

# Direct Reactivation of a Coherent Neocortical Memory of Context

Kiriana K. Cowansage,<sup>1</sup> Tristan Shuman,<sup>3</sup> Blythe C. Dillingham,<sup>1,2</sup> Allene Chang,<sup>1</sup> Peyman Golshani,<sup>3,4,5</sup> and Mark Mayford<sup>1,\*</sup>

<sup>1</sup>Department of Molecular and Cellular Neuroscience, Dorris Neuroscience Center, The Scripps Research Institute, 10550 North Torrey Pines Road, DNC 202, La Jolla, CA 92037, USA

<sup>2</sup>The Kellogg School of Science and Technology

<sup>3</sup>Department of Neurology, David Geffen School of Medicine, University of California, Los Angeles, 710 Westwood Plaza, Los Angeles, CA 90095, USA

<sup>4</sup>UCLA Integrative Center for Learning and Memory

<sup>5</sup>West Los Angeles VA Medical Center, 11301 Wilshire Boulevard, Los Angeles, CA 90073, USA

\*Correspondence: [mmayford@scripps.edu](mailto:mmayford@scripps.edu)

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## SUMMARY

Declarative memories are thought to be stored within anatomically distributed neuronal networks requiring the hippocampus; however, it is unclear how neocortical areas participate in memory at the time of encoding. Here, we use a *c-fos*-based genetic tagging system to selectively express the channelrhodopsin variant, ChEF, and optogenetically reactivate a specific neural ensemble in retrosplenial cortex (RSC) engaged by context fear conditioning. Artificial stimulation of RSC was sufficient to produce both context-specific behavior and downstream cellular activity commensurate with natural experience. Moreover, optogenetically but not contextually elicited responses were insensitive to hippocampal inactivation, suggesting that although the hippocampus is needed to coordinate activation by sensory cues, a higher-order cortical framework can independently subserve learned behavior, even shortly after learning.

## INTRODUCTION

The ability to form lasting associations is a critical evolutionary adaptation, yet relatively little is known about how these experiences are biologically represented within distributed neuroanatomical networks. Since the early studies of amnesic patient H.M., much of memory research has focused on the hippocampus, a subcortical structure known to be essential for the formation of explicit memories, which are generally accessible to conscious recall in humans (Squire, 1986). Although cortical networks are known to undergo activation and communicate with hippocampal circuits during both sensory experience and memory-related activity (Bontempi et al., 1999; Frankland et al., 2004, 2006; Goshen et al., 2011; Maviel et al., 2004; McClelland and Goddard, 1996; Smith et al., 2012; Tse et al., 2011; Wang and Morris, 2010), the consequences of their interactions for memory encoding and retrieval are unknown. To date, several theoretical views of systems-level memory consolidation have emerged

based on the observation that hippocampal damage in both humans and rats causes temporally graded retrograde amnesia, such that newer memories are lost while older memories are spared (Anagnostaras et al., 1999; Bayley et al., 2003; Bontempi et al., 1999; Reed and Squire, 1998). Although different in abstract principles, these ideas converge on a common premise that hippocampal networks provide an index, map, or unified representation of memory based on multiple cortically processed sensory features (Eichenbaum, 2000; Morris, 2006; Nadel and MacDonald, 1980; Nadel et al., 2000; O'Reilly and Rudy, 2001; Tse et al., 2007). According to prevailing models, cortical representations gradually acquire independence from the hippocampus over time (Teng and Squire, 1999; Zola-Morgan and Squire, 1990), either through synaptic strengthening via internal replay of patterned activity or by the formation of separate traces, incorporated into memory during retrieval to generate a flexible and anatomically distributed framework for long-term associations.

Several circuit-based mechanisms could account for the observed interactions between hippocampal and cortical systems: (1) learning-induced cellular activity or plasticity within the hippocampus, itself sufficient to store information and engage the cortex during retrieval; (2) concurrent encoding of task-relevant information in both the hippocampus and cortex, which synergistically retrieve memory within a coordinated network; or (3) direct acquisition of information in neocortical ensembles, with hippocampal neurons providing location-specific input to higher-order circuits during retrieval. Recently published work has reported that sparse neural ensembles are recruited during contextual fear conditioning (Garner et al., 2012) and when targeted in the dentate gyrus (DGs) can be directly stimulated to induce context-specific fear expression (Liu et al., 2012; Ramirez et al., 2013). Although this finding supports the view that learning-related patterns of cellular activity in the hippocampus can be used to artificially recapitulate memory retrieval, these studies do not address whether DG circuits serve as a unique cellular signature with an obligatory function in contextual memory. Here, we investigate if patterns of activity generated in higher-level cortical structures at the time of learning are sufficient to drive contextual memory recall. By optogenetically bypassing the hippocampus, we revealed a targetable circuit in retrosplenial

cortex (RSC), able to drive context-specific behavior less than 2 days after training.

We combined a *c-fos*-based genetic tagging system with optogenetic stimulation to functionally target and “tag” cells recruited during contextual fear conditioning. We focused on the RSC because this region has strong reciprocal connectivity with the hippocampus, entorhinal cortex, and numerous sensory and subcortical areas involved in emotional learning, making it a likely participant in hippocampal learning mechanisms. In addition, this area is essential for spatial cognition and memory, and has been shown to participate in the retrieval of both recent and remote contextual fear memory (Bucci and Macleod, 2007; Corcoran et al., 2011; Keene and Bucci, 2008a, 2008b, 2008c, 2009; Robinson et al., 2011, 2012). We found that stimulation of the neural ensembles activated in the RSC with contextual learning was sufficient to produce fear memory retrieval. Moreover, artificially induced retrieval activated downstream cell populations in the central and basal nuclei of the amygdala that were common to those activated by natural memory recall. Finally, we show that while natural contextual fear memory retrieval was impaired by hippocampal inactivation, optogenetically reactivated memory, through direct stimulation of RSC ensembles, could bypass this requirement, leading to expression of contextual fear even in the absence of hippocampal activity. Taken together, these results are consistent with the idea that a redundant encoding mechanism enables recent contextual memories to drive behavior through dissociable cortical and hippocampal pathways.

## RESULTS

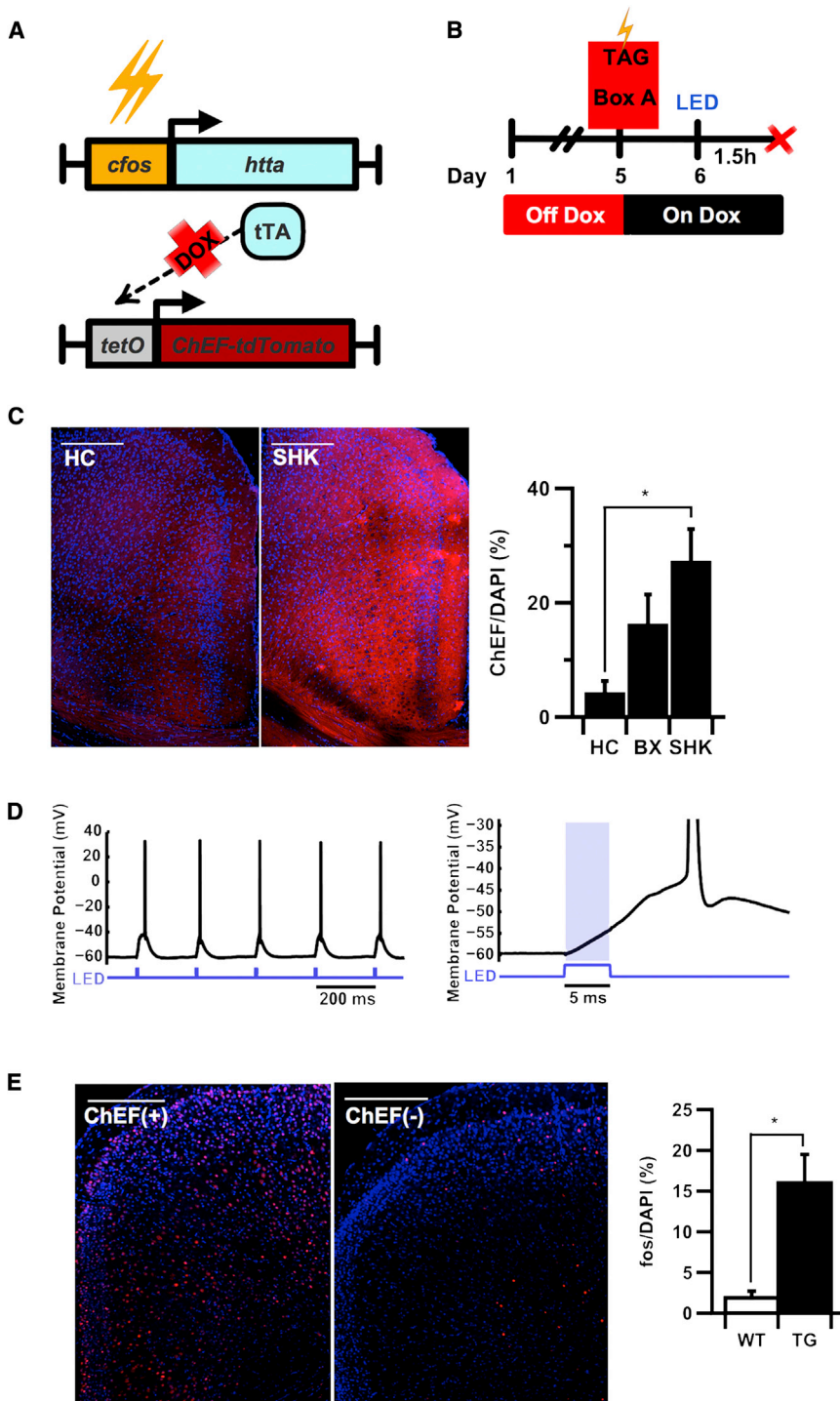
To test the idea that RSC might contribute to a cellular representation of contextual memory, we used the Tet Tag mouse line (Reijmers et al., 2007), which expresses the tetracycline transactivator (tTA) under control of the neural activity-responsive *c-fos* promoter. To generate the tet-off bitransgenic line used in this study, Tet Tag mice were crossed with a tetO-ChEF-tdTomato transgenic line, enabling us to selectively express the channelrhodopsin variant, ChEF (Lin et al., 2009), in active neurons, while also allowing us to use doxycycline (Dox) to globally restrict transgene expression to a defined temporal window. As shown in Figure 1A, mice were removed from Dox for 4 days to open a window for *c-fos*-mediated ChEF transgene expression (Figure 1B). Mice that received a series of footshocks (SHK) on the fifth day after Dox removal showed a significant increase in RSC levels of ChEF-tdTomato compared to home cage controls (HCs) (Figure 1C). We did not observe a significant difference in the total number of RSC cells tagged in box- compared to shock-tagged mice using either of two cell counting thresholds (Figure 1C and see Figure S1A online). These numbers are comparable to those previously reported for hippocampus (Matsuo et al., 2008) and are consistent with physiological data from RSC (Smith et al., 2012). Thus, context-driven activity in RSC appears to engage a cellular ensemble that either overlaps with context-shock-responsive cells or is unique but similar in size. Immunofluorescence confirmed that multiple cell types express *fos*-induced ChEF in RSC after natural learning-related activity; thus the relevant ensemble includes both excitatory and inhibitory contributions (Figures S1E and S1F). Stimulation (5 Hz) of ChEF(+) RSC

neurons with light (454 nm) was sufficient to trigger action potential firing in an acute slice preparation (Figure 1D), and significantly increase *c-fos* protein expression after in vivo delivery of light pulses through a cranial window (Figure 1E). Moreover, analysis of direct overlap between ChEF-expressing neurons tagged during training in Box A and retrieval-induced endogenous *c-fos* expression (Figure S2A) showed a significantly higher percentage of reactivated (*fos*-expressing) ChEF(+) cells after a 24 hr memory test in Box A, compared to mice exposed to novel Box B (Figure S2B). Similarly, 30%–50% percentage of ChEF+ cells colocalized with *c-fos* 90 min after LED stimulation (Figure S1C).

Expression of ChEF protein in transgenic (TG) mice did not affect normal contextual fear memory compared to wild-type (WT) controls (Figure 2A). To determine if RSC ensemble stimulation was sufficient to drive fear-related behavior (freezing), we fear conditioned mice off Dox with four 1 mA shocks (SHK) so as to genetically tag an active ensemble in the RSC of TG mice. Mice were then returned to high-dose Dox chow overnight to suppress further ChEF expression and the next day received either right unilateral or bilateral 5 Hz light stimulation of RSC in a neutral arena (light was delivered by an LED mounted to the skull). Results confirmed that neither unshocked transgenic box controls (TG/BX) nor shocked wild-type (WT/SHK) littermates froze significantly above baseline (Pre) during LED stimulation. In contrast, TG mice that were fear conditioned (SHK) froze significantly more than mice in either of the control conditions during LED stimulation, regardless of whether light was applied bilaterally or right unilaterally (Figure 2B; Table S1). In addition, mean LED-induced freezing among TG/SHK mice was significantly correlated with shock-induced freezing triggered in the first post-shock interval (a measure of initial learning; Figure 2C).

To confirm that the observed behavioral difference was specifically attributable to the expression of ChEF in RSC cells during training, rather than due to fibers of passage or background activity in home cage, we blocked *c-fos*-induced ChEF expression by microinfusion of either Dox or saline directly into RSC 2 hr prior to fear conditioning (Figures 2D and 2E). We reasoned that if a relevant pattern of ChEF was expressed in RSC during training, then acute blockade of learning-related expression would impair the efficacy of LED-induced recall the next day. Consistent with this hypothesis, we observed that animals receiving Dox froze significantly less than saline-infused mice during LED stimulation trials (Figure 2F). These results suggest that RSC participates in the initial phase of context fear memory formation.

Previous studies suggest that some place-responsive neurons in the hippocampus and RSC undergo context-specific shifts in their firing patterns after the location is paired with a salient emotional cue (Moita et al., 2003; Smith et al., 2012). Since our initial experiments involved tagging mice during an aversive experience and comparing responses from shocked mice to those from unshocked mice, our next question was whether RSC encodes a specific representation of the CS (context), the US (shock), or a novel representation of the conjunction of the two stimuli. We therefore tagged active neural ensembles with ChEF prior to conditioning in a neutral (Box A or Box B) and tested LED-induced freezing after subsequent training in Box A (Figure 3A). Thus, instead of tagging mice for 40 min in Box A during training, mice were tagged for 40 min in either Box A or Box B



**Figure 1. The ChEF Bitransgenic Mouse Enables Optogenetic Manipulation of Previously Active Neurons**

(A and B) (A) Schematic of the fos/tTA-tetO/ChEF-tdTomato bitransgenic system and (B) experimental protocol used to tag memory-related circuits in RSC.

(C) Behavioral induction of transgene expression in RSC measured 1 day after training off Dox. Footshock (SHK) induced significantly greater expression of ChEF-tdTomato protein than home cage (HC) (ANOVA,  $F_{(2,20)} = 3.907$ , main effect  $p = 0.037^*$ , post hoc Fisher LSD, FC  $\times$  HC,  $*p = 0.012$ ; BX  $\times$  HC,  $p = 0.188$ , n.s.). Images show ChEF-tdTomato expressed in RSC (red) 24 hr post-induction counterstained with DAPI (blue).

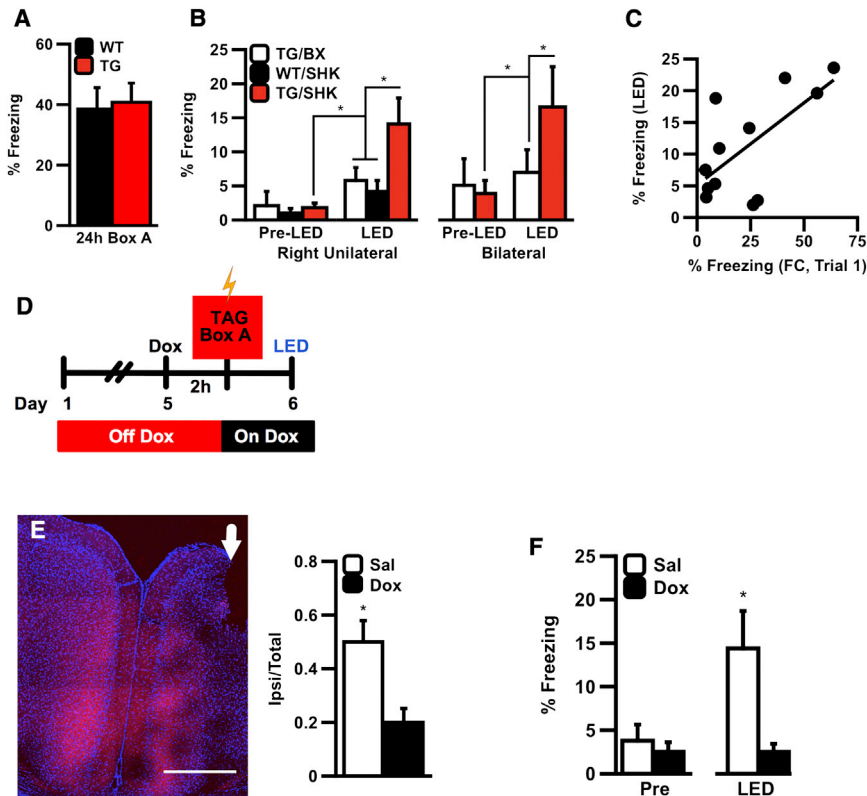
(D) Whole-cell recording of an RSC layer 2/3 pyramidal cell in an acute slice preparation ( $n = 7$  neurons in two mice). Light pulses (5 Hz) evoked large short latency depolarizations (latency  $< 1$  ms) in two regular spiking (RS) neurons (putative pyramidal cells) and reliably evoked action potentials when cells were held at  $-60$  mV (top). The time course of activation indicates direct optical activation of this cell by light (bottom).

(E) Transgenic mice receiving LED stimulation show significantly higher levels of *c-fos* protein 90 min after LED stimulation of RSC (t test,  $t_{[18.512]} = 3.936$ ,  $**p = 0.001$ ). Confocal images depict optical induction of *c-fos* protein (red) with DAPI counterstain (blue) in transgenic and wild-type brains perfused 90 s after LED stimulation. Error bars indicate SEM; scale bar, 250  $\mu$ M; asterisk indicates statistical significance at the level of  $p = 0.05$ ; double asterisk indicates statistical significance at the level of  $p = 0.001$ .

outcomes could be anticipated from this experiment: (1) only A/A mice freeze during LED stimulation, suggesting that the tagged Box A representation is sufficiently stable to be linked to the shock during subsequent conditioning in Box A; (2) both A/A and B/A mice express LED-induced freezing, suggesting that reactivation of any tagged network in RSC is sufficient to reactivate a shock association, suggesting encoding of the US shock component; or (3) neither A/A nor B/A mice express LED-induced freezing, suggesting that the Box A association with shock forms a unique representation that cannot be accessed by reactivating cells tagged in a neutral context.

the day prior to training. Following tagging, mice were returned to Dox to prevent further expression of ChEF, and all mice in both groups received training in Box A the next day, followed by LED stimulation of RSC. Thus, mice receiving the (A/A) sequence were shocked in the same box where contextual tagging (CS only) had occurred, while mice receiving the (B/A) sequence were shocked in a completely different context. Three possible

Analysis of the data from this experiment showed that, consistent with the idea of a context-specific tag, mice in the A/A group froze significantly more than mice in the B/A group (Figure 3B, left). Moreover, the level of LED-induced freezing in A/A mice was not significantly different from natural level of context fear memory observed in WT mice that were similarly pre-exposed to Box A or Box B, trained in Box A, and tested for natural LTM



**Figure 2. Optical Reactivation of a Recent Contextual Representation in RSC Was Sufficient to Induce Behavior**

(A) Natural long-term memory recall in wild-type (WT) and transgenic (TG) mice re-exposed to the fear conditioning chamber (Box A) 24 hr post-training.

(B) Transgenic/Shock (TG/SHK) mice froze significantly more than controls in response to right unilateral (left) and bilateral (right) stimulation of RSC. Pre-LED freezing did not differ between groups (ANOVA, pre-LED freezing,  $F_{(2,17)} = 0.145$ ;  $p = 0.866$ , n.s.), whereas LED-induced freezing was significantly increased only in TG/SHK mice during the LED test (ANOVA, main effect of LED test  $\times$  group  $F_{(2,17)} = 3.939$ , main effect,  $*p = 0.039$ ; test  $\times$  group interaction ( $F_{(2,19)} = 4.94$ ,  $0.02$ ); post hoc Fisher LSD, TG/SHK  $\times$  TG/BX,  $*p = 0.039$ ; TG/SHK  $\times$  WT/SHK,  $*p = 0.020$ ). Post hoc analysis of significant main effects obtained from comparisons of pre versus LED test (main effect,  $F_{(1,20)} = 23.62$ ,  $**p = 0.0002$ ) and group versus LED test revealed a significant interaction ( $F_{(2,19)} = 4.94$ ,  $*p = 0.020$ ) revealed that of the three groups, only TG/SHK mice froze significantly more during LED stimulation, as measured by a within-subject comparison (by group) of the calculated difference between pre-LED and LED freezing (see also Table S1 for all statistics).

(C) Across all uni- or bilaterally stimulated TG/SHK mice ( $n = 12$  total), LED-induced freezing was significantly correlated with trial 1 of learning ( $R = 0.665$ ,  $*p = 0.018$ ).

(D) To assess the anatomical and temporal specificity of the training-induced tag, mice received pretraining intra-RSC microinfusions of Dox (50  $\mu$ g, right side/white arrow).

(E) Microinfusion of Dox significantly reduced expression of ChEF-tdTomato ipsilateral to the site of infusion (t test,  $t_{[13]} = 2.815$ ,  $*p = 0.015$ ).

(F) Mice infused with Dox showed significantly reduced LED-triggered freezing (t test,  $t_{[8.34]} = 2.728$ ,  $p = 0.025$ ) than mice infused with saline. Error bars indicate SEM; scale bars, 1,000  $\mu$ m; asterisk indicates statistical significance at the level of  $p = 0.05$ .

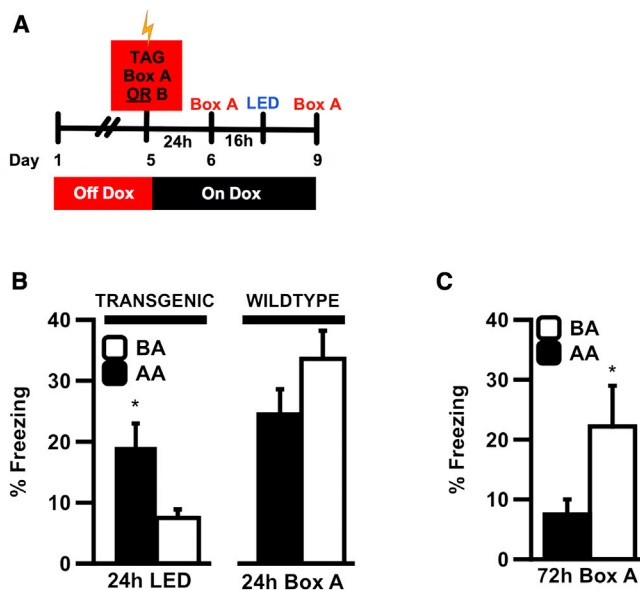
in Box A at the same time point posttraining (Figure 3B, right). These results suggest that even when tagging occurs prior to training, RSC retains a sufficiently stable context representation to permit reactivation of a fear memory formed later in the same context.

If LED stimulation of a ChEF-expressing neural ensemble is sufficient to directly reactivate memory of Box A, then LED-induced retrieval might modify properties of the original memory circuit, just as extinction training (box re-exposure without shock) alters activity in cells previously sensitive to fear-inducing stimuli. To determine if any such change in the original memory occurs, we completed the experiment by testing transgenic animals for natural memory of Box A 48 hr after the LED stimulation. Interestingly, A/A mice froze significantly less than B/A mice (Figure 3C), suggesting that by targeting artificial LED stimulation to cells tagged in Box A, we were able to induce a behavioral modification to the natural Box A association, possibly similar to extinction. In contrast, stimulation of cells tagged in Box B, which remained neutral and unrelated to training Box A, had significantly less effect on the expression of natural LTM in Box A.

Optogenetic activation of neural ensembles differs markedly from natural sensory-based recall in its lack of temporal patterning or synchronization to endogenous rhythms. Although it is difficult

to know the nature of the mouse's experience, our behavioral results imply that local stimulation of a relevant ensemble of RSC neurons is sufficient to produce a complex perceptual experience similar to natural recall. If natural and optogenetic stimulation are indeed similar in terms of their ability to drive behavior via common patterns of cellular activity, then downstream ensembles engaged by both optogenetic and natural recall should overlap, even in areas distant from RSC. To test this hypothesis, we used cellular compartment analysis of temporal activity by fluorescence in situ hybridization (catFISH) (Guzowski et al., 1999, 2006) to determine if bouts of activity triggered by natural recall and artificial RSC stimulation would recruit overlapping neurons in the amygdala, a brain region critical for the storage and regulation of aversive memories (Figure 4A). In addition, we measured mRNA expression in entorhinal, auditory, and piriform cortices.

Mice were context fear conditioned in Box A and the following day received either a 5 min retrieval test in Box A (Ret) or an equivalent novel box exposure (Novel). Twenty-five minutes later, all mice were given LED stimulation and were perfused for catFISH 5 min later. To differentiate cellular activation from the first (natural retrieval) versus the second (optogenetic) bouts of activity, tissue was colabeled with RNA probes directed against either the full-length (somatic) or intronic (nuclear foci)



**Figure 3. The RSC Representation Shows Context Specificity**

(A) Time course for experiments testing the contextual specificity of a tagged neuronal representation. Mice were tagged with exposure to Box A or B, were returned to Dox, and received fear conditioning in Box A 24 hr later.

(B) TG mice pre-exposed to Box A showed significantly more LED-induced freezing than mice pre-exposed to Box B (left) (ANOVA, main effect of group during LED ( $n = 8/6$ ,  $F_{(1,12)} = 5.053$ ,  $p = 0.044$ ) and did not significantly differ from WT mice tested for natural Box A memory at the same time point after training (right) (group  $\times$  genotype interaction,  $*p = 0.001$ ; post hoc  $t$  test for TG versus WT in AA,  $t_{[12]} = -0.955$ ,  $p = 0.358$ , n.s.).

(C) Twenty-four hours after stimulation (72 hr posttraining), transgenic mice in the A/A group froze significantly less than mice in the B/A group ( $t$  test,  $n = 8/6$ ,  $t_{[12]} = -2.246$ ,  $*p = 0.043$ ). Error bars indicate SEM; asterisk indicates statistical significance at the level of  $p = 0.05$ .

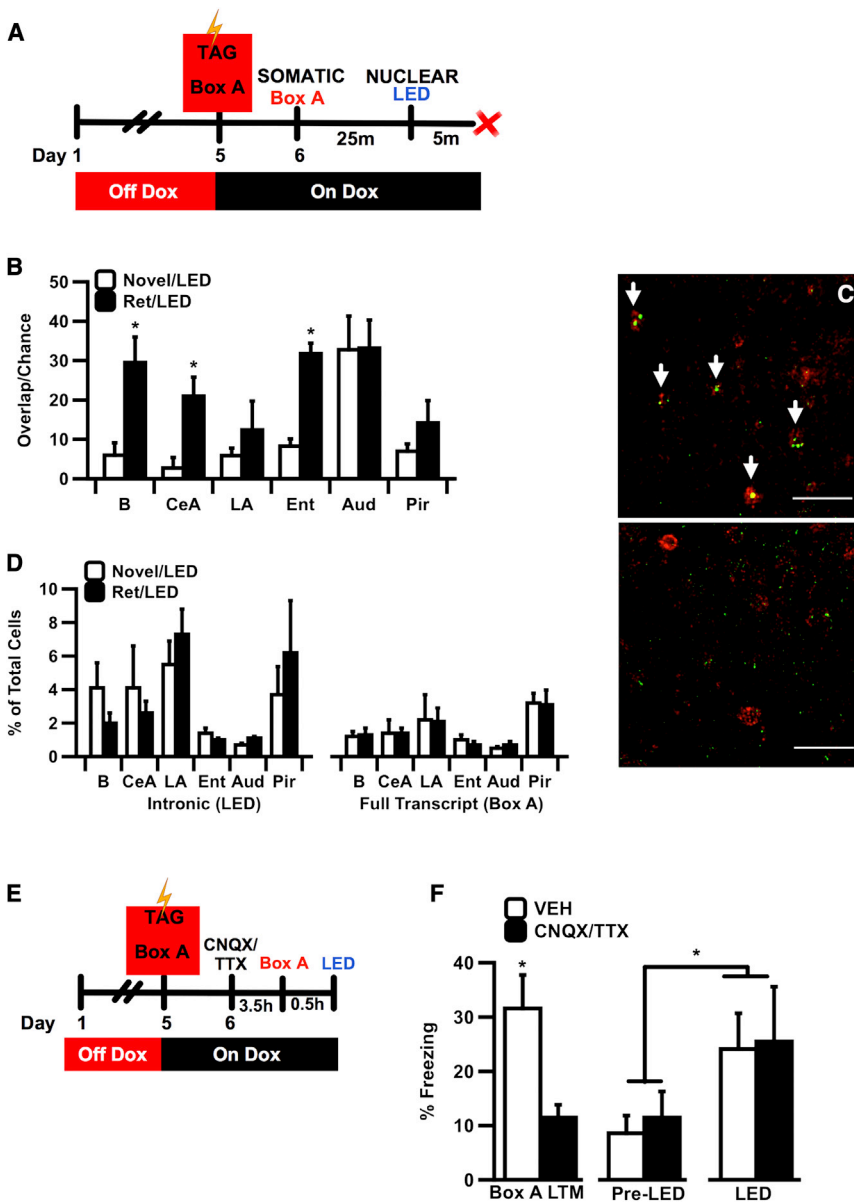
*c-fos* transcript, respectively. Cell counts obtained from confocal scans of hybridized sections revealed that the *c-fos* intronic and full-length mRNA transcripts induced by the sequential bouts of activity overlapped at significantly higher levels in basal amygdala (B), central amygdala (CeA), and entorhinal cortex (Ent) from Ret/LED mice compared to Novel/LED mice (Figures 4B and 4C). In contrast, overall levels of mRNA expression did not differ between groups in any region analyzed at either time point (Figure 4D). Statistical analysis of cells with somatic and/or nuclear mRNA localization revealed significant region-specific differences in the percent overlap of labeled cells compared to overlap expected by chance (Table S2) (Reijmers et al., 2007; Tayler et al., 2013). Labeled cells within amygdala subregions and Ent overlapped significantly above chance in reactivated (Ret/LED) mice, while cellular overlap did not differ from chance in nonreactivated controls (Novel/LED). Interestingly, labeled cells in piriform and auditory cortices overlapped significantly above chance in both experimental groups, perhaps reflecting patterned activity triggered by ambient background stimuli (olfactory or auditory) or other coordinated network activity within these primary sensory areas.

Previous studies have shown that contextual representations are formed in the DGs during learning; however, our data sug-

gest that this subcortical memory is mirrored by a higher-level representation that encodes meaningful context-specific information at the time of initial learning. Based on these findings, we asked if contextual representations in RSC are sufficient, in and of themselves, to drive reactivation of fear memory independent of a hippocampal contribution. Two groups of TG mice were first trained and tagged in Box A, as previously described. The next day, we pharmacologically inactivated the dorsal hippocampus (dHPC) by local microinfusion of the glutamate receptor antagonist, 6-cyano-7-nitroquinoline-2,3-dione (CNQX) and the sodium channel blocker, tetrodotoxin (TTX), which have previously been shown to impair context memory retrieval (Kitamura et al., 2009; Wiltgen et al., 2010). Mice were then tested for LTM in Box A, followed 30 min later by LED stimulation of RSC in a novel context. Consistent with the known involvement of the dHPC in memory retrieval (Amaral et al., 2007; Conejo et al., 2013; Holt and Maren, 1999; Lorenzini et al., 1996; Moser and Moser, 1998; Riedel et al., 1999), CNQX/TTX-infused mice expressed significantly attenuated memory during the natural memory test in Box A (Figure 4F) and expressed a significant reduction in *c-fos* expression in CA1 (Figure S3). However, both drug and vehicle groups froze at equivalent levels during direct RSC stimulation-induced recall (Figure 4F). This result indicates that the pattern of neural activity in the RSC at the time of learning encodes a coherent and independent representation of the context that can drive behavior when stimulated directly but requires hippocampal activity during natural recall.

## DISCUSSION

The role of the hippocampus in declarative memory has been studied extensively; however, its cellular contribution to memory and its involvement in higher-level cortical processing remain matters of controversy in systems neuroscience. Nevertheless, the presence of a cooperative hippocampal-cortical dynamic is widely acknowledged as essential for the formation of conjunctive representations, where individual sensory elements are bound to a global contextual identity that can be uniquely associated with aversive or rewarding events, like those occurring in Pavlovian fear conditioning (Rudy and O'Reilly, 2001). Despite the common view of cortical circuits as primarily important for late-stage consolidation, some intriguing evidence suggests that these ensembles are recruited into the memory circuit within hours of acquisition (Corcoran et al., 2011; Lesburguères et al., 2011). Our findings go further, to suggest that unitary cellular representations of context, which have often been assumed to exist within hippocampal circuits, are concurrently established in RSC at the time of learning or shortly thereafter. Moreover, the finding that direct RSC stimulation is sufficient to drive context-specific responding in the absence of hippocampal engagement suggests that RSC instantiates a coherent cellular framework for memory that can drive behaviorally relevant activity as an independent memory network. Interestingly, the well-documented finding that dorsal hippocampal silencing impairs natural context-elicited behavior remains consistent with the view that, short of direct top-down stimulation, hippocampal processing of ongoing spatial cues is needed to recruit the appropriate cortical circuits.



**Figure 4. Reactivation of the Tagged RSC Representation Engaged the Same Neurons as Natural Recall and Drove Fear Expression Independent of the Hippocampus**

(A) Time course for behaviorally induced synthesis of full-length (somatic) and intronic (nuclear) *c-fos* mRNA transcripts after sequential bouts of activity triggered by natural and optogenetic recall events, respectively.

(B) Compared to animals that were exposed to a novel box prior to LED stimulation (novel/LED,  $n = 3$ ), animals that received a retrieval test in Box A (Ret/LED,  $n = 5$ ) showed significantly higher coexpression (overlap observed/chance) of somatic and nuclear mRNA in basal amygdala (B) (t test,  $t[6] = -2.637$ ;  $*p = 0.039$ ), central amygdala (CeA) ( $t[6] = -2.716$ ;  $*p = 0.035$ ) and entorhinal cortex (Ent) ( $t[4] = -6.272$ ;  $*p = 0.003$ ), whereas no significant group differences were observed in lateral amygdala (LA) ( $t[6] = -0.666$ ,  $p = 0.53$ ), auditory cortex (Aud) ( $t[6] = -0.035$ ,  $p = 0.973$ ), or piriform cortex (layer 2) (Pir) ( $t[6] = 0.949$ ,  $p = 0.379$ ). Extended statistical analysis in Table S2 shows ANOVA and post hoc Fisher LSD test results comparing observed % overlap with chance for each brain region and experimental group. Chance overlap was calculated as  $P_{(intronic)} \times P_{(full\ length)}$ .

(C) Confocal image from basal amygdala tissue in a RET-LED individual. White arrows indicate examples of cellular overlap of somatic (red) with intronic (green) *c-fos* mRNA transcripts in RET mice (top) compared to NR mice (bot), which showed significantly less transcript overlap; scale bar, 50  $\mu$ M.

(D) Total expression of mRNA did not significantly differ between the RET and NR groups in any brain region analyzed.

(E) Schematic of behavioral paradigm used for hippocampal inactivation.

(F) Mice infused with CNQX/TTX expressed significantly less freezing than Veh when tested for natural memory in Box A (ANOVA,  $F_{(1,9)} = 10.414$ ,  $*p = 0.01$ ); however, mice receiving dHPC microinfusions of CNQX/TTX and vehicle froze significantly above baseline (pre-LED) levels during LED stimulation of RSC (repeated-measures ANOVA,  $n = 5/6$ ,  $F_{(1,9)} = 14.925$ ,  $*p = 0.004$ ) with no significant difference between groups. Post hoc comparison of Pre-LED versus LED-induced freezing for each group revealed significantly increased freezing during LED stimulation in both the Veh and Drug treatment conditions (Fisher LSD,  $p = 0.022$ , Veh;  $p = 0.024$ , CNQX/TTX).

B, basal amygdala; CeA, central amygdala; LA, lateral amygdala; Ent, entorhinal cortex; Aud, primary auditory cortex; Pir, layer 2 piriform cortex. dHPC, dorsal hippocampus. Error bars indicate SEM; asterisk indicates statistical significance at the level of  $p = 0.05$ .

Recent reports of contextual recall by optogenetic stimulation of hippocampal ensembles have focused attention on hippocampal networks as the primary cellular site for memory storage. Our results, however, suggest that complex contextual representations may not be exclusive to hippocampal ensembles. Instead, a representation of contextually relevant information memory also appears to occur within higher-level RSC circuits at the time of initial learning. Our data are therefore consistent with the theory of a hippocampal map or index for associations that may be stored redundantly in cortical systems (Moscovitch et al., 2005; Nadel, 1992; O'Keefe, 1990; O'Keefe and Black,

1977). Our findings diverge from these ideas, however, in demonstrating that memory-related information can be directly accessed via anatomically localized cortical stimulation, suggesting that although the hippocampus may participate in retrieval under natural circumstances, it can also be uncoupled from the memory recall process if cortical networks are directly engaged.

Compared to the hippocampus, much less research has focused on the involvement of RSC in memory; yet a surprisingly consistent and compelling body of literature in both humans and rodents has emerged, demonstrating critical functions of RSC in

the ability to retrieve episodic memories and emotional associations (Aggleton, 2010; Katche et al., 2013b; Keene and Bucci, 2008a, 2008c), to integrate relationships among distinct sensory cues (Bucci and Macleod, 2007; Keene and Bucci, 2008b; Smith et al., 2012), to navigate (Auger and Maguire, 2013; Clark et al., 2010; Cooper and Mizumori, 2001), and to identify familiar or meaningful places in space (Auger et al., 2012; Cho and Sharp, 2001; Smith et al., 2012). Moreover, the large surface area of this structure; its densely interconnected organization as a site of anatomical convergence among sensory, limbic, and spatial systems (Shibata et al., 2009); and its known physiological interactions with the medial temporal lobe (Albasser et al., 2007; Cooper and Mizumori, 2001; Dumont et al., 2010; Robinson et al., 2012; Sherrill et al., 2013; Smith et al., 2012) make RSC uniquely suited to host a cellular record of contextual experience. Given the striking level of intracortical connectivity observed in RSC across multiple sensory modalities, this area could even be theorized to serve as an index for contextual memory itself.

Despite major technological advances in optogenetic tools, studies using animal models to study brain circuit function are limited by their reliance on primitive behavioral readouts that do not provide true measures of subjective perceptual experience. As a result, experiments using artificial stimulation to drive behavior often rely on the untested assumption that if natural and artificial patterns of circuit activity produce the same behavioral output, they must do so by a common neural mechanism. How can we confirm that artificially induced freezing triggers patterns of brain activity that accurately recapitulate the stimulus-driven process of memory recall? The current study begins to address this question by examining patterns of overlapping cellular activity in brain areas downstream of the RSC after individual mice were subjected to both optogenetic and natural recall. These data revealed a high degree of overlap, in both amygdala and entorhinal cortex, among cells activated by the optogenetic tag and those activated by the fear-inducing context itself. This observation suggests that, at least at the level of neural circuits, optogenetic stimulation of specific RSC circuits can be validly related to the natural physiological process of memory retrieval. Future studies taking advantage of the CatFISH approach in conjunction with artificial stimulation protocols may provide deeper insights into how best to interpret optogenetically generated behaviors.

Taken together, these data provide evidence that recently formed cortical representations of memory can coherently drive learned behaviors that mimic stimulus-driven retrieval at the level of single cells. In addition, artificial activation of the RSC trace was sufficient to independently initiate an otherwise hippocampus-dependent response, thereby unmasking the presence of a redundant pathway for new memory encoding and maintenance. Moreover, these findings support the idea that information accessed through hippocampal networks is neither exclusive nor unique to that locus, and that cortical circuits are concurrently recruited to establish a stable record of experience (Goshen et al., 2011; Katche et al., 2013a; Smith et al., 2012). Future investigations will be necessary to determine how other cortical connections involving prefrontal, anterior cingulate, and entorhinal cortex also participate in storing and conveying relevant contextual information to subcortical structures like the amygdala.

Finally, these results lend credence to the long-standing yet previously untested assumption that targeted artificial stimulation of relevant pathways can generate behavior through patterns of brain activity that directly converge with those triggered by natural stimuli.

Our data and previous studies in the DG (Liu et al., 2012; Ramirez et al., 2013) demonstrate that the *c-fos*-based tagging system can be used to artificially induce contextual memory recall. This is surprising, given the artificial nature of the activity produced with ChEF, as well as the fact that the genetic tagging system is likely to label a variety of neurons not specific to the context (e.g., persistently active cells, cells associated with home cage, handling, room noise, etc.). Future studies will be needed to determine exactly how many cells are required for retrieval and how much noise is tolerated by given network. The fact that RSC stimulation is sufficient to drive a meaningful and resolvable network signal, despite potential interference from ongoing sensory input, internally generated ensembles, and spontaneous firing, is consistent with the observation that emotional associations are readily excitable and disruption resistant. These data also suggest that memory reactivation is less contingent on precise temporal and spatial firing patterns than previously thought.

## EXPERIMENTAL PROCEDURES

### Subjects

Double transgenic *c-fos*-tTA/tetO-ChEF mice were generated by crossing single transgenic *c-fos*-tTA (Reijmers et al., 2007) and tetO-ChEF-tdTomato mice bred on a C57BL/6J background. Mice used in behavioral experiments were 8–16 weeks old and were group housed on a 12 hr/12 hr light/dark cycle with food and water ad libitum. Control experiments involving wild-type mice were conducted using single transgenic littermates of experimental subjects. All procedures were conducted in compliance with The Scripps Research Institute and National Institutes of Health guidelines for humane care and use of animals.

### LED Attachment

For bilateral stimulation, mice were anesthetized with an oxygen/isoflurane mix and the skull was thinned to create a 1 mm<sup>2</sup> translucent window above RSC (anterioposterior –1.58 mm; mediolateral, ±0.5 mm at window center), extending 0.5 mm into each hemisphere from the midsagittal suture). Area of light penetration was limited by surrounding entire skull surface except for thinned skull window with a layer of opaque black lacquer. Waterproof/heatproof silicon-encased SMD 5050 trichip ultrabright LEDs (oznium.com) were purchased prewired with resistors and affixed with clear superglue over skull window. Light intensity at brain surface ranged from 3.5 to 4.1 mWmm<sup>-2</sup> (mean = 3.8 mWmm<sup>-2</sup>), obtained by pulsing light at 5 Hz through a 1 mm<sup>2</sup> thinned skull window (removed postmortem from an experimental animal) and placed over a light sensor (ThorLabs, S302C) connected to a power meter (ThorLabs, PM100USB). Estimated light spread is ≤250 μm from surface, based on previously published estimates (Huber et al., 2008).

### Drug Infusions

For local infusions of Dox (50 μg dissolved in 0.5 μL 0.9% saline) into RSC, drug or vehicle was unilaterally infused below the center of the LED (anterioposterior –1.5 mm; mediolateral, ±0.5 mm; dorsoventral, 0.5 mm) (Paxinos and Franklin, 2001). Infusions took place over 10 min at a rate of 0.2 μL/minute. For intrahippocampal infusions, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 mM, Wako) and tetrodotoxin (TTX, 3 mM, Tocris) were dissolved 0.9% saline and bilaterally microinfused into the dHPC at a rate of 0.2 μL/min (0.5 μL per side, anteroposterior –2.0 mm; mediolateral, ±1.7 mm; dorsoventral, 1.5 mm; Paxinos and Franklin, 2001).

### Behavior/Optical Stimulation

Four days after removal from low-dose Dox chow (40 mg/kg), mice were context fear conditioned (Med Associates). Training occurred over 40 min and began with a 4 min period of chamber acclimation, followed by four 1 s footshocks (1 mA, mean ITI = 100 s). After training (15–20 hr), mice received LED stimulation (5 ms pulses, 5 Hz) in an open-field arena. Stimulation was preceded by 6 min of context acclimation, followed by three 1 min trials of light pulses, each separated by a 1 min period of light off. After stimulation, mice were returned to their home cages and perfused for immunofluorescence 90 min later. For A/A-B/A experiments, mice were tagged during a single 40 min exposure to Box A (fear conditioning chamber) or Box B (novel arena B). Mice were trained (on Dox) in Box A 16 hr later (4 × 1 s 1 mA footshocks, 525 s total session) and received LED stimulation in Box C (novel arena C) 24 hr posttraining.

### Immunofluorescence

Following completion of all behavioral procedures, mice were deeply anesthetized with isoflurane and transcardially perfused with 4% paraformaldehyde. Brains were postfixed in PFA overnight and were sectioned by vibratome at 50  $\mu\text{M}$ . Sections were blocked in 10% normal goat serum/0.2% Triton X and were probed with an anti-cFos IgG (Chemicon, 1:750) followed by an anti-rabbit Alexa 488 or Alexa 647 fluorescent dye-conjugated secondary antibody (Invitrogen, 1:700). Sections were mounted, counterstained with DAPI, and coverslipped before imaging (Invitrogen, Slowfade with DAPI).

### In Situ Hybridization

Brains were perfused with 4% paraformaldehyde, and 40  $\mu\text{M}$  free-floating sections were incubated in formamide hybridization buffer (KPL) with Digoxigenin (DIG)- and fluorescein isothiocyanate (FITC)-labeled RNA probes targeting the *c-fos* first intron and full-length mRNA transcripts. Primer sequences for the intronic probe were 5'-CTTTGTGTAGCCGCCAGGTC-3' (forward) and 5'-AA AAAGAGGAAAGCGGAGGTGAGC-3' (reverse), and primers for the full-length probe were 5'-GGCTCTCTGTCAACACACA-3' (forward) and 5'-TAAGTA GTGCAGCCCGGAGT-3' (reverse). Signals were amplified using Cy5- and FITC-tyramide (PerkinElmer) and visualized using Cy5- and FITC-conjugated antibodies (Roche). Sections were mounted using SlowFade Gold Antifade Reagent with 4',6-diamidino-2-phenylindole (DAPI) as a general nuclear counterstain (Invitrogen).

### Microscopy and Cell Counting

Sections (50  $\mu\text{M}$ ) were imaged at 20× magnification using an A1 Nikon Confocal microscope. Whole coronal stitched images were acquired as single-plane optical sections at (6.5  $\mu\text{M}$ ) at a scale of 0.62 pixels/ $\mu\text{M}$ . All imaging was done using standardized laser settings held constant for samples from the same experimental data set. Quantifications derive from cell counts averaged across mice, where the value for each subject represents the mean of 1–2 single-plane ROIs (left and right hemisphere). The z plane for each section was adjusted to the level where DAPI emission was highest. ROIs were determined blind to experimental group using the DAPI channel and published anatomical guidelines (Paxinos and Franklin, 2001). Two different methods were used to confirm estimates ChEF-tdTomato cell quantifications in RSC (see Supplemental Experimental Procedures). For *c-fos* nuclear counts, an ImageJ macro was used to apply a standard MIT to images in a data set. An ImageJ macro was applied to count nuclei above this threshold that overlapped with in-plane DAPI+ nuclei. For counts of overlap between ChEF and *c-fos* (see representative example of colocalization in Figure S1) (Taylor et al., 2013), only those cells identified as positive for both ChEF and *c-fos* were counted as “overlapping” % overlap by chance was calculated by  $[\text{ChEF/DAPI} \times \text{fos/DAPI} \times 100]$ , % overlap was calculated by  $[\text{overlap/DAPI} \times 100]$ , and % overlap was normalized to % chance overlap by  $[\text{overlap/chance} \times 100]$ . Calculation of % ChEF+ cells reactivated (i.e., % of ChEF+ cells that coexpress *c-fos*) was obtained by  $[\text{overlap/total ChEF} \times 100]$ .

### Acute Brain Slice Preparation

Mice were anesthetized with isoflurane and the brain quickly removed and placed into a sucrose-based cutting solution (in mM): sucrose 222, D-glucose 11, NaHCO<sub>3</sub> 26, NaH<sub>2</sub>PO<sub>4</sub> 1, KCl 3, MgCl<sub>2</sub> 7, CaCl<sub>2</sub> 0.5, aerated with 95% O<sub>2</sub>,

5% CO<sub>2</sub>. Coronal slices (300  $\mu\text{M}$ ) containing RSC were cut with a Leica VT1000S Vibratome. Slices were allowed to recover at 37°C for 30 min, and then at room temperature in ACSF (in mM): NaCl 124, KCl 2.5, NaHCO<sub>3</sub> 26, NaH<sub>2</sub>PO<sub>4</sub> 1.25, D-glucose 10, sucrose 4, CaCl<sub>2</sub> 2.5, MgCl<sub>2</sub> 2, aerated with 95% O<sub>2</sub>, 5% CO<sub>2</sub>.

### Electrophysiology/Optical Stimulation

Cells were visualized using an upright Olympus BX51 microscope with a 40× water-immersion lens using epifluorescence and DIC illumination. Whole-cell recordings of fluorescent cells were made in current clamp mode or voltage clamp mode using a Multiclamp (Molecular Devices) patch-clamp amplifier under visual guidance using DIC optics and epifluorescence with an Olympus BX51 microscope equipped with a custom made analog-controlled LED illumination system. Recordings were performed at 31°C–33°C. The internal solution contained (in mM) KGluc 115, KCl 20, HEPES 10, phosphocreatine 10, ATP-Mg<sup>2+</sup> 4, GTP-Na<sup>+</sup> 0.3. Micropipettes were 3–5 MOhms in resistance. Series resistance was below 30 MOhms and fully compensated in current clamp mode. Electrophysiological signals were low-pass-filtered at 4 kHz and digitized at 10 kHz using NIDAQ boards controlled by Strathclyde WinWCP software. After a whole-cell recording was established, pulsed blue light stimuli (470 nm) were delivered through the epifluorescence pathway of the microscope using a custom-made LED light array driven by analog pulses delivered through the NIDAQ card. The illumination intensity was measured through the objective as 1–7 mWmm<sup>-2</sup>.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, two tables, and Supplemental Experimental Procedures and can be found with this article at <http://dx.doi.org/10.1016/j.neuron.2014.09.022>.

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