROI Manager. Only DCX-positive cells in a 600 μm × 100 μm ROI were counted. Cells in the dorsal and ventral blades of the dentate gyrus were counted separately and then averaged.

2.11. dDG target verification and ensemble size quantification

In all cohorts, immunoreactivity for eYFP (by proxy of anti-GFP staining) was examined to ensure bilateral expression of the virus in targeted regions. Animals that did not show eYFP immunoreactivity bilaterally in the target region were excluded from analysis. Activity-dependent ensemble size was determined using a subset of animals in each group; the number of eYFP-positive cells in a 600 μm × 100 μm ROI in the dorsal blade of dDG was manually quantified using FIJI software as described above.

2.12. Statistical methods

Calculated statistics are presented as means ± SEM. To analyze differences, we used two-way repeated measures (RM) ANOVAs (Between subject factor: Group; Within-subject factor: Time). When time was not a factor, we used one-way ANOVAs. All data were tested for normality using the Shapiro-Wilk test and homogeneity of variance was assessed with Levene's test. In the case of the necessity of non-parametric statistics, Kruskal Wallis tests were used. Data were analyzed using GraphPad Prism 8.0 and SPSS Statistics v26 software. Alpha was set to 0.05. All tests were two-tailed.

2.13. Data availability

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

3. Results

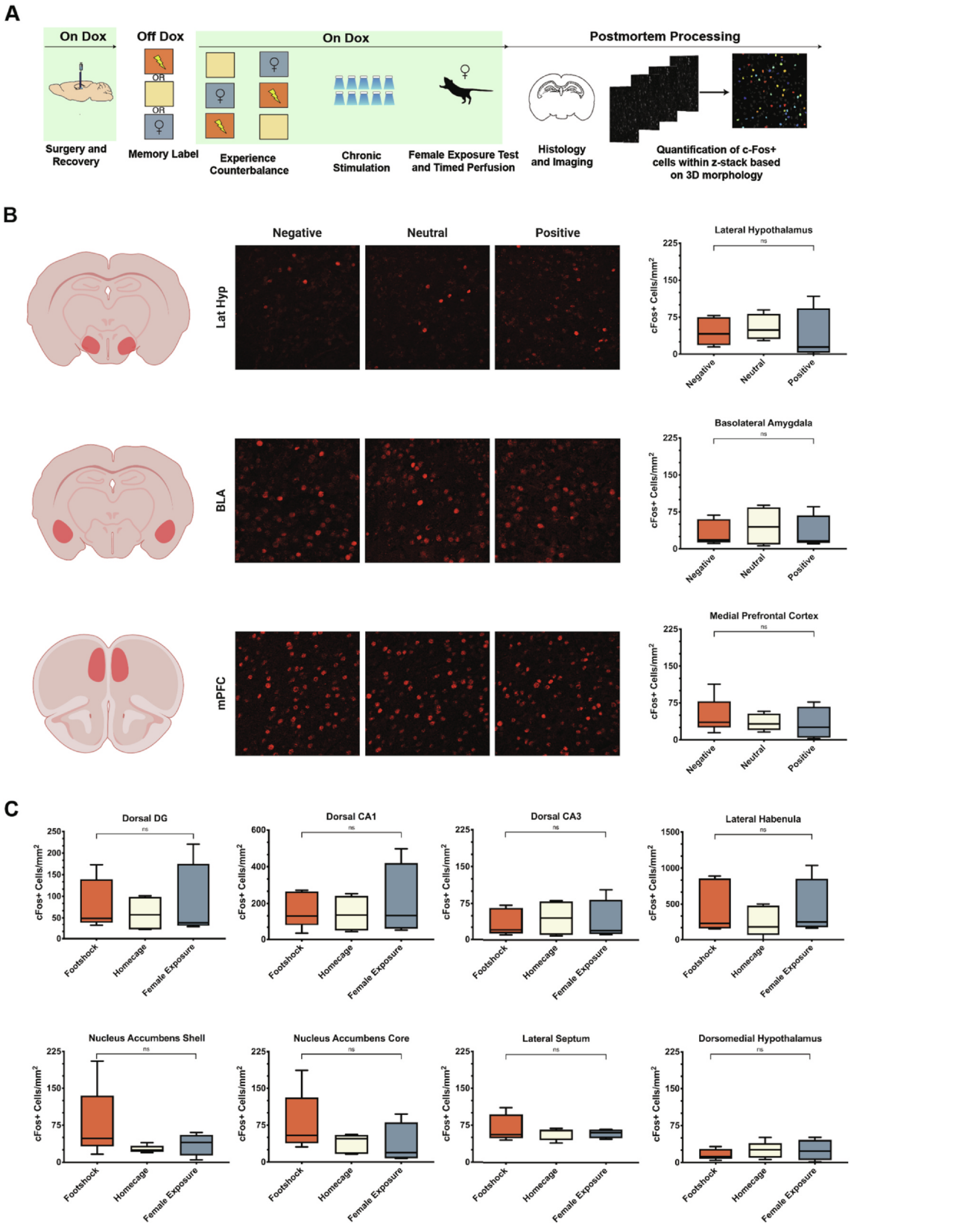
Hippocampal cells were tagged during either a positive, negative, or neutral behavioral epoch, a design that was implemented to allow for stimulation of similarly sized cellular ensembles encoding experiences of different valences. Mice showed no differences across groups in the number of eYFP + dDG cells (One-Way ANOVA, $F_{2,13} = 1.392$, $p = 0.2834$) (Fig. 1C-D). Animals then underwent a previously established (Chen et al., 2019; Ramirez et al., 2015) 10-minute optogenetic stimulation protocol twice daily for 5 days, followed by a behavioral test assessing social behaviors (Fig. 1E).

To test the effects of chronic stimulation on social behaviors, male mice underwent a female exposure test after chronic stimulation (Felix-Ortiz, Burgos-Robles, Bhagat, Leppla, & Tye, 2016) (Fig. 1F). All mice showed increased interaction with the female at the post-stimulation time point relative to the pre-stimulation time point (Fig. 1F; Two-Way RM ANOVA, Main Effect of Time, $F_{1,\ 21} = 22.30$, $p = 0.0001$). Interaction time was not modulated by the type of reactivated hippocampal ensemble at either time point (Two-Way RM ANOVA, No Main Effect of Group, $F_{2,21} = 0.0303$, $p = 0.9702$). While we note that increases in social behavior pre- versus post- stimulation was particularly pronounced in the positive and negative groups (Fig. 1F), there was no interactive Time × Group Effect ($F_{2,21} = 0.8950$, $p = 0.4236$).

We next assessed if chronically reactivating hippocampus-mediated memories affected social behavior involving only males using two additional tests: social interaction and resident intruder (Fig. 1G-H). Chronic optogenetic stimulation of dDG cells involved in the encoding of a positive, negative, or neutral behavioral epoch did not result in group differences in time spent interacting with a novel, juvenile conspecific male in the social interaction test (One-Way ANOVA for time interacting $F_{2,25} = 0.09415$, $p = 0.9105$ and difference score $F_{2,25} = 0.1382$, $p = 0.8716$) or the resident intruder test (One-Way ANOVA $F_{2,23} = 0.3150$, $p = 0.7329$). Notably, as mice were not administered a baseline test of these measures prior to chronic stimulation due to the nature of our "tagged" experience, these comparisons could only be made at the post-stimulation time point. Therefore, within-group changes in social interaction or resident intruder behaviors could not be assessed.

We also sought to determine whether chronic stimulation of ensembles encoding negative, neutral, or positive memories had lasting effects on regional brain activity during social behaviors. We quantified the mean number of c-Fos + cells in various brain regions implicated in processing social interaction and valence (Fig. 2B) and found no group differences in the mean number of c-Fos + cells per area in all brain regions observed (see figure legend for statistics in each brain region).

Previous studies have found that chronic optogenetic stimulation of cells encoding female exposure, but not cells encoding a novel context, rescues stress-induced deficits in neurogenesis in the DG and social behaviors are known to modulate levels of neurogenesis (Gheusi et al., 2009; Opendak et al., 2016; Ramirez et al., 2015). Therefore, we sought to determine whether chronic optogenetic stimulation of distinct hippocampal ensembles drives differences in expression in a manner dependent on the identity of the ensemble. We quantified cells expressing doublecortin, a neuronal marker for immature neurons (Fig. 3B-E). Chronically stimulating positive, neutral, or negative dDG ensembles had no effect on neurogenesis in the dorsal (One-Way ANOVA, $F_{2,10} = 0.4617$, $p = 0.6430$) or ventral DG ($F_{2,10} = 0.1272$, $p = 0.8819$).



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Our findings that chronic stimulation of dDG cells processing a positive, neutral, or negative experience do not drive between-group differences in brain activity during social behavior or in neurogenesis are consistent with our behavioral findings. In all cases, the identity of the reactivated hippocampal ensemble (cells processing a positive vs. neutral vs. negative experience) does not modulate social or cellular effects.

Fig. 2. Chronically stimulating dDG ensembles encoding a footshock, novel homecage, or female exposure experience does not differentially affect c-Fos across multiple brain regions. (**a**) Behavioral schedule and groups used to examine brainwide c-Fos activation during female exposure after chronic stimulation of different dDG ensembles. Green regions depict periods in which dox was present in the diet, and white regions depict regions where dox was removed to tag active cells ("memory label"). The orange box with a shock symbol represents a four-shock protocol (Negative, n = 5), the tan-colored box represents a clean homecage exposure (Neutral, n = 5) and the gray box with a female symbol represents exposure to a female conspecific (Positive, n = 4). (**b**) Representative images depicting expression in the lateral hypothalamus (Lat Hyp), basolateral amygdala (BLA) and medial prefrontal cortex (mPFC) and quantification of c-Fos activation during female exposure after chronic stimulation of dDG negative, neutral and positive ensembles. (One-Way ANOVAs, Lat Hyp: $F_{2,10} = 0.2025$, $p = 0.8200$; BLA: $F_{2,10} = 0.2163$, $p = 0.8091$; mPFC: $F_{2,11} = 0.3545$, $p = 0.7093$) (**c**) Mean c-Fos + per area during post-stimulation female exposure for dorsal DG (dDG, One-Way ANOVA: $F_{2,10} = 0.1437$, $p = 0.8679$), dorsal CA1 (dCA1, One-Way ANOVA: $F_{2,10} = 0.2$, $p = 0.8220$), dorsal CA3 (dCA3, One-Way ANOVA: $F_{2,10} = 0.07539$, $p = 0.9279$), lateral habenula (LHb One-Way ANOVA $F_{2,11} = 0.5030$, $p = 0.6180$), nucleus accumbens shell and core (NAc Shell, One-Way ANOVA: $F_{2,11} = 1.581$, $p = 0.2492$; NAc Core, One-Way ANOVA: $F_{2,11} = 1.356$, $p = 0.2976$), Lateral Septum (LS, Kruskal-Wallis test, $H = 0.28$, $p = 0.8791$), and dorsomedial hypothalamus (dmHyp, One-Way ANOVA: $F_{2,11} = 0.4055$, $p = 0.6762$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

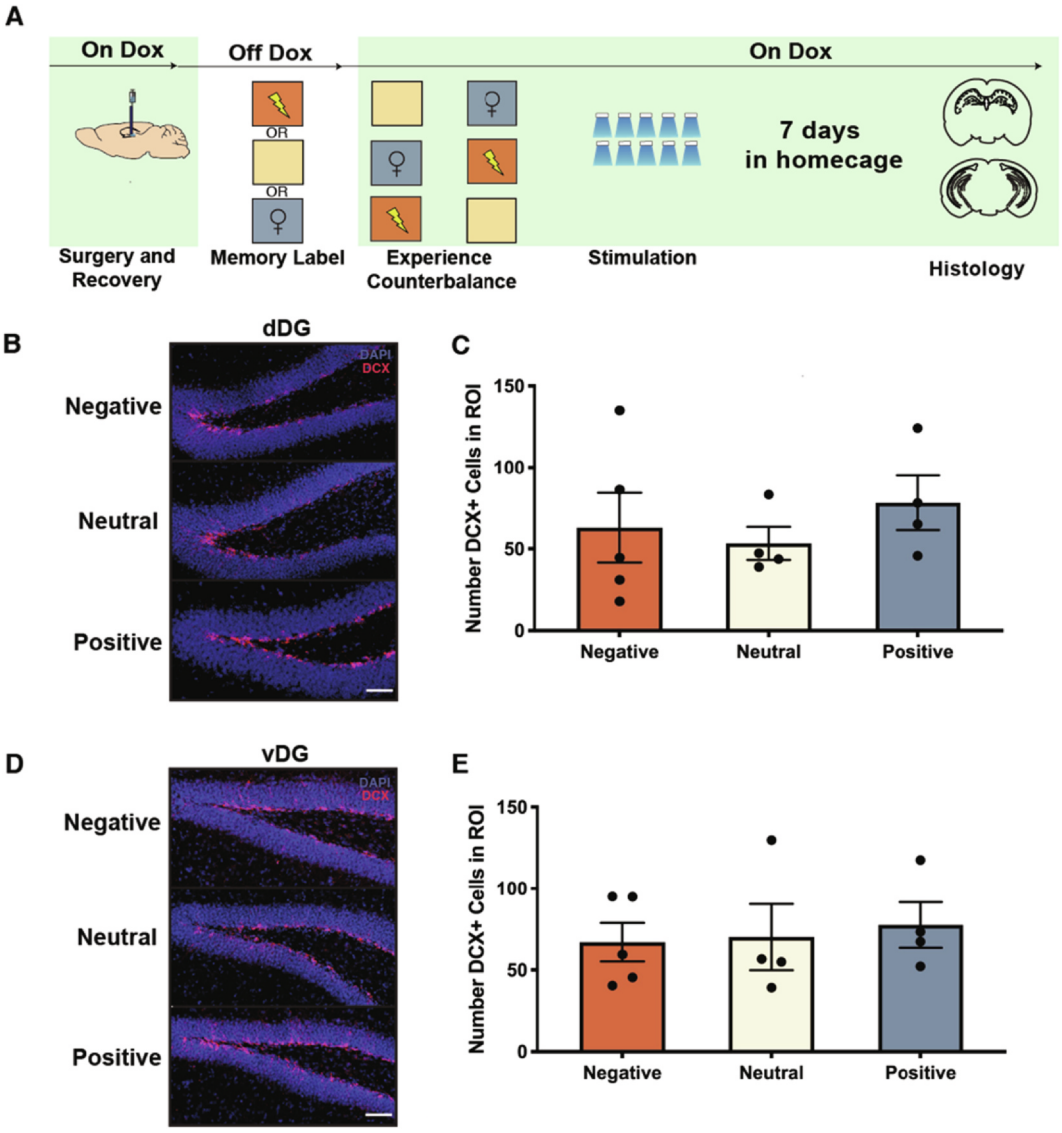

Fig. 3. Chronically stimulating dDG ensembles encoding a footshock, novel homecage, or female exposure experience does not differentially affect neurogenesis in dDG or vDG. (**a**) Behavioral schedule and groups used to examine neurogenesis induced by the chronic stimulation protocol. Green regions depict periods in which dox was present in the diet, and white regions depict regions where dox was removed to tag active cells ("memory label"). The orange box with a shock symbol represents a four-shock protocol (Negative, n = 5), the peach-colored box represents a clean homecage exposure (Neutral, n = 4) and the gray box with a female symbol represents exposure to a female conspecific (Positive, n = 4). (**b**) Representative images of dDG (**c**) and quantification of doublecortin-positive cells (red) in the dDG for each group (One-Way ANOVA, $F_{2,10} = 0.4617$, $p = 0.6430$). (**d**) Representative images of vDG and quantification (**e**) of doublecortin-positive cells (red) in the vDG for each group (One-Way ANOVA, $F_{2,10} = 0.1272$, $p = 0.8819$). Data are presented as mean ± s.e.m. Scale bar represents 100 μm. Dorsal dentate gyrus (dDG), ventral dentate gyrus (vDG). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Our findings demonstrate that chronic stimulation of dDG neurons involved in the encoding of distinct behavioral experiences did not drive differential social or cellular effects post-stimulation. After five days of chronic stimulation, we observed no differences in the amount of time experimental mice interacted with a conspecific mouse in the social interaction test or a novel male mouse in the resident intruder test. Although mice interacted more with the female mouse in a female exposure assay post-stimulation versus pre-stimulation, this effect was not dependent on the identity of the hippocampal ensemble (cells processing positive vs. neutral vs. negative experience). Together, our results point to the need for future research aimed at understanding the varying effects of chronic stimulation on different brain areas or specific sets of cells stimulated. For instance, stimulation of differently valenced dDG ensembles failed to differentially affect social behavior across groups during post-stimulation social interactions, and this may be due to dorso-ventral differences in the encoding of contextual, emotional or social information, which underscores the ventral DG's prominence in processing similar types of information (Ciocchi et al., 2015; Kheirbek et al., 2013; Okuyama et al., 2016), its influence on neurogenesis (Anacker et al., 2018), and its putative promise as a future target for chronic stimulation.

While we observed an increase in female interaction time over the course of our chronic stimulation protocol, our experimental design did not permit us to determine whether this enhancement across time and stimulation extended to other social behaviors between male mice. Future experiments may examine whether there is an increase in male-male social interactions before and after a chronic optogenetic stimulation protocol, as we observed for the female interaction groups. Furthermore, and surprisingly, stimulation of cells encoding an aversive (footshock), socially appetitive (female encounter), or neutral (homecage) experience all drove enhancement of subsequent female interaction, suggesting that reactivation of cells that were active during experiences of opposite or neutral valence modulated behavior similarly. Notably, previous work has established valence-specific effects of dDG manipulations. For instance, chronic stimulation of hippocampal ensembles can bi-directionally modulate fear responses (freezing) in a manner dependent on the valence associated with the specific dDG memory ensemble targeted (Redondo et al., 2014), and chronic re-activation cells encoding a positive (female exposure) but not neutral (homecage) experience can rescue depression-like behavior following stress (Ramirez et al., 2015). Such valence-specific modulation of downstream neural circuits after chronic-reactivation may explain our present findings. In particular, chronically re-activating a female exposure-related ensemble in the dDG may have reinforced the downstream responses promoting female interaction or mate-seeking behavior, while the same re-activation of fear memory ensembles in the dDG may have extinguished fear responses, potentially decreasing general anxiety and promoting social interaction.

We also do not report group differences in neuronal activity during social behavior across a number of brain regions. However, this does not necessarily rule out that chronic optogenetic stimulation of distinct hippocampal ensembles results in distinct network-activation effects. For instance, a recent study reported that while excitation or inhibition of DG parvalbumin cells did not affect the number of c-Fos + cells in hippocampal subregions, increased coordination between the activated ensembles was observed (Carames et al., 2020). Therefore, although we found no differences in the number of c-Fos + cells across groups, it is possible that the chronic stimulation of different dDG ensembles still had effects on the coordination of the neuronal ensembles in those brain regions, at timescales of neural activity shorter than c-Fos expression.

It is also possible that increased interaction with the female was due to more general, valence-independent, mechanisms. For instance, multiple exposures to the female interaction assay may have resulted in increased engagement with the (novel) female, although, a previous study which re-exposed male mice to a female exposure test did not report differences in interaction time across tests. However, it is important to note that several methodological differences exist between our behavioral paradigm and that described by D'Amato and colleagues, and further studies are needed to characterize the effect of multiple exposures to the female interaction test. Alternatively, the observed effects may be due to a valence-independent mechanism in which reactivation of hippocampal populations modulates general arousal levels (Fastenrath et al., 2014; Kensinger and Corkin, 2004), resulting in increased social behavior in the female exposure test.

Previous studies report that optogenetic stimulation of hippocampal ensembles drives changes in behavior, and that the direction and magnitude of these changes are modulated by the valence of the experience which is encoded by the reactivated ensemble (Ramirez et al., 2015, Redondo et al., 2014, Zhang et al, 2019). The present results add nuance to these findings; in the present case, reactivating dDG ensembles encoding three distinct experiences did not drive different behaviors in several social assays. This discrepancy between previous studies and these results may arise from several differences in experimental protocol, including the prior experience of the animal (e.g., stressed vs. unstressed), the timing of the stimulation protocol (e.g., during or after a behavioral session), the length of the stimulation protocol (e.g., acute vs. chronic), or the tagged cellular experience (e.g., hippocampal population encoding foot shock vs. social defeat epoch). Indeed, chronic stimulation may affect behaviors between groups only following chronic stress, and acute optogenetic stimulation may alter behavior during a stimulation session but may not be sufficient to induce lasting structural or functional changes supporting enduring behavioral effects between groups (Ramirez et al., 2015; Redondo et al., 2014). Similarly, stimulating memory ensembles while mice are engaged in a social interaction (rather than beforehand in a chronic paradigm, as done in the present study) could determine if acute stimulation affects social behaviors in real time. Future studies may examine whether alternative protocols could reveal valence-dependent effects of chronic ensemble reactivation on social behavior.

Our results shed further light on the relationship between optogenetic stimulation and neurogenesis in the DG. In our previous reports, chronic activation of a positive memory reversed the effects of stress on neurogenesis, highlighting putative differential effects of stimulating hippocampal cells in a stressed or unstressed rodent (Ramirez et al., 2015). Our results indicate that chronic stimulation of dDG ensembles alone is not sufficient to alter neurogenesis, suggesting that changes in neurogenesis induced by chronic optogenetic stimulation may occur only when administered after stress. It is possible that stress reduces cognitive flexibility, which chronic optogenetic stimulation is sufficient to circumvent and that such perturbations in a healthy rodent have already reached a "ceiling effect" in their capacity to modulate the production of adult-born cells (Anacker & Hen, 2017). Our recent work suggested a bidirectional role for the dorsal and ventral hippocampus in respectively suppressing or enhancing context-specific memories, and we speculate that the capacity for chronic stimulation of hippocampal cells to alter neurogenesis levels too may depend on the site stimulated (Anacker et al., 2018; Chen et al., 2019). Intriguingly, a recent study (Johnston et al., 2020) reported that AAVs in the DG lead to cell death of immature neurons in mice. This study found dramatic reductions in DCX + cells such that almost no cells were detected by 4 weeks post-AAV injection. Though our animals were sacrificed 26 days post injection and we still detected DCX + cells, it is possible that we failed to detect small group differences in DCX + neurons if our AAVs blunted neurogenesis or reduced the DCX + population to reach a floor level of expression. Though a previous study by our group was able to detect group differences in DCX + cells after chronic optogenetic stimulation and utilizing a similar design as these experiments, we believe this was due to the effects of the stimulation in combination with chronic stress, which was sufficient to reset the levels of adult-born cells to a pre-stress baseline (Ramirez et al., 2015).

Finally, a number of recent studies have leveraged the effects of repeatedly activating various brain regions and circuits to note their enduring effects on behavior. Optogenetic-induced long-term depotentiation was sufficient to lastingly impair a memory while subsequent induction of long-term potentiation restored the expression of the memory (Nabavi et al., 2014). Additionally, high-frequency spike trains that lasted for 10 min were sufficient to alter excitation/inhibition balance and spine levels in the hippocampus and also facilitated the extinction of a contextual memory (Mendez et al., 2018), which points to the power of prolonged optogenetic strategies in modifying the structural and functional properties of the hippocampus. Various groups have also utilized optogenetic-inspired deep-brain stimulation strategies to provide a translational approach to enduringly reprogram a brain out of a maladaptive state (Creed et al., 2019), and we propose that artificially manipulating hippocampal engrams provides a conceptual means by which to sculpt neural activity and behavior.

Overall, our data suggest that chronic stimulation of hippocampus-mediated memory engrams may affect social behaviors. These behavioral effects are independent of the valence of the reactivated engram, overall levels of neurogenesis post-stimulation, as well as multi-region c-Fos-based cellular activity during social assays. These data reinforce the importance of considering multiple factors such as the stimulation parameters, the specific behavioral assays used, and measures of neural changes when implementing chronic stimulation protocols.

CRediT authorship contribution statement

E.D., E.M., H.L., A.M., C.C., J.L., S.L.G., and S.R. designed and performed the experiments. E.D., E.M., A.M., H.L., and S.R. wrote the paper. All authors edited and commented on the manuscript.

Declaration of Competing Interest

The authors declare no competing interests.

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Appendix A. Supplementary material

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