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Amnesia for context fear is caused by widespread disruption of hippocampal activity

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Abstract

The hippocampus plays an essential role in the formation and retrieval of episodic memories in humans and contextual memories in animals. However, amnesia is not always observed when this structure is compromised. To address this issue, we compared the effects of several different circuit manipulations on memory retrieval and hippocampal activity. Mice were first trained on context fear conditioning and then we used optogenetic and chemogenetic tools to alter activity during memory retrieval. We found that retrieval was only impaired when manipulations caused widespread changes (increases or decreases) in hippocampal activity. Widespread increases occurred when pyramidal cells were excited and widespread decreases were found when GABAergic neurons were stimulated. Direct hyperpolarization of excitatory neurons

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Credit Author Statement

Jamie N. Krueger – data curation; formal analysis; investigation; methodology; visualization; writing – original draft; writing – review & editing

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Brian J. Wiltgen - conceptualization; formal analysis; funding acquisition; project administration; resources; supervision; validation; writing – review & editing

only moderately reduced activity and did not produce amnesia. Surprisingly, widespread decreases in hippocampal activity did not prevent retrieval if they occurred gradually prior to testing. This suggests that intact brain regions can express contextual memories if they are given adequate time to compensate for the loss of the hippocampus.

Keywords

Context fear; retrieval; memory; hippocampus; optogenetics; chemogenetics

1. INTRODUCTION

The hippocampus is important for encoding and retrieving episodic memories in humans and contextual memories in animals. However, whether or not amnesia is observed when this structure is damaged depends on many different factors. These include the age of the memory being tested, how often it was recalled, how vivid and detailed it is, as well as the extent of hippocampal damage (Casagrande et al., 2018; Frankland & Bontempi, 2005; Wiltgen & Tanaka, 2013; Winocur et al., 2010). For example, bilateral damage to the hippocampus produces profound amnesia in humans while unilateral damage has little to no effect on memory (Milner & Penfield, 1955; Scoville & Milner, 1957). This suggests that episodic memories can be encoded and retrieved by spared hippocampal tissue and do not require the entire structure to be functional. Other brain regions can also compensate when the hippocampus is damaged and make contributions to learning and memory. This is evidenced by the fact that amnesia is most severe when damage is found in surrounding cortical areas and in distal regions like the prefrontal cortex (Bayley et al., 2006; Rempel-Clower et al., 1996; Squire et al., 2004). These findings suggest that intact brain regions can work together to support learning and memory when damage to the hippocampus is restricted or incomplete.

Memory loss in animals also correlates with the amount of damage and/or dysfunction in the hippocampus (Li et al., 1999). For example, partial lesions of the rodent hippocampus produce less severe retrograde amnesia for context fear than complete lesions (Anagnostaras et al., 2001; Sutherland et al., 2010). Pharmacological inactivation, which typically affects less hippocampal tissue, produces even milder deficits (Holt & Maren, 1999; Maren & Holt, 2004; Resstel et al., 2008) but see (Matus-Amat et al., 2004; Raybuck & Lattal, 2014). Lesions may be particularly disruptive because they cause significant damage to distal structures (Jarrard, 2002). In fact, some studies have reported a 40–50% reduction in cortical volume following excitotoxic lesions of the hippocampus (Anagnostaras et al., 2002; Logue et al., 1997). Therefore, similar to episodic memory in humans, robust amnesia for context fear appears to require widespread changes in hippocampal activity that prevent spared tissue, and other brain regions, from compensating.

Given that lesions and pharmacological manipulations lack temporal precision, we used optogenentic and chemogenetic tools to manipulate hippocampal activity during memory retrieval. Widespread inactivation was produced when GABAergic neurons were stimulated with ChR2 while limited silencing occurred when pyramidal cells were directly

hyperpolarized (Babl et al., 2019; Daie et al., 2019). We found that memory retrieval for context fear was significantly impaired by the former but not the latter. These results are consistent with the idea that amnesia requires extensive changes in hippocampal function. Unexpectedly, some inhibitory manipulations led to increases in excitatory activity. Effects like these have been reported in other studies and are thought to be caused, in part, by reduced activation of inhibitory neurons (López et al., 2016; Stefanelli et al., 2016). When we observed increases in activity, they typically spread throughout the hippocampus and caused severe memory impairments.

Activation of GABAergic neurons with excitatory DREADDs led to widespread increases in inhibitory activity, but unlike optogenetic stimulation, did not impair memory retrieval. We hypothesize that this is due to the temporal differences between optogenetic and chemogenetic silencing methods. The former produces an immediate decrease in excitation while the latter can take tens of minutes (Ryan et al., 2015; Zhu et al., 2014). It is possible that longer delays between hippocampal inactivation and testing gives spared tissue enough time to compensate and retrieve memory (Goshen et al., 2011).

2. METHODS

2.1 LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Brian Wiltgen (bjwiltgen@ucdavis.edu). This study did not generate any unique reagents.

2.2 EXPERIMENTAL MODEL AND SUBJECT DETAILS

Subjects: All procedures were approved by the Animal Care and Use Committee at UC Davis. Mice were maintained on a 12/12-light/dark schedule and were given free access to food and water. Animals were group-housed until surgery, after which they were single-housed for the remainder of each experiment. Littermates of the same sex were randomly assigned to experimental groups. Surgeries were performed at 8 weeks of age, with behavioral experiments beginning at approximately 10–12 weeks of age. Both C57/B6J and F1 hybrids (C57/B6J:129SvEv) were used for these experiments, as described below.

2.3 METHOD DETAILS

2.3.1 Stereotaxic Surgeries.—All viruses were infused into the dorsal hippocampus (dHPC) (−2.0mm AP; +/−1.5mm ML; −1.25mm DV) using a MicroSyringe pump controller at 2nL/second. For DREADD experiments, animals received 1μl bilateral infusions into the dHPC of *AAV5 CaMKII-hM4Di-IRES-mCitrine* (2.7×10^12 virus molecules/mL) (UNC Vector Core), *AAV5 hSyn-hM4Di-IRES-mCitrine* (1.12×10^12 virus molecules/mL) (UNC Vector Core), *AAV5 CaMKII-hM3Dq-IRES-mCitrine* (3.75×10^14 or 3.75×10^13 virus molecules/mL) (Addgene plasmid #50466, a gift from Bryan Roth, assembled and packaged with AAV5 helper plasmid by the UC Davis Molecular Construct and Packaging core facility. The virus was gradient purified, checked by SDS-PAGE and titered using qRT-PCR by standard methods (Flannery & Visel, 2013), or *AAV5-hDlx-GqDREADD-dTomato* (3.15×10^15 virus molecules/mL) (Addgene plasmid # 83897, a gift from Gordon Fishell,

assembled and packaged with AAV5 helper plasmid by the UC Davis Molecular Construct and Packaging core facility. The virus was gradient purified, checked by SDS-PAGE and titered using qRT-PCR by standard methods (Flannery & Visel, 2013). Dlx-enhanced viruses expressed red fluorophores (dTomato/mCherry) instead of green (mCitrine/eGFP/eYFP) due to commercial availability. For channelrhodopsin optogenetic experiments, animals received 0.25µl bilateral infusions of *AAV9 CaMKII-ChR2-eGFP* (8.96×10^11 virus molecules/mL) (Penn) or *AAV9 CaMKII-eGFP* (3.49×10^11virus molecules/mL) (Penn). For all other optogenetic experiments, animals received 0.5 µl bilateral infusions of *AAV5 CaMKII-ArchT-eGFP* (5.2×10^11 virus molecules/mL)(UNC), *AAV5 CaMKII-eYFP* (5.3×10^12 virus molecules/mL)(UNC), *AAV9 CaMKII-NpHR3.0-eYFP* (2.5×10^13 virus molecules/mL)(Penn), *AAV9 CaMKII-eGFP* (3.45×10^12 virus molecules/mL)(Penn) or *AAV5-hDlx-ChR2-mCherry* (7.6 ×10^14 virus molecules/mL) (Addgene plasmid # 83898, a gift from Gordon Fishell, assembled and packaged with AAV5 helper plasmid by the UC Davis Molecular Construct and Packaging core facility. The virus was gradient purified, checked by SDS-PAGE and titered using qRT-PCR by standard methods (Flannery & Visel, 2013).

Following virus infusion, the injection needle remained in place for five minutes. The scalp of DREADD experimental animals was closed with surgical glue (Vetbond). For optogenetic experiments, fibers were implanted into the dHPC. Optic fibers were constructed and polished as previously described (Sparta et al., 2011). Briefly, a 200um diameter optic fiber (Thorlabs) was stripped and inserted into a plastic ferrule (PlasticOne). The convex side of ferrule was polished and the optical fiber scored with a ruby knife to extend 1.2 mm from the tip. Fibers were implanted into the dorsal hippocampus (−2.0mmAP; +/− 1.5mm ML; −1.1mm DV). Following implantation, the skull was scored with a surgical blade and covered in C&B Metabond (Parkell). A dental acrylic headcap (Harry J. Bosworth Company) was constructed to hold the fibers in place and seal the incision. Animals recovered for two weeks prior to the beginning of behavioral experiments to allow for recovery and sufficient receptor/opsin expression.

2.3.2 Behavioral Apparatus: The contextual fear conditioning equipment used in all experiments was previously described (Tanaka et al., 2014; Tayler et al., 2013). Briefly, mice were trained and tested in conditioning chambers (30.5 cm x 24.1 cm x 21.0 cm) located within sound-attenuated boxes. The chambers consisted of a stainless-steel grid floor with overhead LED lighting (providing broad spectrum light). Sessions were recorded with a scanning charge-coupled device video camera (Med Associates). The chamber and drop pan were cleaned with 70% ethanol before each behavioral session. Detailed training procedures are provided below for specific optogenetic and DREADD experiments. Memory was assessed the following day by placing the mice in the context and measuring the freezing response. Freezing measurements were made using the automated Video Freeze System (Med Associates) as previously described (Stephan G. Anagnostaras et al., 2010).

DREADD Behavioral Procedures: C57/B6J:129SvEv mice were ordered or bred in-house (Taconic). 8 groups were used in these experiments: CaMKII-hM4Di-CNO (3m, 3f); CaMKII-hM4Di-Vehicle (3m, 3f), hSyn-hM4Di-CNO (3m, 3f), Syn1-hm4Di-Vehicle (3m, 3f), CaMKII-hM3Dq-CNO (5m), CaMKII-hM3Dq-Vehicle (5m), Dlx-GqDREADD-CNO

(7m, 3f), Dlx-GqDREADD-Vehicle (6m, 3f). After recovery, animals were handled for 5 minutes a day for five days prior to contextual fear conditioning for habituation to the experimenter. During training, following a three-minute baseline period, animals were given three two-second 0.4mA shocks with 60 second inter-trial intervals. The following day, animals were given IP injections of either 0.5 (CaMKII-hM3Dq) or 5mg/kg (all other groups) CNO (Toronto Research Chemicals) (10mg dissolved in 100μL DMSO, diluted to 0.1 or 1mg/mL CNO in 0.9% saline) or vehicle (1% DMSO in 0.9% saline), one hour prior to re-exposure to the fear conditioning chamber. A 0.5mg/kg CNO concentration was used for the CaMKII-hM3Dq group as pilot experiments with 5mg/kg produced seizures (data not shown). Animals were tested in the absence of shock for 30 minutes and freezing behavior was analyzed during the first 12 minutes of the session. Only the first 12-minutes were analyzed so the data could be compared with the optogenetic retrieval experiments, which were 12-minutes in duration.

2.3.3 Optogenetic Behavioral Procedures: C57/B6J: mice used for these experiments were ordered or bred in-house (Taconic). Nine groups were used for these experiments: CaMKII-NpHr3.0 (3m, 3f), CaMKII-eGFP (3m, 3f), CaMKII-ArchT (3m, 3f), CaMKII-eYFP (3m, 3f), CaMKII-ChR2 20Hz (3m, 2f), CaMKII-ChR2 4Hz (4f, 1m), (CaMKII-eGFP (3m, 2f), Dlx-ChR2 (8f, 9m). Animals were handled for 2–5 minutes a day for five days prior to contextual fear conditioning. During handling, animals used for these studies were also connected to the optic fiber cable. Animals were placed in a Med Associates fear conditioning chamber with fiber implants connected to a splitting 200um optic fiber (Doric lenses). The fiber was attached to the conditioning chamber through a rotating commutator (Doric lenses) and coupled to a 473/532/561 nm 200 mW solid-state laser diode (OEM laser systems) with 15 mW output. Prior to each experiment, laser output intensity was measured with an optical power meter (Thorlabs). Stereotaxic coordinates were used to place optic fibers directly above dorsal CA1, directing laser stimulation to this region. Following a three-minute baseline period, animals were given four 2s 0.75mA shocks with 60-second inter-trial intervals. The following day, animals were returned to the chamber for a 12-minute testing session. Using Doric studio to trigger the laser, mice underwent laser stimulation twice for 3 minutes over the testing session. The session began with a 3-minute baseline period, then the laser (473nm (ChR2), 532nm (ArchT), 561nm (NpHR3.0), was turned on for 3 min (20Hz, 15ms pulse width: ChR2; continuous stimulation NpHR3.0/ArchT) at 10 or 15mW. This was repeated once. Dlx-ChR2 animals underwent an additional testing session 24 hours later consisting of 12 minutes of laser stimulation.

2.3.4 Tissue Collection and Immunohistochemistry.—Ninety minutes following re-exposure to the chamber (DREADD experiments) or first laser on epoch (optogenetic experiments), animals were perfused using ice-cold PBS and 4% paraformeldahyde (PFA). Brain tissue was collected and stored overnight in 4% PFA. Slices were taken at 40nm using a Leica Vibratome. Tissue was stored in slice storage solution (100mL 10x tris-buffered saline, 300mL ethylene glycol, 300mL glycerol, 300mL dH_2O) at −20°C until staining. Slices were washed 3 × 5 minutes in PBS, stained overnight in rabbit anti-cFos (1:5000, Millipore ABE457) in donkey blocking buffer. Slices were washed in 3 × 5 minutes in PBS,

counterstained in biotynilated donkey anti-rabbit (Jackson) (1:500) for 1 hour, washed 3 × 5 minutes in PBS, stained with Cy3/Cy5 (Jackson) (1:500) for 45 minutes, washed in 3 × 5 minutes PBS and counterstained for 10 minutes with a DAPI nuclear stain (1:10000). Slices were mounted on SuperFrost slides with VectaShield mounting media.

2.3.5 Microscopy and Cell Counting.—Three to four coronal sections surrounding the −2.0 AP coordinate of each mouse were selected for c-Fos quantification. For optogenetic experiments, tissue was selected from beneath the fiber tip. Tissue slices were scanned in a 35um z-stack using an Olympus Slide Scanner at 20x magnification. Exposure times per channel (DAPI, FITC, TRITC) for each animal were set under saturation. Images were further cropped to the intermediate dCA1 for analysis. Fluorescent images were imported into ImageJ in grayscale and separated by channel. Cell counts were obtained using the multipoint tool on ImageJ with the experimenter blind to groups. An estimate of total dorsal CA1 cells per section was calculated by using 3D Object Counter in ImageJ and dividing the obtained volume by an average single nucleus volume for the group. Approximately 500 cells were counted from each slice, and 6 hemispheres were counted from each animal (~ 3,000 cells). The percentage of c-Fos+ neurons was calculated for each animal and then normalized to the mean of the appropriate control group (individual percent c-Fos+/control group percent c-Fos+). For Dlx experiments, we calculated both the percentage of c-Fos+ pyramidal cells and Dlx+ inhibitory neurons in each animal. These values were normalized to the appropriate control group. To estimate the degree of inhibition produced in the Dlx experiments, we quantified the number of c-Fos+ cells in the stratum oriens, stratum radiatum and stratum lacunosum-moleculare at 3 different AP coordinates (anterior (−1.2 to −1.5 AP), intermediate (−2.5 to −2.8 AP) and posterior (−3.2. to −3.5 AP). Anterior to posterior Dlx+ cell quantification was performed in single-plane images, and cell counts shown as number of c-Fos+ cells per ~.45mm^2.

2.4 QUANTIFICATION AND STATISTICAL ANALYSIS

Freezing data during memory testing were analyzed using repeated measures ANOVA with the p value set to 0.05. Post-hoc comparisons were made using Fisher's LSD. Cell counts were normalized to control groups and analyzed using a t-test with the p value set to 0.05. All data are represented as mean +/− SEM. Statistical details can be found within the results section.

2.4.1 Excluded data: For the optogenetic experiments, subjects with incorrect fiber placements were excluded from all analyses (1 animal from CaMKII-NpHR3.0 and CaMKII-ArchT experimental groups). Brain tissue that was damaged during extraction and/or slicing was excluded from c-Fos analyses (1 CaMKII-hM4Di (CNO); 2 CaMKII-eGFP (NpHR control group); 1 CaMKII-eGFP (ChR2 control group); 1 CaMKII-eYFP (ArchT control group). Four laser-on animals in the Dlx-ChR2 experiment did not express any c-Fos in inhibitory interneurons (indicating that stimulation did not work) and were excluded. One subject was excluded from the Dlx-ChR2 control group because the optic fiber caused significant damage to the hippocampus.

2.4.2 Figures: Training/testing paradigm images (Figures 2–3) were created using *BioRender*. All data were plotted and analyzed using *GraphPad Prism 8*.

2.5 DATA AND CODE AVAILABILITY

This study did not generate/analyze datasets/code. Original/source data for figures in the paper is available by request.

3. RESULTS

3.1 Acute activation of inhibitory neurons impairs memory retrieval for context fear

To examine the relationship between neural activity and memory retrieval, we trained mice on a context fear conditioning task that requires the hippocampus (Tanaka et al., 2014; Wiltgen et al., 2010). We targeted the dorsal segment of this structure because damage to this area produces reliable amnesia for context fear 1-day after learning (Anagnostaras et al., 1999; Frankland et al., 1998; Kim & Fanselow, 1992; Maren et al., 1997).

Widespread inactivation of the dorsal hippocampus was achieved by stimulating inhibitory neurons with ChR2 or hM3D. These excitatory proteins were selectively expressed in GABAergic neurons using the Dlx enhancer (Dimidschstein et al., 2016) (Figures 1A–1C). In the first experiment, mice received infusions of AAV-Dlx-ChR2 and then underwent context fear conditioning 2-weeks later (Figure 2A). Animals were returned to the conditioning chamber the next day for a long-term memory test (Figure 2B). After a 3-minute baseline period, inhibitory neurons were stimulated with ChR2 (473nm, 20Hz, 10mW, 180s). Stimulation occurred a second time 3-minutes later. Dlx-ChR2 mice (n = 10) froze significantly less during the laser stimulation periods than control animals (n = 6) (Figure 2C) (Group x laser interaction $F(1,15)$, $p = .0012$, Fisher's LSD post-hoc tests, Laser off ($p = .54$), Laser on ($p = .0064$)).

To verify that laser stimulation inhibited pyramidal cells and activated interneurons, we quantified c-Fos expression in CA1. This was done after a second context test, during which, ChR2 was stimulated throughout the session (i.e. no laser off periods). Compared to control animals, c-Fos expression was decreased in pyramidal neurons (Figure 2D) ($t = 7.366$, $df = 13$, $p < 0.0001$) and increased in inhibitory neurons in Dlx-ChR2 mice (Figure 2E) ($t = 4.226$, $df = 13$, $p = .0010$). These data indicate that acute stimulation of inhibitory neurons reduces excitation in the dorsal hippocampus and impairs memory retrieval for context fear.

In the next experiment, mice received infusions of AAV-Dlx-hM3Dq and were trained on context fear conditioning 2-weeks later (Figure 3A). Injections of 5mg/kg CNO (n=10) or a vehicle solution (n=9) were administered one hour before testing (Figure 3B). Pilot experiments found that this concentration did not affect context fear in wild-type animals (Data not shown, No effect of group $F(1,8) = 1.274$, $p = .2917$). Stimulation of inhibitory neurons with CNO did not affect memory retrieval in Dlx-hM3Dq mice compared to controls (Figure 3C) (No effect of group $F(1,17) = 1.931$, $p = .1826$). This was despite the fact that CNO decreased c-Fos expression in pyramidal neurons (Figure 3D) ($t = 11.68$, $df = 17$, $p < 0.0001$) and increased expression in inhibitory cells (Figure 3E) ($t = 11.44$, $df = 17$, $p < 0.0001$).

To estimate the spatial extent of inhibitory activity produced by stimulation of ChR2 and hM3Dq, we quantified c-Fos expression in GABAergic neurons at 3 different anteroposterior (AP) coordinates that spanned most of the hippocampus (ChR2 n=5, control n=5; hM3Dq CNO n=4, vehicle n=4). Quantification was restricted to SO, SR and SLM cell layers in CA1 where inhibitory cells could be easily identified and distinguished from pyramidal neurons located in SP (Figure 4A). When ChR2 was stimulated, we observed increased c-Fos expression in GABAergic neurons at multiple sites within the dorsal (anterior and intermediate) but not ventral hippocampus (posterior) (Figure 4B) (Group x AP coordinate interaction, $F(2,16) = 6.12$, $p = .0106$), Fisher's LSD post-hoc tests, Anterior ($p = .042$), Intermediate ($p = .0048$), Posterior ($p = .3294$)). Based on these data, we estimate that optogenetic stimulation of GABAergic neurons produces inhibitory activity that extends at least 1mm AP from the tip of the optic fiber (Babl et al., 2019; N. Li et al., 2019).

When GABAergic neurons were stimulated with hM3Dq, we found that the spatial extent of inhibitory activity was similar to that observed with ChR2 (Figure 4C) (Group x AP coordinate interaction, $F(2,12) = 42.2$, $p = <.0001$; Fisher's LSD post-hoc tests, Anterior ($p <.0001$), Intermediate ($p=.0003$), Posterior ($p=.1060$)). This suggests that the retrieval deficit caused by ChR2 stimulation did not result from more extensive inhibition of the dorsal hippocampus. We hypothesize that the prolonged stimulation period required to activate hM3Dq (tens of minutes) may have given other brain regions time to compensate prior to testing.

3.2 Both acute and prolonged stimulation of excitatory neurons impairs memory retrieval

Amnesia can also be produced by widespread increases in hippocampal activity. Patients with temporal lobe epilepsy, for example, are significantly impaired at forming and retrieving autobiographical memories from their lives (Viskontas et al., 2000; Zhao et al., 2014). Rodent models of epilepsy produce profound amnesia for spatial and contextual memories as do other manipulations that lead to large increases in hippocampal excitability (Butler et al., 1995; Palop et al., 2007). The current experiments examined the effects of increasing hippocampal activity on memory retrieval by stimulating pyramidal neurons with excitatory opsins or DREADDs (Figure 5A).

First, AAV containing hM3Dq was infused into the dorsal hippocampus and expressed in excitatory neurons using the CaMKII promoter. Two weeks later, mice were trained on context fear conditioning and memory was tested the following day. One hour before testing, animals received an IP injection of CNO (0.5mg/kg) (n=5) or vehicle solution (n=5) (same behavioral design as Figure 3A). Compared to controls, stimulation of pyramidal neurons with hM3Dq produced profound impairments in memory retrieval (Figure 5B) (Significant group x time interaction ($F(3, 24) = 9.39$, $p = .0003$; Main effect of group, $F(1, 8) = 17.9$, $p = 0.003$). To confirm that stimulation of hM3Dq increased excitatory activity during the context test, we quantified c-Fos expression in CA1 pyramidal neurons (Figure 5C). As expected, CNO injections produces a large increase in c-Fos labeling compared to the vehicle-treated group ($t(8) = 12.13$, $p < 0.0001$).

In order to acutely activate pyramidal neurons, we expressed ChR2 under control of the CaMKII promoter (Figure 5A). Two-weeks later, mice were trained on context fear

conditioning and received a memory test the following day. After a 3-minute baseline period, pyramidal neurons in CA1 were stimulated with ChR2 (473nm, 10mW, 180s). Stimulation occurred a second time 3-minutes later (same behavioral procedure as Figure 2A). Excitatory neurons were stimulated at 4Hz (n = 6) or 20Hz (n = 5) because low frequency stimulation has been shown to induce freezing in CA1 “engram cells” (Roy et al., 2019; Ryan et al., 2015). However, we did not observe a difference between these frequencies, so the data were combined for statistical analyses. When pyramidal neurons were stimulated after the baseline period, freezing levels decreased significantly in CaMKII-ChR2 mice relative to CaMKII-eGFP animals (Figure 5C) (Stimulation period x Group interaction $F(1, 15) = 5.938$, $p = .0278$), Fisher’s LSD post-hoc tests, ChR2 ($p < .0001$), eGFP ($p = .1386$). Freezing did not recover in CaMKII-ChR2 mice after the laser turned off and remained low for the remainder of the session (Main effect of group $F(1, 15) = 36.45$, $p < .0001$, No effect of stimulation period $F(1, 15) = .024$, $p = .8786$, No group x stimulation period interaction $F(1, 15) = .608$, $p = .4476$). Quantification of c-Fos revealed that ChR2 stimulation increased expression in CA1 pyramidal neurons as expected ($t(8) = 14.23$, $p<0.0001$) (Figure 5D). These data indicate that non-selective increases in excitatory activity lead to severe memory deficits in context fear.

3.3 Direct hyperpolarization of excitatory neurons has variable effects on memory retrieval

In the next set of experiments, we inhibited excitatory neurons directly and examined the effects on memory retrieval. To do this, we expressed inhibitory DREADDs or opsins in CA1 pyramidal neurons using the CaMKII promoter (Figure 6A). For the optogenetic manipulations, mice were trained and tested as in our other experiments, except that yellow light was used to stimulate NpHR (561nM, 15mW) (n = 6) and green light was used for ArchT (532nM, 10mW) (n = 6). In the DREADD experiment, animals received an IP injection of 5mg/kg CNO (n=7) or vehicle (n=6) one hour before the context test. We used the same behavioral procedures described in Figures 2A/3A.

When pyramidal neurons were hyperpolarized with hM4Di, we found that memory retrieval for context fear was not affected (No effect of treatment, $F(1, 11) = 0.077$, $p = 0.785$; Main effect of time $F(3, 33) = 6.98$, $p = .0009$, No treatment x time interaction $F(3, 33) = .216$, $p = .8844$) (Figure 6B). Quantification of c-Fos revealed that hM4Di stimulation reduced expression in CA1 pyramidal neurons during testing although the size of this decrease was modest ($t(10) =2.95$, $p = 0.01$) (Figure 6C). This result is consistent with our Dlx-hM3Dq data and suggests that prolonged inhibition of dorsal CA1 neurons does not prevent the expression of context fear.

In contrast to CaMKII-hM4Di, acute hyperpolarization with ArchT produced large deficits in freezing relative to CaMKII-eGFP animals (n = 5) (Figure 6D) (Group x Stimulation period interaction $F(1, 9) = 9.254$, $p = .014$, Fisher’s LSD post-hoc tests, Laser off (p .384) Laser on ($p = .049$)). Similar to our CaMKII-ChR2 data, freezing did not recover when ArchT stimulation was terminated and remained low for the remainder of the session (Main effect of group $F(1, 9) = 14.39$, $p = .0043$, No effect of Stimulation period $F(1,9) = 1.476$, $p = .2554$), No Group x Stimulation period interaction $F(1, 9)$ 1.539, $p = .2461$). Given the

similarity between these results, we quantified c-Fos expression after the test and found a large increase in excitatory activity rather than a decrease ($t(8) = 5.92$, $p=0.0004$) (Figure 6E). This unexpected result may have been caused by rebound excitation; transient increases in firing rate that are often observed after hyperpolarization (Li et al., 2019). If pyramidal neurons were activated each time ArchT stimulation ended, it could produce an increase c-Fos expression during testing.

To determine if other inhibitory opsins produce similar effects, we used NpHR to hyperpolarize CA1 neurons during testing. Unlike ArchT, this manipulation had no effect on memory retrieval (No effect of group, $F(1,9) = 0.05$, $p = 0.8182$; Main effect of time, $F(3, 27) = 9.23$, $p = 0.0002$; No group x stimulus period interaction, $F(3,27) = 0.8$, $p = 0.47$) (Figure 6F) and did not increase c-Fos expression relative to CaMKII-eGFP ($n = 6$) ($t(7) = 0.11$, $p = 0.92$) (Figure 6G). Because laser off periods can obscure decreases in pyramidal cell activity, we conducted a second test where NpHR and ArchT were stimulated during the entire session (Figure 6). In this case, both opsins reduced c-Fos expression around the tip of the optic fiber (Figure 6I,J). This decrease was not observed in CaMKII-eYFP control animals suggesting that it was not caused by prolonged laser stimulation. In addition to reducing activity near the optic fiber, ArchT stimulation also increased in c-Fos expression outside of this area. This increase in activity was extensive and could be observed in CA3 and the dentate gyrus as well as CA1. Large increases in c-Fos expression were not observed following NpHR stimulation. Therefore, we hypothesize that activation of ArchT impaired memory retrieval because it produced increases in excitatory activity that spread throughout the dorsal hippocampus (similar to CaMKII-ChR2).

ArchT is a proton pump and prolonged stimulation of this opsin (< 1 min) can raise the intracellular pH of synaptic terminals and increase spontaneous glutamate release (Mahn et al., 2016). Stimulation can also decrease the extracellular pH and induce action potentials in neighboring neurons by activating acid-sensing ion channels (ASICs) (Baron et al., 2002; T. Li et al., 2014). Because these excitatory effects take time to emerge, we determined how quickly freezing began to decrease after ArchT was stimulated. To do this, we analyzed the behavioral data from the test session in 20s bins (Figure 7A). We found that decreases did not occur until ArchT had been stimulated for ≈100–140s. For comparison, we examined changes in freezing when inhibition was produced by stimulating GABAergic neurons with ChR2. In contrast to ArchT, this manipulation produced an immediate decrease in freezing (Figure 7B) (N. Li et al., 2019). Another difference was that freezing returned to control levels after Dlx-ChR2 was stimulated but did not recover in ArchT animals. The latter was also observed when we activated pyramidal neurons with CaMKII-ChR2 (Figure 5D). These data strongly suggest that context fear is impaired by prolonged ArchT stimulation because it increases excitatory activity in the dorsal hippocampus. It is possible that freezing did not recover when the laser turned off because stimulation caused place cells to remap (Diamantaki et al., 2018; Trouche et al., 2016). Remapping would make it difficult for animals to recognize the training context, which should increase exploration and decrease freezing as we observed.

3.4 Hyperpolarizing inhibitory neurons increases activity and impairs memory retrieval

A recent study found that stimulation of hM4Di in the dorsal hippocampus increased c-Fos expression and impaired object recognition memory (López et al., 2016). In this case, a neuron specific promoter (Syn1) was used to drive hM4Di expression in both excitatory and inhibitory neurons. Hyperpolarization of the latter reduced GABAergic tone and led to a net increase in excitatory activity. In the current experiment, we determined if the same manipulation would increase c-Fos expression in CA1 pyramidal neurons and impair the retrieval of context fear. Similar to the published report, we found that injections of CNO in Syn1-hM4Di mice produce significant memory deficits and increased c-Fos expression in excitatory neurons relative to controls (Figure 7C–E). Together with our ArchT data, these results demonstrate that some inhibitory manipulations impair memory retrieval by increasing the amount of excitatory activity.

4. DISCUSSION

In the current study, we determined if altered hippocampal activity produces amnesia for recently acquired context fear. Reductions in activity were achieved by activating inhibitory neurons or by hyperpolarizing pyramidal cells directly. Similar to recent reports, we found that the former produced much more robust and widespread silencing than the latter (Babl et al., 2019; Li et al., 2019). When inhibitory neurons were stimulated with ChR2 during testing, memory retrieval was significantly impaired. In contrast, when the same neurons were activated with the excitatory DREADD hM3Dq, retrieval was not affected. This dissociation was not due to differences in inhibition, as both manipulations activated interneurons and reduced excitation throughout the dorsal hippocampus (as indexed by c-Fos expression). Therefore, we hypothesize that the retrieval deficit caused by ChR2 stimulation is due to an immediate reduction in hippocampal activity that does not provide enough time for other brain regions to compensate. Stimulation of DREADDs, on the other hand, produces a gradual loss of excitation in the hippocampus that can take up to 30 minutes to reach asymptote (Ryan et al., 2015; Zhu et al., 2014). This appears to be a sufficient amount of time for extra-hippocampal structures to become engaged and express context fear.

A similar difference between acute and prolonged hippocampal inactivation was observed during remote memory retrieval (Goshen et al., 2011). In that case, it was assumed that cortical regions could express context fear during prolonged silencing because systems consolidation had taken place. However, we find that the same dissociation exists 1-day after learning. It is possible, therefore, that cortical regions are able to express both recent and remote context fear provided they have enough time to compensate for the loss of the hippocampus. It should be noted that the brain areas mediating memory retrieval in each of these situations may or may not be the same. The ACC is important for the expression of remote context fear while the PFC, entorhinal, perirhinal, postrhinal and retrosplenial cortices contribute to recent and remote memory retrieval (Burwell et al., 2004; Coelho et al., 2018; Cowansage et al., 2014; de Sousa et al., 2019; Frankland et al., 2001; Kitamura et al., 2017; Zelikowsky et al., 2014). The ventral hippocampus is also likely to play an important role as it projects to the amygdala and influences the expression of context fear (Chen et al., 2019; Cullen et al., 2015; Huckleberry et al., 2018; Wiltgen et al., 2006).

The type of context memory that is expressed during prolonged inactivation of the dorsal hippocampus may differ from the one that is retrieved in control animals. For example, context memories are often less precise when they are retrieved without the hippocampus (Wiltgen et al., 2010; Wiltgen & Silva, 2007; Winocur et al., 2007). This possibility can be addressed in future work by using discrimination procedures to examine memory specificity (Wang et al., 2009). However, it is important to note that damage to the dorsal hippocampus impairs retrieval on the same type of context recognition tests that were used in the current experiments (Anagnostaras et al., 1999; Frankland et al., 1998; Kim & Fanselow, 1992; Maren et al., 1997). In the case of lesions, this deficit could result from consolidation impairments because memory is tested several days after training and surgery. The retrieval deficit could also be caused, in part, by damage that is caused to distal structures. For example, excitotoxic lesions of the hippocampus produce a substantial loss of cortical tissue that may prevent other brain regions from compensating during memory tests (Anagnostaras et al., 2002). Consistent with this idea, episodic memory deficits in patients with hippocampus damage are strongly correlated with tissue loss in distal structures (Argyropoulos et al., 2019).

In contrast to prolonged inactivation, we found that rapid optogenetic silencing of the dorsal hippocampus produced significant deficits in memory retrieval. Given than other brain regions are able to express context fear, this result suggests that optogenetic inactivation somehow prevents them from doing so. One possibility is that retrieval is impaired by acute silencing because memory is tested at the exact same moment hippocampal activity is disrupted. Optogenetic perturbations have been shown to change local activity and simultaneously disrupt the function of distal brain regions (Allen et al., 2015; Otchy et al., 2015). These off-target effects could prevent connected areas from compensating for the loss of the hippocampus during memory retrieval. In contrast, when inactivation occurs over an extended period, other brain regions may be able to recover by the time memory is tested (Otchy et al., 2015).

Consistent with a recent report, we found that direct hyperpolarization of pyramidal cells produces less extensive inhibition than stimulation of interneurons (Li et al., 2019). Limited inhibition is likely the reason the former did not impair memory retrieval. Surprisingly, some inhibitory manipulations (CaMKII-ArchT and hSyn-hM4Di) produced off-target effects that increased the total amount of hippocampal activity. When this occurred, memory expression was severely impaired. In addition, the deficits appeared similar to those observed when pyramidal cells were activated with ChR2 or hM3Dq. In the case of ArchT, we hypothesize that the prolonged stimulation period (3-min.) increased excitatory activity by altering internal and/or external pH (Baron et al., 2002; Li et al., 2014). Shorter stimulation periods are recommended to reduce these effects (Mahn et al., 2016). Reduced expression levels may also help as widespread increases in excitatory activity were not reported in previous ArchT studies that confined silencing to “engram cells” (Denny et al., 2014; Lacagnina et al., 2019; Tanaka et al., 2014). It is clearly essential to determine how different optogenetic and chemogenenic manipulations affect network activity in order to understand their effects on learning and memory (Allen et al., 2015; Otchy et al., 2015). Unfortunately, efficacy is often confirmed only in cells that express the protein of interest and changes in network activity are not examined.

Taken together, our results suggest that amnesia is caused by widespread disruption of hippocampal activity (Table 1). Memory is not affected by localized changes, presumably because spared tissue can compensate during learning and retrieval. Compensation is most effective when decreases in hippocampal activity occur over a prolonged period. In these cases, memories can be formed and retrieved even after extensive changes in activity. However, memory quality and precision are likely to be compromised in these situations (Frankland et al., 1998; Wiltgen et al., 2010; Yonelinas, 2013).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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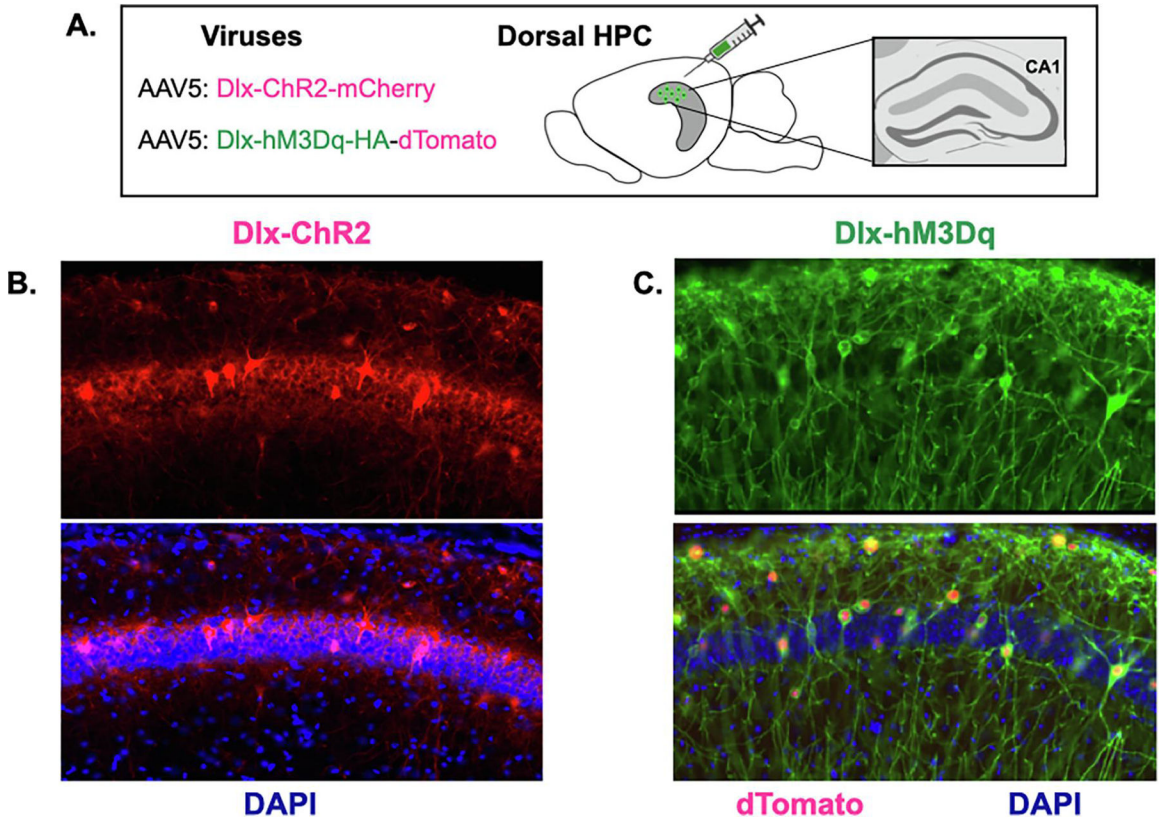

Figure 1. Expression of ChR2 and hM3Dq in inhibitory neurons.

A. Dlx-ChR2-mCherry or Dlx-hM3Dq-dTomato were expressed in CA1 of the dorsal hippocampus (image adapted from (Moser & Moser, 1998). B. *Left top*: Dlx-ChR2 expression (red) in dorsal CA1. *Left bottom*: Dlx-ChR2 expression merged with DAPI (blue) in dorsal CA1. C. *Right top*: Dlx-hM3Dq HA-tag expression (green) in dorsal CA1. *Right bottom*: Dlx-hM3Dq HA-tag expression (green) and Dlx-hM3Dq dTomato nuclear expression (red) merged with DAPI (blue) in dorsal CA1.

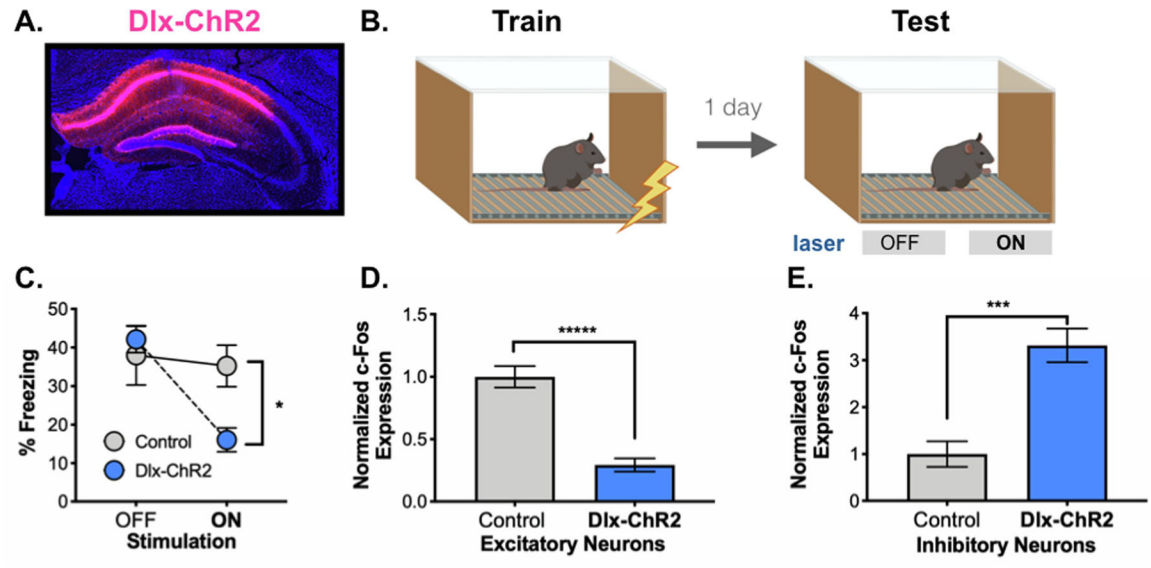

Figure 2. Acute activation of inhibitory neurons.

A. Dlx-ChR2 expression (pink) in the dorsal hippocampus. B. Behavioral paradigm. Dlx-ChR2 animals were trained in contextual fear conditioning (4 x .75mA shocks). One day later animals were returned to the testing chamber for a 12-minute test. Animals received a 3-minute baseline, then 3 minutes of blue laser (473nm, 20Hz, 10mW) stimulation. This was repeated once. C. Dlx-ChR2 animals (blue) froze less during Laser ON periods than controls (gray). D. c-Fos expression was lower in excitatory pyramidal neurons in dCA1 of Dlx-ChR2 (blue) vs. control (gray) animals. E. c-Fos expression was higher in inhibitory neurons in dCA1 of Dlx-ChR2 (blue) vs. control (gray) animals. All data are expressed as mean +/− SEM.

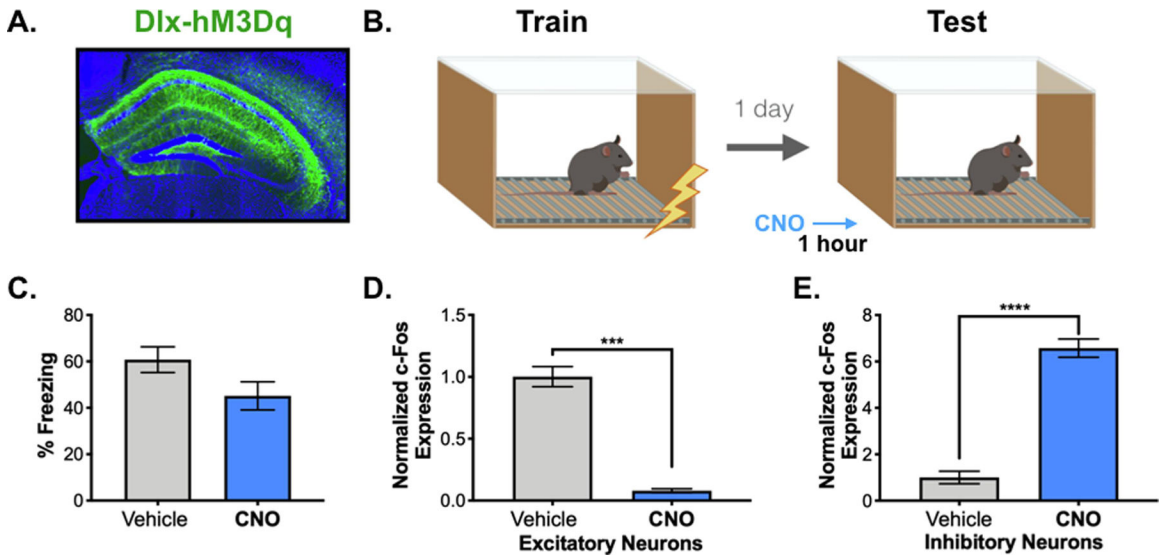

Figure 3. Prolonged activation of inhibitory neurons.

A. Dlx-hM3Dq expression (green, HA tag) in the dorsal hippocampus. B. Behavioral paradigm. Dlx-hM3Dq animals were trained in contextual fear conditioning (3 x .4mA shocks). One day later, animals were returned to the chamber for a 12-minute memory test following a 5mg/kg CNO or vehicle IP injection. C. There was no difference in freezing over the 12-minute testing period between the vehicle (gray) and CNO-treated (blue) animals. D. c-Fos expression was lower in excitatory pyramidal neurons in dCA1 of CNO-treated (blue) vs. vehicle-treated (gray) animals. E. c-Fos expression was increased in inhibitory neurons in dCA1 of CNO-treated (blue) vs. vehicle-treated (gray) animals. All data are expressed as mean +/− SEM.

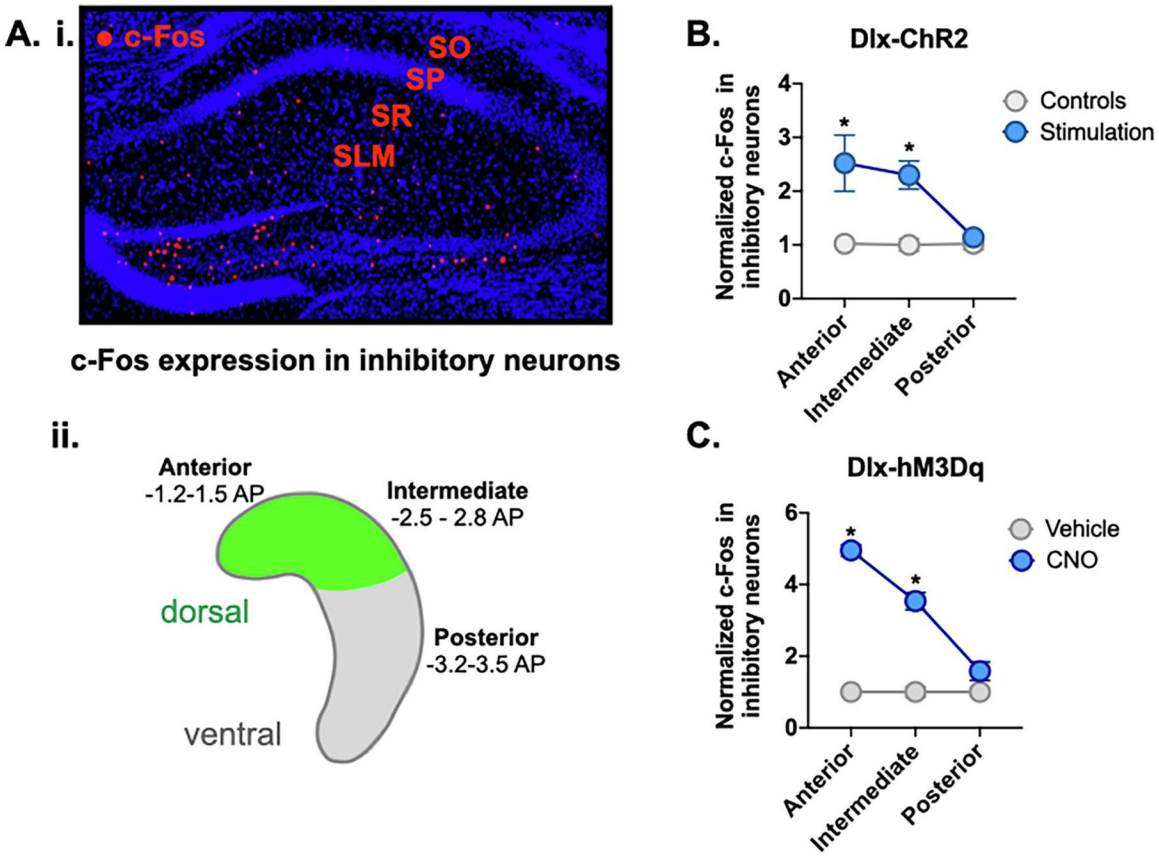

Figure 4. Widespread activation of inhibitory neurons in the dorsal hippocampus.

A. i. Anterior dHPC of representative CNO-treated Dlx-hM3Dq animal showing c-Fos expression (red) in inhibitory strata: stratum oriens (SO), stratum radiatum (SR), stratum lacunosum-moleculare (SLM). A. ii. Schematic of HPC showing location of virus expression (green) in dorsal HPC. Representative slices were taken for c-Fos analysis from anterior (−1.2 to −1.5 AP), intermediate (−2.5 to −2.8 AP) and posterior/ventral HPC (−3.2 to −3.5 AP). B. c-Fos expression in inhibitory neurons of anterior and intermediate HPC was increased in Dlx-ChR2 laser-stimulated animals (blue). There was no difference in c-Fos expression in posterior HPC. C. c-Fos expression in inhibitory neurons of anterior and intermediate hippocampus was increased in Dlx-hM3Dq CNO-treated animals (blue). There was no difference in c-Fos expression in posterior HPC. All data are expressed as mean +/− SEM.

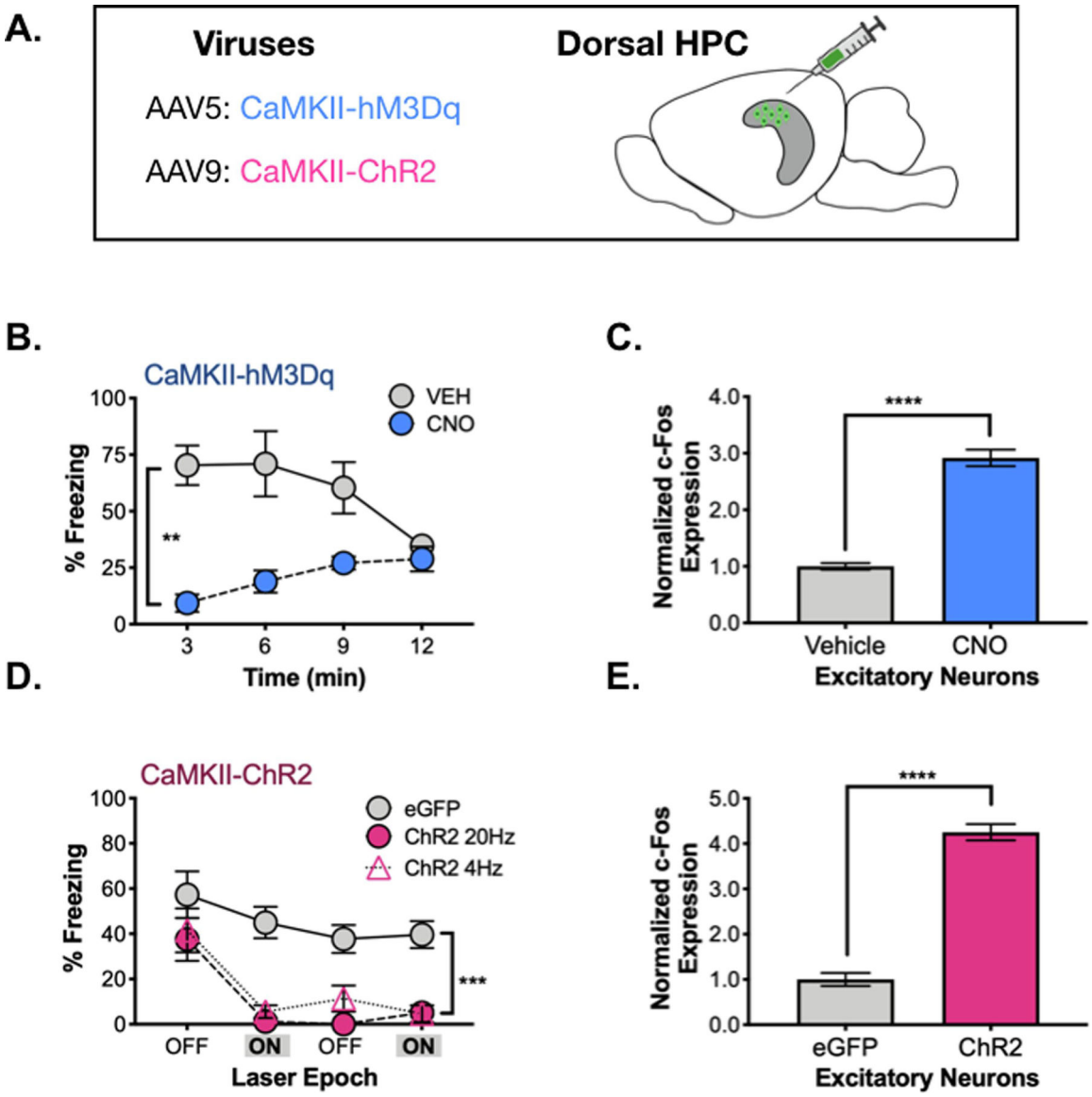

Figure 5. Prolonged vs. acute activation of excitatory neurons.

A. CaMKII-ChR2 or CaMKII-hM3Dq were infused into dHPC. B. During memory testing, hM3Dq CNO-treated animals (pink) froze less than their vehicle-treated counterparts (gray). C. c-Fos expression in excitatory neurons of dCA1 was elevated in hM3Dq CNO-treated animals (pink) compared to their vehicle-treated counterparts (gray). D. During memory testing, ChR2 (20Hz – pink circles, 4Hz – pink triangles) animals and eGFP animals (gray) did not differ during the first 3-minute laser OFF epoch. Following laser stimulation, 20 and 4Hz stimulated animals froze less than controls over the remainder of the testing period. E. c-Fos expression was elevated in excitatory neurons of dCA1 in ChR2 20Hz-stimulated animals (pink) compared to controls (gray). All data are represented as mean +/− SEM.

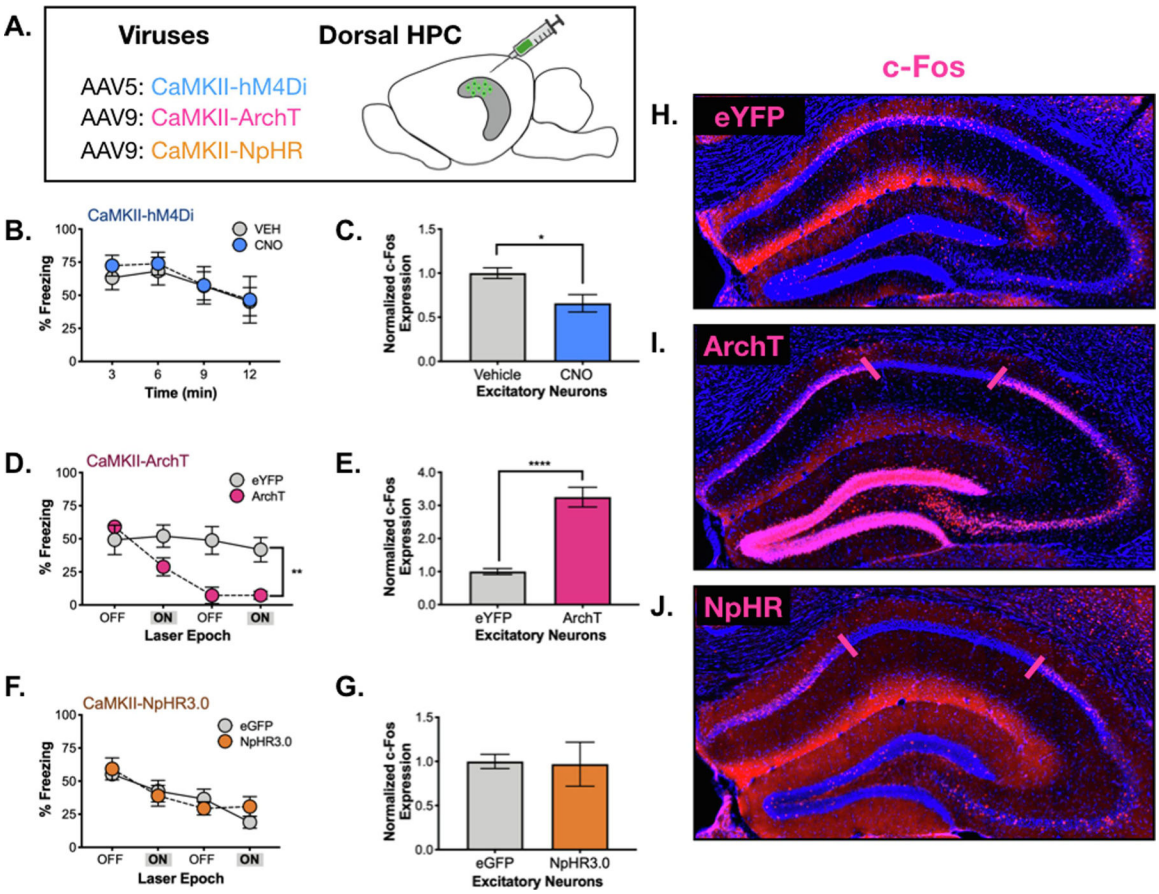

Figure 6. Prolonged vs. acute inhibition of excitatory neurons.

A. CaMKII-hM4Di, CaMKII-ArchT or CaMKII-NpHR were infused into the dHPC in order to silence excitatory neurons. B. There was no difference in freezing during testing between hM4Di CNO (blue) and vehicle-treated (gray) animals. C. There was a small, but significant decrease in dCA1 c-Fos expression in CNO-treated hM4Di expressing animals (blue). D. There was no difference in freezing during the initial 3-minute laser OFF period between ArchT (orange) and eYFP (gray) expressing animals. Following the first laser ON epoch, ArchT animals froze less than controls over the remainder of the testing period. E. There was a significant **increase** in dCA1 c-Fos expression in ArchT animals (orange) compared to controls. F. There was no difference in freezing during laser OFF or ON epochs in NpHR vs. control animals. G. There was no difference in dCA1 c-Fos expression between NpHR and control animals. H. c-Fos expression (pink) in dHPC of a control animal (CaMKII-eYFP). I. c-Fos expression (pink) in an ArchT-expressing animal. There is no c-Fos expression underneath the laser (denoted between pink lines), but expression is elevated throughout dentate gyrus, CA3 and CA1 surrounding the area of silencing. J. c-Fos expression (pink) in dHPC of a NpHR-expressing animal. c-Fos is absent in the area underneath the laser (denoted between pink lines), but it not elevated outside of this zone. Data are represented as mean +/− SEM.

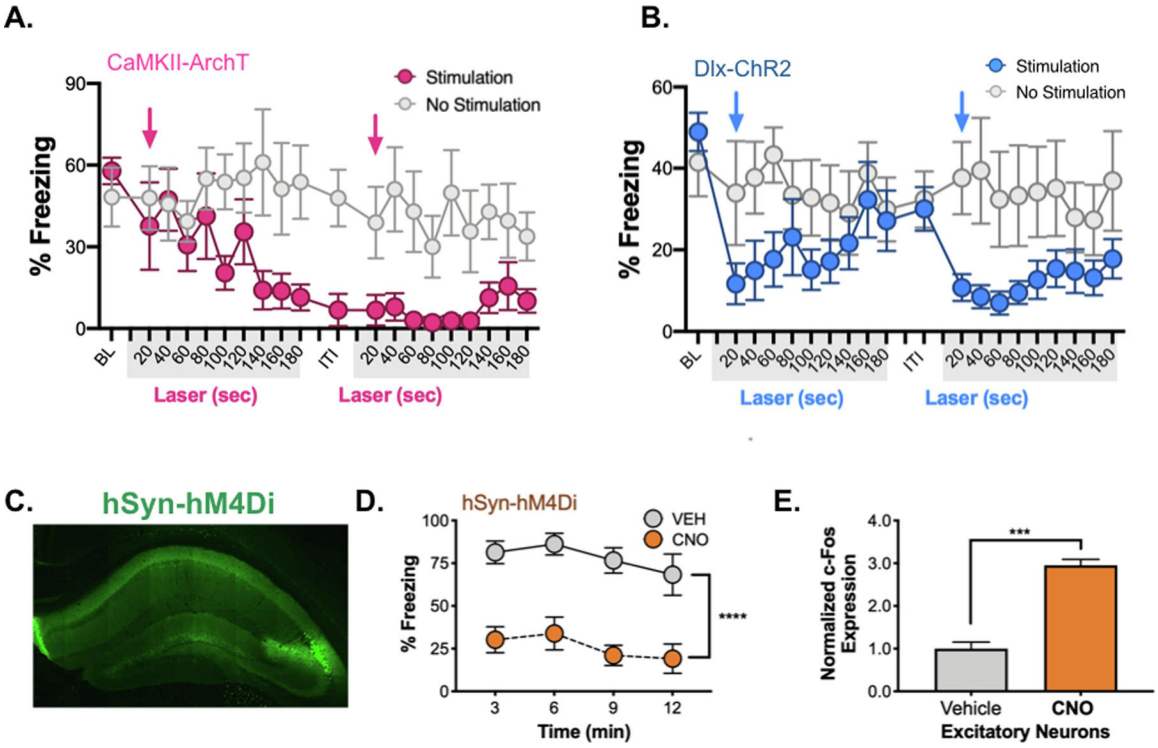

Figure 7. Inhibitory manipulations that increase excitation and impair memory.

A. 20 second bins of freezing activity in ArchT (blue) vs. control animals (gray) during laser ON periods. ~140s into laser stimulation, ArchT animals ceased freezing, this was not recovered over the remainder of the testing period. Blue arrows denote onset of laser stimulation. B. 20 second bins of freezing activity in Dlx-ChR2 laser-stimulated (blue) vs. control animals (gray) during laser ON periods. Dlx-ChR2 animals ceased freezing during laser onset (blue arrows), but freezing recovered during laser OFF periods. C. hSyn-hM4Di virus expression (green) in dHPC. D. During the 12-minute testing period, hSyn-hM4Di CNO-treated animals froze less than vehicle treated animals. E. c-Fos expression was increased in excitatory neurons of dCA1 in CNO-treated animals. Data are expressed as mean +/− SEM.

Table 1.
Manipulation of dHPC activity and the effects on memory retrieval.

Summary of the effects of acute vs. prolonged manipulations on memory retrieval and excitatory activity in the hippocampus.

		Activation of inhibitory neurons	
Virus	**Time course**	**Memory Retrieval**	**Excitatory activity**
Dlx-ChR2	acute	***impaired***	widespread decreases
Dlx-hM3Dq	prolonged	intact	widespread decreases
		Activation of excitatory neurons	
Virus	**Time course**	**Memory Retrieval**	**Excitatory activity**
CaMKII-ChR2	acute	***impaired***	widespread increases
CaMKII-hM3Dq	prolonged	***impaired***	widespread increases
		Inhibition of excitatory neurons	
Virus	**Time course**	**Memory Retrieval**	**Excitatory activity**
CaMKII-NpHR 3.0	acute	intact	moderate decreases
CaMKII ArchT	acute	***impaired***	*widespread increases**
CaMKII-hM4Di	prolonged	intact	moderate decreases
		Inhibition of excitatory and inhibitory neurons	
Virus	**Time course**	**Memory Retrieval**	**Excitatory activity**
Syn1-hM4Di	prolonged	***impaired***	*widespread increases**