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Neurons Are Recruited to a Memory Trace Based on Relative Neuronal Excitability Immediately before Training

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SUMMARY

Memories are thought to be sparsely encoded in neuronal networks, but little is known about why a given neuron is recruited or allocated to a particular memory trace. Previous research shows that in the lateral amygdala (LA), neurons with increased CREB are selectively recruited to a fear memory trace. CREB is a ubiquitous transcription factor implicated in many cellular processes. Which process mediates neuronal memory allocation? One hypothesis is that CREB increases neuronal excitability to bias neuronal recruitment, although this has not been shown experimentally. Here we use several methods to increase neuronal excitability and show this both biases recruitment into the memory trace and enhances memory formation. Moreover, artificial activation of these neurons alone is a sufficient retrieval cue for fear memory expression, showing that these neurons are critical components of the memory trace. These results indicate that neuronal memory allocation is based on relative neuronal excitability immediately before training.

INTRODUCTION

Although different brain regions may specialize in storing different types of memories, computational and observational findings suggest that only a small portion of neurons within a given region is necessary to encode any particular memory (Guzowski et al., 1999; Kanerva, 1988; Reijmers et al., 2007; Rolls and Treves, 1990; Wilson and McNaughton, 1993). For instance, it is generally agreed that the amygdala, in particular the lateral nucleus of the amygdala (LA), is important for auditory fear (Davis, 1992; Duvarci and Pare, 2014; Fanselow and Gale, 2003; Maren, 2003) or threat (LeDoux, 2014) conditioning, in which a tone is paired with an aversive shock. However, while over 70% of pyramidal/principal neurons in the rodent LA respond to both tone and shock presentation (suggesting these neurons are "correctly wired" and therefore eligible to become part of the memory trace), only a small portion (~10%–30%) of these eligible neurons seem to be recruited into any one fear memory trace (Han et al., 2007; Reijmers et al., 2007; Repa et al., 2001; Rumpel et al., 2005). Similarly, there is evidence for a stable sparse fear memory trace in the human amygdala (Bach et al., 2011). Here we examine the mechanisms that help determine which particular neurons are selected or allocated (Zhou et al., 2009) to a sparsely encoded fear memory trace in the mouse LA.

Previously, we and others showed that LA neurons compete against one another for allocation to a fear memory trace, and furthermore that it was possible to bias the outcome of this competition by manipulating CREB (cAMP/Ca^{2+} *r*esponsive *e*lement *b*inding protein) function in individual LA neurons. Neurons with relatively higher CREB function were more likely to be included, whereas neurons with relatively lower CREB function were more likely to be excluded from the memory trace (Han et al., 2007, 2009; Zhou et al., 2009). Importantly, the overall size of the LA memory trace remained stable despite these various CREB manipulations, and indeed did not vary with the strength of the fear memory. This suggests that there is a limit or constraint on the overall size of the LA fear memory trace. Interestingly, decreasing CREB function in a small population of random LA neurons did not disrupt memory formation. This result is likely because the small population of neurons with decreased CREB function was excluded from the memory trace (which was composed of nonmanipulated neurons). In contrast, increasing CREB function in a similar small portion of LA neurons was sufficient to enhance memory formation (Han et al., 2007). Together, these results suggest that neuronal competition is important for neuronal allocation and memory formation, and that neurons with relatively higher CREB are

competitively advantaged (and therefore, more likely to "win" this competition).

CREB is a ubiquitous transcription factor implicated in many diverse cellular processes, including proliferation, survival, apoptosis, differentiation, metabolism, glucose homeostasis, and neuronal excitability (Lonze and Ginty, 2002). For instance, CREB bidirectionally modulates neuronal excitability (increasing CREB function increases the propensity of neurons to fire action potentials, while decreasing CREB function decreases neuronal excitability) (Dong et al., 2006; Viosca et al., 2009; Zhou et al., 2009). Which of these CREB-mediated processes is important for neuronal allocation during memory formation? One plausible mechanism is that neurons with high levels of CREB are preferentially recruited to a memory trace because these neurons are more excitable than their neighbors (a postsynaptic neuron that is more excitable than its neighbor would be "primed" for allocation into a given memory trace). Although an increase in excitability has been proposed as a mechanism mediating neuronal allocation during memory formation (Kim et al., 2013; Zhou et al., 2009), this idea has not been directly tested experimentally. Here we used three different methods to determine whether relatively higher excitability before training confers a competitive advantage for neuronal allocation to a fear memory.

RESULTS

HSV Microinjected into the LA Preferentially Infects Excitatory Pyramidal/Principal Neurons

To manipulate excitability in a small, arbitrarily chosen subpopulation of LA neurons, we used replication-defective herpes simplex viral (HSV) vectors. To phenotype the type of LA cells infected by HSV, we microinjected mice with HSV expressing GFP (to allow for easy visualization of infected cells) into the LA and performed immunohistochemistry for different cell markers (NeuN, neuronal nuclei, as a marker of neurons; αCaMKII, alpha Ca^{2+}/calmodulin-dependent protein kinase II, as a marker of excitatory pyramidal/principal neurons; GFAP, glial fibrillary acidic protein, as a marker of astrocytes; and GAD67, glutamate decarboxylase 67, as a marker of inhibitory neurons). We observed complete overlap between LA cells infected by HSV (GFP^+) and cells expressing a neuronal marker ($NeuN^+$), but no overlap between infected cells and cells expressing GFAP, confirming that HSV is neurotropic (Cole et al., 2012; Fink et al., 1996). Moreover, HSV predominantly (~98%) infected pyramidal/principal excitatory ($\alpha CaMKII^+$) neurons in the LA, with only a very small number (<2%) of infected neurons costaining with the inhibitory neuronal marker GAD67 (Figures 1A and 1B). Therefore, consistent with previous reports, we found that following microinjection into the LA, HSV preferentially infects pyramidal/principal neurons (Cole et al., 2012). Because of this tropism, we used HSV to manipulate excitability in a portion of pyramidal/principal neurons in the LA.

Manipulating Neuronal Excitability Using Voltage-Dependent K^+ Channels

Neuronal excitability is determined by the composition, distribution, and properties of ion channels (e.g., Na^+, K^+, and Ca^{2+}) in the plasma membrane. Increasing CREB function increases neuronal excitability, in part, by decreasing voltage-gated K^+ currents (Dong et al., 2006; Lopez de Armentia et al., 2006; Viosca et al., 2009; Zhou et al., 2009). This, in turn, inhibits the postburst afterhyperpolarization (AHP, a hyperpolarizing current which mediates the "undershoot phase" or refractory period following an action potential). Two members of the voltage-dependent K^+ family of channels, KCNQ2 and related KCNQ3, help mediate the AHP and function as molecular brakes on neuron firing (Delmas and Brown, 2005; Gu et al., 2005). Expression of a dominant-negative KCNQ2 mutant (hQ2-G279S; dnKCNQ2) coassembles with native KCNQ2/3 subunits, disrupts their function, and thereby increases neuronal excitability (Peters et al., 2005; Schroeder et al., 1998; Wuttke et al., 2007). We observed that LA neurons in adult mice endogenously express KCNQ2-containing channels (Figure 1C), suggesting that expression of the dnKCNQ2 construct could be a viable method for increasing excitability in LA neurons. To test this, we first transfected primary hippocampal neurons with the dnKCNQ2 construct. The dnKCNQ2 construct was also observed near the axon initial segment (Figure 1D), consistent with the notion that dnKCNQ2 coassembles with, and blocks the function of, endogenous KCNQ2/3 channels. Therefore, as our first method to increase excitability without directly manipulating CREB function, we used HSV vectors to express dnKCNQ2. To decrease excitability without directly decreasing CREB function, we used HSV to express Kir2.1, an inwardly rectifying K^+ channel, which reduces neuronal input resistance and decreases evoked action potential firing (Dong et al., 2006).

To verify that expression of our excitability constructs indeed manipulated neuronal excitability, we assessed excitability (as measured by firing rate) of cultured hippocampal neurons transfected with GFP, CREB, dnKCNQ2, Kir2.1, and CREB+Kir2.1 constructs. Consistent with previous reports, we found that expression of dnKCNQ2 (Peters et al., 2005) or CREB (Dong et al., 2006; Han et al., 2006; Lopez de Armentia et al., 2006; Viosca et al., 2009; Zhou et al., 2009) increased excitability, while Kir2.1 decreased (Dong et al., 2006) excitability. Moreover, the CREB-induced increase in excitability was blocked by coexpression with Kir2.1 in the same neurons (Figure 2A; one-way ANOVA on firing rate of transfected/not-transfected neurons with between-group factor Transgene [GFP, CREB, dnKCNQ2, Kir2.1, and CREB+Kir2.1], $F_{4,28} = 7.18$, $p < 0.001$, posthoc Newman-Keuls tests showed that dnKCNQ2 and CREB increased excitability over GFP, whereas CREB+Kir2.1 condition was not different from GFP). To confirm that HSV-CREB and HSV-dnKCNQ2 increased excitability in infected LA neurons, we also assessed excitability in ex vivo LA slices following microinjection of HSV expressing GFP, CREB, or dnKCNQ2 into mice. LA neurons overexpressing dnKCNQ2 or CREB showed higher firing rates than LA neurons expressing GFP (Figure 2B; one-way ANOVA conducted on firing rate of infected/not-infected neurons with between-group factor Vector (GFP, CREB, and dnKCNQ2), $F_{2,21} = 4.15$, $p < 0.05$, LSD posthoc tests showed that both neurons with dnKCNQ2 or CREB fired more action potentials than neurons expressing GFP and did not differ from each other). Therefore, although we did not directly determine excitability following our manipulations in vivo, results from cell culture and ex vivo LA neuron slices confirm previous reports that dnKCNQ2 and CREB enhance excitability.

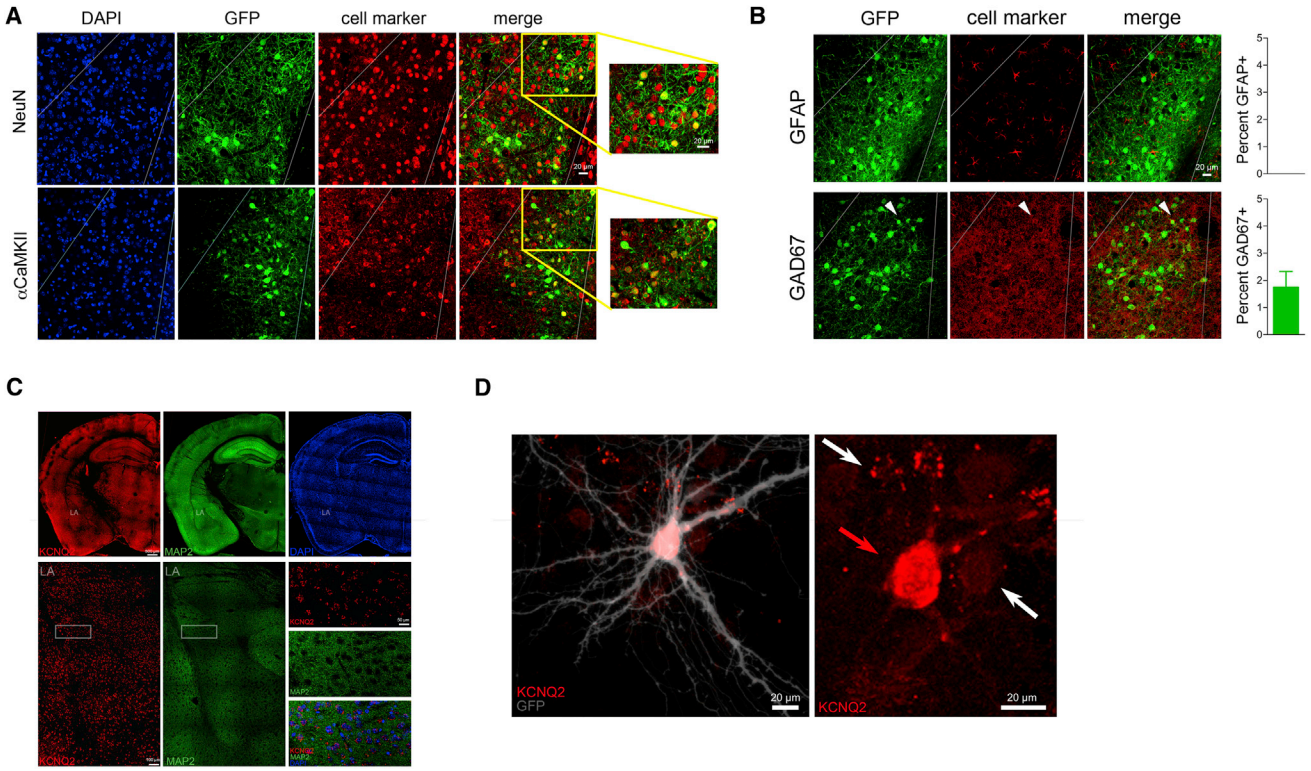

Figure 1. Using Replication-Defective HSV Viral Vectors to Manipulate Neuronal Excitability

(A and B) Following microinjection into the LA, HSV vectors preferentially infect pyramidal/principal neurons. Mice were microinjected with HSV expressing GFP into the LA, and 4 days later infected cells were phenotyped using immunohistochemistry for various cell markers. Example images from 4 days postinjection (dpi). (A) DAPI (blue, nuclear stain), GFP (green, infected cell), various cell markers (red). Top: NeuN was used as a marker of neurons. All infected cells (GFP^+) were neurons (also $NeuN^+$). Bottom: αCaMKII was used as a marker of excitatory pyramidal/principal neurons. The vast majority of infected cells (roughly 98%) were excitatory pyramidal neurons (also $\alpha CaMKII^+$). (B) Top: we found no (0%) HSV-infected cell that coexpressed endogenous markers typical of astrocytes (GFAP, glial fibrillary acidic protein) and (bottom) only very rare HSV-infected cells that coexpressed inhibitory neurons (GAD67, glutamate decarboxylase 67) (roughly 1.7%). Detailed quantification cell phenotype infected by HSV-GFP in six mice shows that following microinjection into the LA, HSV overwhelmingly infects excitatory pyramidal neurons.

(C) Endogenous KCNQ2 is widely expressed in adult mouse brain neurons (including LA) and localized to cell body and proximal parts of neurites (near axon initial segment). Left: low-power (upper panel) and high-power (lower panel) immunofluorescence images of adult mouse brain stained with antibody directed against KCNQ2 (red). Middle (upper and lower panels): MAP2 (microtubule-associated protein 2, neuronal cytoskeletal protein, green) used to visualize dendritic processes. Right (top panel): Sections were counterstained with DAPI (blue) to visualize nuclei. Lower panels: higher-power images showing KCNQ2 (upper, red) and MAP2 (middle, green) staining. Bottom panel: triple labeling shows KCNQ2 localized to cell body and proximal parts of neurites, as expected.

(D) Left: primary hippocampal neurons transfected with dnKCNQ2 vector that also expresses GFP (GFP, gray; KCNQ2, red). Right: magnification of transfected neuron shows increased KCNQ2 protein (KCNQ2, red) in the cell body and proximal parts of neurites, as expected. Red arrow points to transfected neuron, depicting that dnKCNQ2 is localized to correct neuronal region. White arrows point to representative nontransfected neurons and the endogenous KCNQ2 expression. Data presented are mean ± SEM.

Memory-Enhancing Effects of Increasing CREB Levels in a Small Portion of LA Neurons Are Mimicked by dnKCNQ2 and Blocked by Coexpression of Kir2.1

We previously showed that increasing CREB function in a small, random population of LA neurons by microinjecting HSV-CREB was sufficient to enhance conditioned fear memory formation, and that these neurons overexpressing CREB were selectively allocated to the fear memory trace (Han et al., 2007, 2009). To examine if the memory-enhancing effects of CREB overexpression in these experiments could be attributed to CREB increasing neuronal excitability, we microinjected HSV vectors expressing GFP (as a control), CREB (to increase excitability), dnKCNQ2 (to increase excitability), Kir2.1 (to decrease excitability), or CREB+Kir2.1 (to counteract the increase in excitability produced by CREB) into the LA 2 days before weak auditory fear conditioning (in which a tone conditioned stimulus [CS] was paired with low-intensity, 0.3 mA shock). Importantly, following microinjection, these viral microinjections infect roughly 10% of LA principal neurons (as determined by stereological counting), and the pattern of infection appears random (Figure 2C; see Supplemental Experimental Procedures). In agreement with previous findings (Han et al., 2007, 2009; Rexach et al., 2012; Zhou et al., 2009), we observed that increasing CREB in a small portion of LA neurons enhanced memory formation (Figure 2D). Similarly, expressing dnKCNQ2 in roughly 10% of LA neurons produced a memory enhancement that was strikingly similar to

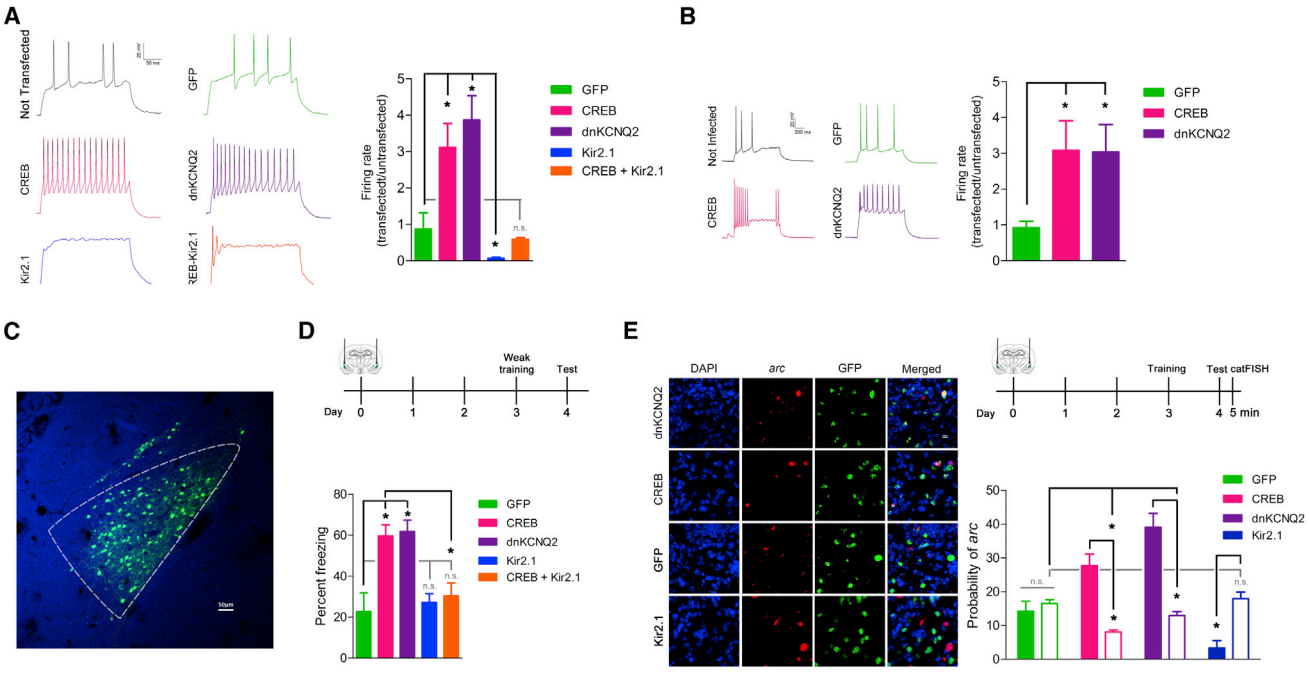

Figure 2. Increasing Intrinsic Excitability by Overexpressing CREB or dnKCNQ2 in a Small, Random Portion of LA Principal/Pyramidal Neurons Enhances Fear Memory Formation; Neurons with Relatively Higher Excitability Are Preferentially Allocated to this Fear Memory Trace

(A) Primary hippocampal neurons transfected with CREB or dnKCNQ2 construct show increased firing rates (relative to not-transfected neighboring neurons), whereas neurons transfected with GFP show no increase in firing. Neurons expressing Kir2.1 show decreased firing rates, whereas neurons coexpressing both CREB and Kir2.1 are not different from neurons expressing GFP. Left: example traces from injecting 120 pA current. Right: quantification. GFP n = 7 neurons, CREB n = 6 neurons, dnKCNQ2 n = 12 neurons, Kir2.1 n = 6 neurons, CREB+Kir2.1 n = 2 neurons.

(B) Ex vivo LA neurons from mice microinjected with CREB or dnKCNQ2 vector show increased firing rates relative to not-infected neurons (or neurons expressing GFP control vector, which do not show differences from not-infected neighbors). Left: example traces. Right: quantification. GFP n = 9 neurons, CREB n = 7 neurons, dnKCNQ2 n = 8 neurons.

(C) Microinjecting HSV vector infects ~10% of LA principal neurons (based on stereological counting). Example image from mouse brain 4 days postinjection (dpi). DAPI (blue, nuclear stain), GFP (green, infected neuron). LA outlined with dotted line.

(D) Expressing CREB or dnKCNQ2 vector in a small portion of LA principal neurons 2 days before weak auditory fear conditioning enhances fear memory formation. Expressing Kir2.1 vector in a similar portion of neurons does not affect fear memory. However, the memory enhancement produced by CREB vector was prevented by coexpressing Kir2.1 in the same neurons. GFP vector n = 7 mice, CREB n = 7 mice, dnKCNQ2 n = 15 mice, Kir2.1 n = 10 mice, CREB+Kir2.1 n = 8 mice.

(E) Neurons are allocated to the memory trace based on relative excitability. In mice microinjected with CREB or dnKCNQ2 vector, infected neurons were more likely to be part of the memory trace (active *arc*$^+$ neurons assessed 5 min after the memory test) than their noninfected neighbors. In mice microinjected with GFP vector, infected neurons were equally likely to be *arc*$^+$, while in mice microinjected with Kir2.1 vector, infected neurons were less likely to be *arc*$^+$ than their noninfected neighbors. Left: example images. DAPI (blue, nuclear stain), *arc*$^+$ (red, active neuron following memory test), GFP (green, infected neuron). Right: quantification of images. GFP vector n = 8 sections from 5 mice, CREB n = 8 sections from 5 mice, dnKCNQ2 n = 14 sections from 6 mice, Kir2.1 n = 4 sections from 4 mice. Data presented are mean ± SEM. n.s., not statistically different.

that observed following CREB overexpression. However, coexpression of CREB and Kir2.1 in the same LA neurons blocked the memory enhancement produced by CREB vector alone (one-way ANOVA, $F_{4,42} = 14.75$, $p < 0.001$, mice microinjected with CREB or dnKCNQ2 vector froze more than mice with GFP vector, but were not different from each other; mice microinjected with CREB+Kir2.1 vector froze less than mice with CREB vector alone, but were not different from mice with GFP vector [posthoc Newman-Keuls]).

The Memory-Enhancing Effects of Increasing CREB or Blocking KCNQ2 Function Are Behaviorally Specific

These results suggest that increasing excitability in a small portion of LA neurons before fear conditioning enhanced the formation of a fear memory. To more thoroughly examine this effect, we conducted a series of control experiments. First, we examined the phase of memory which was important in the memory enhancement produced by CREB or dnKCNQ2 expression. To determine whether the higher freezing during the tone test observed in mice microinjected with HSV-CREB or HSV-dnKCNQ2 was due to an effect on acquisition or expression of conditioned fear memory, we microinjected our viral vectors 1 day after training (rather than 2 days before training). As a control for the possible effects of surgery, we compared freezing levels on the test day to no-surgery control mice. We observed that microinjecting our viral vectors 1 day after either weak (0.3 mA shock) or strong (0.6 mA shock) training did not affect freezing levels during the tone test (Figure 3A; strong training,

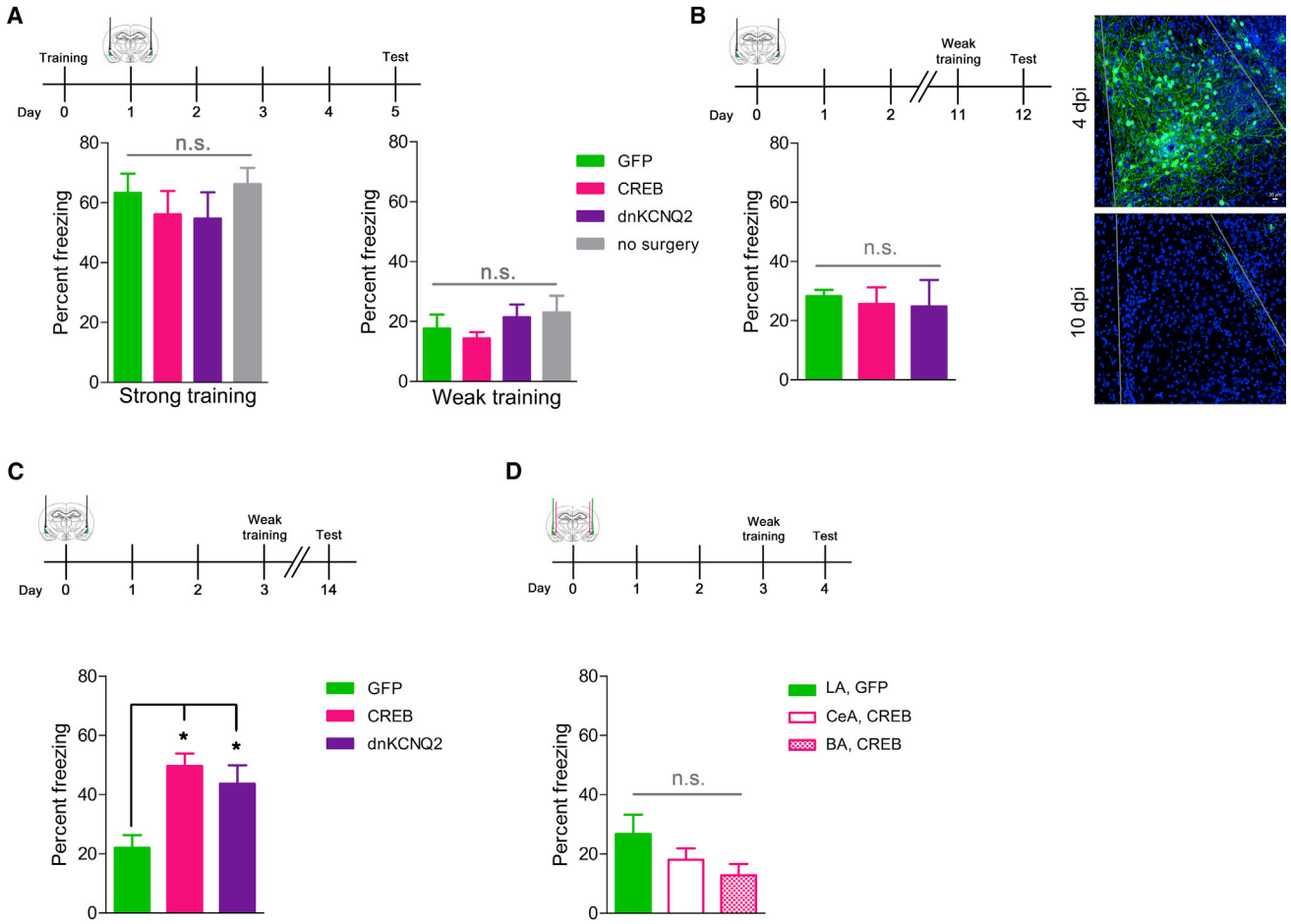

Figure 3. The Memory Enhancement Produced by Overexpressing CREB or dnKCNQ2 in the LA Prior to Fear Conditioning Is Specific

(A) Microinjecting CREB or dnKCNQ2 vector after strong or weak training has no effect on expression of a previously acquired fear memory. Strong training; GFP n = 10 mice, CREB n = 11, dnKCNQ2 n = 11, no surgery n = 8. Weak training; GFP n = 8, CREB n = 8, dnKCNQ2 n = 8, no surgery n = 8.

(B) Microinjecting CREB or dnKCNQ2 vector 11 days before weak auditory fear conditioning (such that mice were trained after transgene expression dissipated) does not enhance memory. n = 8 mice per group. Right: little to no LA transgene expression 10 days postinjection (dpi, bottom) compared to high levels of transgene expression 4 dpi (top).

(C) Microinjecting CREB or dnKCNQ2 vector 2 days before weak auditory fear conditioning produces an enduring memory enhancement that outlasts transgene expression. n = 8 mice per group.

(D) Microinjecting CREB vector into the central amygdala (CeA) or basal amygdala (BA) 2 days before weak auditory fear conditioning fails to enhance memory, showing that the memory enhancement is anatomically specific. LA, GFP n = 7; CeA, CREB n = 8; and BA, CREB n = 8. Data presented are mean ± SEM. n.s., not statistically different.

$F_{3,36} = 0.52$, $p > 0.05$; weak training, $F_{3,28} = 0.81$, $p > 0.05$). These findings indicate that increasing excitability in a small subset of LA neurons did not increase freezing by enhancing the expression of a previously acquired fear memory. Instead, these results are consistent with the interpretation that CREB and dnKCNQ2 enhanced the formation of a fear memory.

Second, we asked when an increase in neuronal excitability resulted in enhanced fear memory. Transgene expression using this HSV system begins hours following microinjection, peaks within 2–3 days later, and dissipates within 7–12 days (Figure 3B, right) (Sekeres et al., 2012). We took advantage of this transgene expression time course by microinjecting CREB or dnKCNQ2 vectors 11 days before training (such that mice were trained at a time when the transgenes were no longer expressed). Microinjecting dnKCNQ2 or CREB vector 11 days before training had no effect on subsequent memory formation, suggesting that the enhanced memory was dependent on increased neuronal excitability at the time of training (Figure 3B, left; $F_{2,20} = 0.09$, $p > 0.05$). Third, we examined the duration of memory enhancement produced by CREB or dnKCNQ2 vector. We found that the memory enhancement produced by high levels of CREB or dnKCNQ2 at the time of training (mice were microinjected with vectors 2 days before training) was enduring and even continued beyond transgene expression (14 days after microinjection) (Figure 3C; $F_{2,21} = 8.44$, $p < 0.05$, mice microinjected with CREB or dnKCNQ2 vector continued to freeze higher than mice microinjected with GFP vector [but did not differ from each other] even 14 days following microinjection [Newman-Keuls]). Finally, we

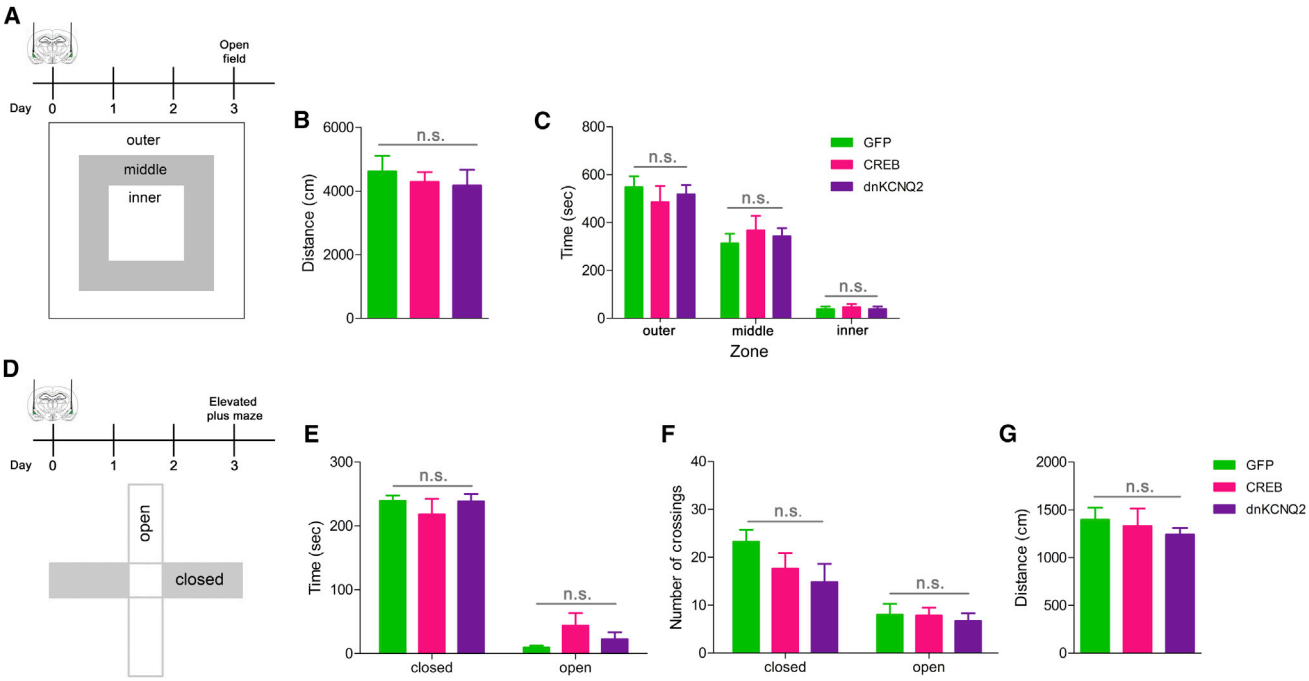

Figure 4. Overexpressing CREB or dnKCNQ2 in the LA Does Not Increase Overall Anxiety-like Behavior

(A–G) Increasing excitability by overexpressing CREB or dnKCNQ2 in a random small portion of LA neurons does not impact basal anxiety-like behavior as assessed in the open field (A–C) or elevated plus maze (D–G). We observed no effect of microinjecting CREB or dnKCNQ2 vectors into LA on open-field behavior (either total distance traveled [B] or time spent in outer, middle, and inner zone [C]). GFP vector n = 9 mice, CREB n = 8 mice, dnKCNQ2 n = 9 mice. We observed no effect of microinjecting excitability vectors into the LA on elevated plus maze behavior (time in open versus closed arms [E], crossings in open versus closed arms [F], or total distance traveled [G]). GFP vector n = 8 mice, CREB n = 5 mice, dnKCNQ2 n = 6 mice. Data presented are mean ± SEM. n.s., not statistically different.

examined the anatomical specificity of the memory enhancement induced by overexpression of CREB. To this end, we microinjected CREB vector into the neighboring basal amygdala (BA) or central amygdala (CeA) 2 days prior to training. We observed no memory enhancement with CREB overexpression in these neighboring amygdala nuclei (Figure 3D; one-way ANOVA conducted on three groups, GFP vector in LA, CREB vector in CeA, or CREB vector in BA, $F_{2,20} = 2.58$, $p > 0.05$), suggesting that the memory-enhancing effects of CREB overexpression are anatomically specific. Together, these results indicate memory formation was enhanced if, and only if, CREB or dnKCNQ2 was expressed in a small population of LA neurons at the time of training. Once formed, however, this enhanced memory endured and no longer required continued overexpression of CREB or dnKCNQ2.

Although we observed no difference in baseline (pretone) freezing in our experiments (Figures S1A–S1D available online), we next asked whether increasing excitability in a small portion of LA neurons enhanced freezing during the tone test by nonspecifically increasing overall basal anxiety-like behavior. We microinjected CREB or dnKCNQ2 vector into the LA 2 days before testing in the open field (Figure 4A) or on an elevated plus maze (Figure 4D). In the open field, a decrease in total distance traveled and/or an increase in time spent in the periphery of the open field are commonly used phenotypic markers of anxiety-like behavior in rodents (Crawley et al., 1997; Prut and Belzung, 2003). Neither CREB nor dnKCNQ2 vector decreased total distance traveled (Figure 4B; $F_{2,23} = 0.07$, $p > 0.05$) or increased the amount of time spent in the periphery of the open field (Figure 4C; no significant effect of Vector X Zone [time spent in outer, middle, and inner zones of the open field], $F_{4,46} = 0.03$, $p > 0.05$; or Vector, $F_{2,23} = 0.89$, $p > 0.05$; but as expected, significant effect of Zone, $F_{2,46} = 72.96$, $p < 0.001$, with all groups of mice tending to spend more time in the outer zone). Anxiety-like behavior in the elevated plus maze is often inferred from a decrease in the time spent in the open arm and/or a decrease in the number of open arm crossings (Pellow and File, 1986; Rodgers and Dalvi, 1997). Consistent with our findings using the open-field test, microinjecting CREB or dnKCNQ2 vector did not increase the amount of time spent in the open versus closed arms (Figure 4E; no significant Vector X Arm interaction, $F_{2,16} = 1.32$, $p > 0.05$; or effect of Vector alone, $F_{2,16} = 1.30$, $p > 0.05$; as expected, significant effect of Arm with all groups tending to spend more time in the closed arm, $F_{1,66} = 214.12$, $p < 0.001$), crossings in open versus closed arms (Figure 4F; no significant Vector X Arm interaction, $F_{2,16} = 1.25$, $p > 0.05$; or effect of Vector alone, $F_{2,16} = 1.69$, $p > 0.05$; significant effect of Arm only, $F_{1,16} = 29.12$, $p < 0.001$), or total distance traveled (Figure 4G; $F_{2,16} = 0.25$, $p > 0.05$) in the elevated plus maze. These results verify that the increase in freezing observed following microinjection of CREB or dnKCNQ2 vectors in auditory fear conditioning cannot be attributed to a nonspecific increase in anxiety-like behavior.

Neurons with Relatively Increased Excitability at the Time of Training Are Preferentially Recruited/Allocated to a Memory Trace

We observed robust memory enhancement despite infecting a small population of LA neurons (~10%, roughly 16,000–20,000 neurons; see Supplemental Experimental Procedures) with CREB or dnKCNQ2 vector, suggesting that these infected neurons (with increased excitability) were preferentially allocated to the memory trace. To examine this, we assessed whether neurons with CREB or dnKCNQ2 vector were overrepresented in the memory trace. To visualize neurons that may be part of the fear memory trace, we used cellular compartment analysis of temporal activity by fluorescent in situ hybridization (catFISH), a technique that takes advantage of the unique transcriptional time course of the activity-dependent gene *arc* (*a*ctivity-*r*egulated *c*ytoskeleton-associated protein) (Guzowski et al., 1999, 2001). Under basal, quiet conditions, neurons typically show no (or very low levels of) *arc* RNA. Neural activity induces a rapid but transient burst of *arc* transcription such that *arc* RNA is observed in the nucleus within 5 min of neuronal activity. However, this *arc* RNA is transported to dendrites such that roughly 40 min after neuronal activity, neurons again show low levels of *arc* in the nucleus. In this way, the spatial localization of *arc* RNA in a neuron (nucleus, cytoplasm, both, or neither) may serve as a molecular activity time stamp for any particular neuron, with *arc* localized to the nucleus serving as a visual marker of a recently active neuron. To identify infected neurons, we used the GFP expressed by our viral vectors.

Specifically, we examined the brains of mice 5 min following a conditioned fear (tone) memory test and assessed the overlap of active (arc^+, neurons with *arc* localized to the nucleus) and infected (GFP^+) versus noninfected (GFP^-) neurons in mice microinjected with GFP, CREB, dnKCNQ2, or Kir2.1 vector 2 days before training. Consistent with our previous finding, we observed that the overall size of the arc^+ memory trace (number of overall LA neurons that were arc^+) was stable across experiments, regardless of vector microinjected or memory strength ($F_{3,30} = 2.06$, $p > 0.05$). However, the distribution of arc^+ neurons (in infected versus noninfected neurons) differed significantly between vector groups.

In mice microinjected with either CREB or dnKCNQ2 vector, infected neurons were three times more likely to be arc^+ than their noninfected neighbors following a memory test. This suggests that these neurons with CREB or dnKCNQ2 were preferentially recruited to the memory trace, paralleling our previous findings with CREB vector (Han et al., 2007, 2009). The opposite pattern emerged from mice microinjected with Kir2.1 vector; infected neurons were over five times less likely to be arc^+ than noninfected neurons following a memory test (Figure 2E; Vector (GFP, CREB, dnKCNQ2, Kir2.1) X Neuron Infection Status (infected, noninfected) ANOVA, $F_{3,30} = 18.78$, $p < 0.001$, in mice with GFP vector, there was no difference between the probability of arc^+ nuclei in infected versus noninfected neurons). Importantly, mice microinjected with Kir2.1 vector showed normal memory (Figure 2D), suggesting that the majority of noninfected neurons were sufficient to support normal memory. The relative exclusion of neurons with Kir2.1 vector from the arc^+ memory trace (and normal memory) is strikingly similar to our previous finding in which we similarly microinjected a dominant-negative CREB vector (HSV-mCREB). We previously observed that neurons with mCREB were also less likely to be allocated to the memory trace, but that mice microinjected with mCREB vector showed normal memory (Han et al., 2007). It is of interest to note that similar to Kir2.1, mCREB expression decreases neuronal excitability (Dong et al., 2006; Han et al., 2006). These findings support the notion that neurons are chosen for the memory trace based on their relative excitability.

Interestingly, following a memory test, noninfected neurons were less likely to be arc^+ in mice microinjected with CREB or dnKCNQ2 vector than in mice microinjected with GFP vector (one-way ANOVA on the probability of noninfected neurons being arc^+ in the three Vector groups [GFP, CREB, dnKCNQ2] showed a significant difference, $F_{2,27} = 11.87$, $p < 0.05$; the likelihood of noninfected cells being arc^+ was lower in mice microinjected with CREB or dnKCNQ2 vector than in mice microinjected with GFP vector [Newman-Keuls]). Together, these data are consistent with the interpretation that neurons compete against one another for inclusion in a memory trace, and that neurons with relatively higher levels of excitability are more likely to win this competition. In addition, these winning neurons may also actively inhibit "loser" neurons, to maintain the stability of the overall size of the memory trace. Indeed, our experimental data were correctly forecast in an elegant modeling study (Kim et al., 2013), which showed that neuronal competition for memory allocation involves both relative neuronal excitability and a disynaptic inhibition process.

Using Genetically Encoded Mediators of Neural Excitability to Transiently Increase Excitability in a Small Portion of LA Neurons before, but Not Immediately after, Training Enhances Fear Memory Formation

In the above experiments, we used HSV to express different transgenes that affect excitability. Due to the nature of transgene expression using the present HSV vector, mice were trained and tested with high levels of transgene expression. As such, these experiments do not address precisely when an increase in neural excitability is important in the observed memory enhancement. To gain finer temporal control over neuronal excitability, we used two different systems to genetically manipulate neural excitability over a relatively brief time. First, we used HSV to express the DREADD (*d*esigner *r*eceptors *e*xclusively *a*ctivated by *d*esigner *d*rug) hM3Dq. hM3Dq is an evolved Gq-coupled muscarinic receptor, which has no constitutive activity and does not bind to the endogenous ligand (ACh) (Armbruster et al., 2007; Nichols and Roth, 2009). However, binding of the synthetic ligand, clozapine-N-oxide (CNO, an otherwise pharmacologically inert compound), to hM3Dq receptors increases neuronal excitability (Armbruster et al., 2007; Nichols and Roth, 2009). We first verified this in cultured hippocampal neurons. Specifically, we found that neurons transfected with hM3Dq show increased excitability only in the presence of CNO, and that CNO without the hM3Dq receptor did not alter excitability (Figure 5A; two-way ANOVA on membrane potential as a measure of excitability, with within-group factor CNO Application [before versus after CNO application] and between-group factor Transfection [transfected with hM3Dq versus untransfected]

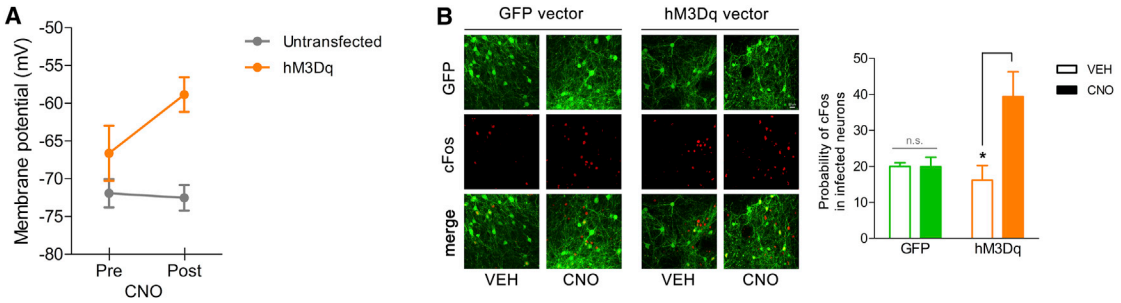

Figure 5. Using hM3Dq DREADD to Increase Neuronal Excitability

(A) Primary cultured hippocampal neurons transfected with hM3Dq (but not neighboring untransfected neurons) show depolarization only after (and not before) CNO administration. hM3Dq construct, n = 5 neurons; untransfected, n = 5 neurons.

(B) LA neurons infected with hM3Dq vector show increased activity (cFos expression) following systemic injection of CNO. Mice microinjected with hM3Dq or GFP vector were not trained but received systemic administration of CNO or VEH. cFos immunohistochemistry was examined 90 min later, and the number of infected neurons positive for cFos was quantified. hM3Dq vector + CNO administration n = 6 mice, hM3Dq+VEH n = 5 mice, GFP+CNO n = 4 mice, GFP+VEH n = 4 mice. Data presented are mean ± SEM. n.s., not statistically different.

revealed a significant interaction, $F_{1,8} = 8.78$, $p < 0.05$, as well as significant main effect of Transfection, $F_{1,8} = 8.56$, $p < 0.05$, and CNO Application, $F_{1,8} = 6.44$, $p < 0.05$; post hoc Newman-Keuls tests performed on the significant interaction revealed that neurons transfected with hM3Dq were significantly more excitable after CNO administration, while all other groups did not differ). In addition, in mice microinjected with hM3Dq vector into the LA, we observed an increase in expression of an activity marker in infected neurons only following systemically administered CNO. Specifically, neurons with hM3Dq vector had higher levels of cFos expression, a marker of neuronal activation (Morgan and Curran, 1991), only following administration of CNO, while CNO on its own produced no effect in neurons with the control GFP vector (Figure 5B; significant Vector X CNO Injection interaction, $F_{1,15} = 5.11$, $p < 0.05$; posthoc Newman-Keuls tests confirmed that infected neurons showed a higher probability of being $cFos^+$ in the hM3Dq+CNO group than in all other groups, which did not differ from each other).

To transiently increase excitability in a small (~10%), random portion of LA principal neurons, specifically in the minutes before (and during) fear training, we microinjected hM3Dq (or GFP) vector as above (2 days before training), and systemically administered CNO (or vehicle [VEH]) before weak fear training. Mice were tested 24 hr later, drug free (in the absence of CNO). In this way, neuronal excitability was increased only in the minutes before training. During the memory test, we observed an increase in freezing only in mice microinjected with hM3Dq vector and administered CNO before training. We observed no memory enhancement by administration of CNO alone (in mice with GFP vector) or expression of hM3Dq alone (in mice administered VEH). Therefore, increasing neuronal excitability in a random, small subset of neurons before training was sufficient to enhance memory formation (Figure 6A; Vector X CNO Injection, $F_{1,29} = 8.57$, $p < 0.05$; mice microinjected with hM3Dq vector and administered CNO before training froze more than all other groups [Newman-Keuls]). Importantly, administering CNO immediately after training failed to enhance memory in mice microinjected with hM3Dq vector (Figure 6B; no significant effect of Vector, CNO Injection, or Vector X CNO Injection interaction, $F_{1,28} = 0.22$, $p > 0.05$). This finding suggests that increased neuronal excitability at the time of memory encoding, rather than during consolidation, determines the strength of memory formation.

To examine whether this small portion of LA neurons that had increased excitability only during the minutes before training was preferentially allocated to the memory trace, we used catFISH for *arc* RNA following a memory test (as above). Before this memory test, no CNO was administered. Specifically, we microinjected mice with hM3Dq vector, administered CNO or VEH prior to training, and 5 min following a drug-free memory test, examined the overlap of *arc* (to identify neurons in the memory trace) and GFP (to identify infected neurons). In mice administered CNO before training, hM3Dq-expressing neurons were 3.8 times more likely to be included in the memory trace than their noninfected neighbors, whereas hM3Dq-expressing and noninfected neighboring neurons were equally likely to be arc^+ in mice administered VEH before training (Figure 6C; CNO Injection X Neuron Infection Status, $F_{1,18} = 14.52$, $p < 0.05$, in mice microinjected with hM3Dq vector and administered CNO, infected neurons were more likely to be arc^+ than noninfected neurons; however, infected and noninfected neurons were equally likely to be arc^+ in mice microinjected with hM3Dq vector but administered VEH [Newman-Keuls]). This result parallels the findings from mice microinjected with CREB or dnKCNQ2 vector, and suggests that neurons with increased excitability in the minutes before (and during) training are preferentially allocated to the memory trace.

Synthetic Activation of Neurons Allocated to a Memory Trace Is Sufficient to Serve as a Retrieval Cue

To determine whether these neurons with increased excitability at the time of training became critical components of the memory trace, we next asked whether artificial activation of just these neurons was sufficient to serve as a memory retrieval cue. In our previous experiments, we probed conditioned fear memory by placing mice in a novel context and presenting an external retrieval cue (presentation of the tone CS that was paired with shock during training). In this experiment, however, we microinjected mice with hM3Dq (or GFP) vector and administered CNO

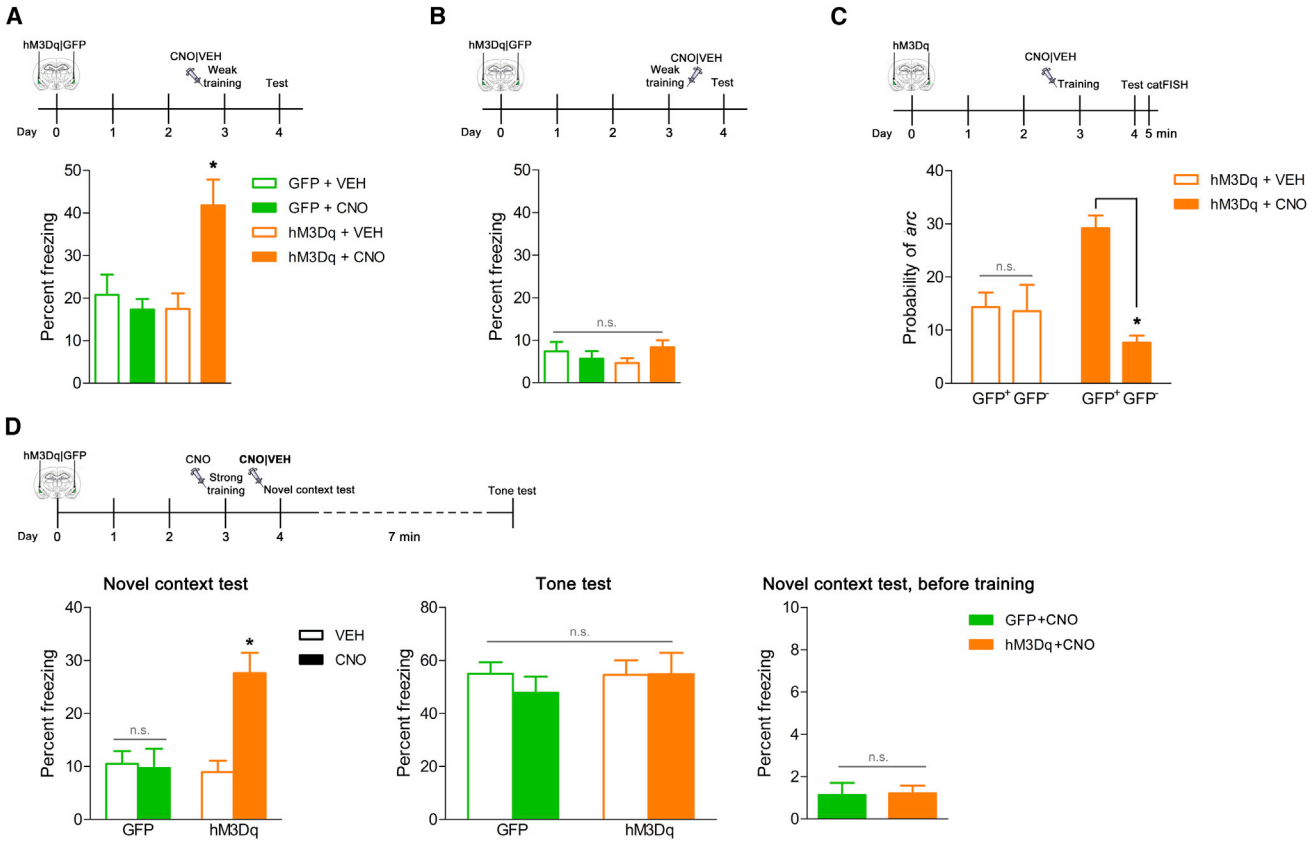

Figure 6. Increasing Excitability Using hM3Dq DREADD in a Small Portion of LA Neurons Immediately before, but Not Immediately after, Training Enhances Fear Memory Formation; These Neurons Are Preferentially Allocated to Memory Trace; Artificial Reactivation of These Neurons Alone Serves as a Sufficient Memory Retrieval Cue

(A and B) Transiently increasing excitability in a small portion of LA neurons before, but not after, training enhances fear memory formation. Microinjecting hM3Dq vector 2 days before weak auditory fear conditioning and systemically administering CNO before, but not immediately after, (B) training enhances fear memory formation. CNO before training; GFP vector + CNO administration n = 7 mice, GFP+VEH n = 8 mice, hM3Dq+CNO n = 10 mice, hM3Dq+VEH n = 8 mice. CNO after training; n = 8 mice for each group.

(C) In mice microinjected with hM3Dq vector, infected (GFP^+) were more likely than noninfected neighbors (GFP^-) to be allocated to arc^+ memory trace if CNO was administered before training. No difference between distribution of arc^+ in infected and noninfected neurons in mice microinjected with hM3Dq vector but administered VEH. CNO administered only before training (not before test). hM3Dq vector + CNO administration n = 15 sections from 8 mice, hM3Dq+VEH n = 5 sections from 5 mice.

(D) Artificially activating neurons that had increased excitability before strong training (hM3Dq+CNO) is sufficient to serve as a memory retrieval cue in the absence of an external retrieval cue. Mice with hM3Dq vector administered CNO before training and again before a test in a novel context show higher levels of freezing than control groups (left). However, all groups froze at equivalent levels when an appropriate external cue was replayed (middle, the tone previously paired with shock). GFP vector + CNO administration n = 8 mice, GFP+VEH n = 8 mice, hM3Dq+CNO n = 8 mice, hM3Dq+VEH n = 8 mice. This enhancement in freezing following CNO administration is specific to fear memory; we observed no increase in freezing in these mice when similarly microinjected with hM3Dq vector, administered CNO, and placed in a novel context, but not trained (right, before tone-shock pairing). GFP vector + CNO n = 16 mice, hM3Dq+CNO n = 16 mice. Data presented are mean ± SEM. n.s., not statistically different.

to all groups before strong training. Rather than assessing memory by exposing mice to the tone (CS), we probed memory by placing mice in a novel context following administration of CNO (to reactivate the neurons that we hypothesize are part of the memory trace) and measured freezing. Activation of these hM3Dq-expressing neurons alone (without external tone retrieval cue) was sufficient to induce freezing (Figure 6D, left; Vector X CNO Injection at Test, $F_{1,28} = 9.80$, $p < 0.05$, mice with hM3Dq vector administered CNO before test froze more than all other groups [Newman-Keuls]). All groups froze at equivalently high levels when an external cue (the tone previously paired with shock) was replayed during a subsequent memory test (Figure 6D, middle; Vector X CNO Injection at Test, $F_{1,28} = 0.37$, $p > 0.05$), indicating that all groups were equally capable of strongly expressing the memory if presented with a retrieval cue that closely matched the training cue (Koutstaal et al., 2001). Importantly, increased freezing was not observed if mice with hM3Dq vector were administered CNO and placed in a novel context before the training session, indicating that simply activating a small population of LA neurons alone does not induce freezing (Figure 6D, right; no significant effect of Vector [hM3Dq versus GFP] [all mice administered CNO before

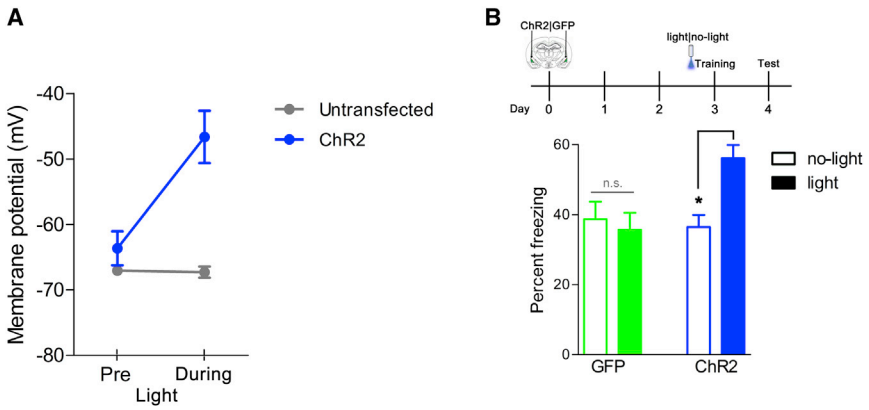

Figure 7. Increasing Excitability in a Small Portion of LA Neurons Using Optogenetics Immediately before Training Enhances Memory Formation

(A) Primary hippocampal neurons transfected with ChR2 (but not neighboring untransfected neurons) show depolarization during, but not before, illumination with a blue light. ChR2 construct, n = 9 neurons; untransfected, n = 4 neurons.

(B) Optogenetically activating a small portion of LA neurons immediately before training enhanced memory formation. GFP vector + no light n = 6 mice, GFP vector + light n = 6 mice, ChR2 vector + no light n = 9 mice, ChR2 vector + light n = 11 mice. Data presented are mean ± SEM. n.s., not statistically different.

training], $F_{1,30} = 0.01$, $p > 0.05$). These data support the interpretation that nonspecifically increasing activity in a random, small population of LA neurons (that are not part of the fear memory trace) does not induce freezing. Instead, these results indicate that artificially reactivating a key component of the fear memory trace (those neurons with increased excitability at the time of training) is sufficient to induce memory recall. The finding that freezing was lower following artificial reactivation of a memory trace than following exposure to the CS that predicted shock is in keeping with the encoding specificity principle, which suggests that memory retrieval is most effective when information available at encoding is also present at retrieval (Tulving and Thomson, 1973).

Using Optogenetic Mediators of Neural Excitability to Transiently Increase Excitability in a Small Portion of LA Neurons Immediately before Training Enhances Fear Memory Formation

To gain further temporal precision over neuronal excitability, we used HSV to express channelrhodopsin-2 (ChR2), a light-activated cation channel, in a small portion of LA neurons. When activated by blue light, ChR2 induces depolarization (Deisseroth, 2011; Sparta et al., 2013; Tye and Deisseroth, 2012). We confirmed this effect in cultured hippocampal neurons (Figure 7A; two-way ANOVA on membrane potential as a measure of excitability with within-group factor Light [before versus during] and between-group factor Transfection [transfected with ChR2 versus untransfected] revealed a significant interaction, $F_{1,11} = 9.47$, $p < 0.05$; posthoc Newman-Keuls tests revealed there was no difference between untransfected hippocampal culture depolarization before and during blue light; however, neurons transfected with ChR2 showed significantly more depolarization during light).

We microinjected HSV expressing ChR2 or control GFP into the LA of mice as above. Immediately before training (in which a tone CS was paired with a 0.5 mA shock), we illuminated the LA with blue (473 nm) light pulses (continuous train at 20 Hz light pulses, with a 5 ms pulse for 30 s immediately before tone-shock pairing). Additional groups were similarly microinjected with ChR2 or GFP vector but were trained with the light off. All mice were given a tone test (without light) 24 hr after training. Optogenetically activating a small portion of LA neurons immediately before training was sufficient to enhance memory formation (Figure 7B; Vector [ChR2 versus GFP] X Light condition immediately before training (light versus no light) ANOVA showed a significant interaction, $F_{1,28} = 6.83$, $p < 0.05$; only mice microinjected with ChR2 and trained immediately after light on showed enhanced memory; there was no difference in freezing levels between mice with GFP vector in either the light or no-light condition or mice with ChR2 vector in the no-light condition [Newman-Keuls]). Together, these data support the conclusion that increased neural excitability at the time of training mediates memory formation and neuronal allocation to a memory trace.

DISCUSSION

Together, these experiments indicate that increasing excitability (without directly manipulating CREB function) of a small, random population (~10%) of LA pyramidal/principal neurons immediately before training was sufficient both to bias neuronal memory allocation and enhance memory formation. Moreover, the memory-enhancing effect of increasing CREB function was prevented by decreasing excitability specifically in these neurons. Artificial reactivation of neurons that were highly excitable during training subsequently served as a memory retrieval cue in the absence of external retrieval cues, indicating that activation of this small population of neurons was sufficient for memory expression. Therefore, these results indicate that memory allocation is at least partially based on relative neuronal excitability at the time of training.

Our finding that increasing excitability in LA neurons mediates neuronal fear memory allocation is also consistent with previous findings examining avoidance learning in the piriform cortex (Choi et al., 2011). Choi and colleagues used lentivirus to express ChR2 in a random population of roughly 10% of excitatory neurons in the piriform cortex. Optogenetic activation of this small population of neurons was paired with a shock in an avoidance paradigm. In a memory test, activation of these neurons alone was sufficient to induce avoidance, suggesting that these neurons were allocated to the avoidance memory trace, and that subsequent activation of these neurons alone could serve as a retrieval cue for this memory.

Our finding that neurons are recruited to a memory trace based on neuronal excitability was predicted by a recent

biophysical modeling study. Kim and colleagues (Kim et al., 2013) used a 1,000-LA-cell conductance-based model, which mirrored the known relative composition of principal neurons and interneurons in the LA. To test the prediction that neurons were allocated to a memory trace based on their relative excitability, principal neurons were randomly divided into three categories that differed only with respect to their intrinsic excitability (low, medium, or high excitability produced by differential expression of K^+ currents) and not to their initial type or number of inputs. The network was trained (''auditory fear conditioned'') and the proportion of principal neurons assigned to the low, medium, or high excitability categories which showed plasticity to the tone CS (neurons with increased CS responsiveness after training, as a proxy of neurons recruited into the memory trace) compared. Similar to our experimental results, this model found a much larger proportion of plastic cells (analogous to our *arc*$^+$ memory trace neurons) among the principal cells assigned to the high excitability category (analogous to our neurons infected with either CREB or dnKCNQ2 vector) than would be expected by chance. Specifically, while only 1% of model LA neurons with low excitability became part of the memory trace, over 40% of the more excitable neurons (medium and high excitability) were allocated to the memory trace.

Here we observed that CREB and dnKCNQ2 expression not only increased the likelihood that the infected neurons were allocated to the memory trace, but these manipulations also decreased the likelihood that noninfected neighboring neurons were allocated to the memory trace. Consistent with this, we observed that the overall size of the LA memory trace (neurons *arc*$^+$ after a memory test) did not vary with any of our manipulations or level of freezing during the test. Indeed, we previously showed that a similar size of memory trace supports both strong and weak fear memories (Han et al., 2007). This finding indicates that the strength of memory is not coded by number of neurons in the LA fear memory trace. In addition, this observation implies that although competition takes place between neurons with high versus low intrinsic excitability, there is an additional form of competition that may also play a critical role in neuronal allocation to a memory trace. Kim et al. suggest that the substrate of this additional form of competition is disynaptic inhibition. This modeling study showed that the outcome of neuronal competition is also mediated by the distribution of excitatory connections between principal cells and the amount of disynatpic inhibition they generate in other projection cells. That is, collections of principal LA neurons band together to form a Hebbian ensemble, which suppresses plasticity in other principal cells via the recruitment of inhibitory interneurons.

Intrinsic excitability is regulated by several mechanisms (Aizenman et al., 2003; Daoudal and Debanne, 2003), including learning. Across several species, learning transiently increases intrinsic excitability of some neurons (Alkon, 1974, 1984; Alkon et al., 1985; Moyer et al., 1996; Oh et al., 2003; Thompson et al., 1996) by downregulating K^+ currents which mediate the AHP (Saar et al., 2002). In the present experiments, we artificially modified intrinsic excitability by overexpressing CREB, manipulating K^+ channel function, or using chemicogenetics or optogenetics. However, endogenous changes in intrinsic excitability are linked to learning in a variety of species (from *Hermissenda* [Alkon, 1974] to rodents [Thompson et al., 1996]), suggesting that the current manipulations, while artificial in nature, tap into and mimic an endogenous underlying fundamental memory process. Moreover, this process of neuronal allocation based on relative excitability may also play a role in ''preplay,'' a recently described phenomenon in which the emergence of hippocampal place cell firing is predicted by neuronal activity patterns that occur in the minutes before actual exposure to a novel spatial context (Dragoi and Tonegawa, 2011, 2013).

EXPERIMENTAL PROCEDURES

Mice

Adult (<12 weeks of age) male and female F1 hybrid (C57BL/6NTac X 129S6/SvEvTac) mice were used for all experiments, except where noted. All procedures were conducted in accordance with policies of the Hospital for Sick Children Animal Care and Use Committee and conformed to both the Canadian Council on Animal Care (CCAC) and NIH Guidelines on the Care and Use of Laboratory Animals.

HSV Vectors

Wild-type full-length CREB (kindly provided by Dr. Satoshi Kida, Tokyo University of Agriculture, Tokyo, Japan), dnKCNQ2 (hKCNQ2-G279S, kindly provided by Dr. Dirk Isbrandt, DFG Heisenberg Team Experimentelle Neuropädiatrie), Kir2.1 (kindly provided by Dr. Eric Nestler, Mt. Sinai, NY), hM3Dq (kindly provided by Dr. Bryan Roth, University of North Carolina), and ChR2 (fused with YFP, kindly provided by Dr. Karl Deisseroth, Stanford) cDNAs were subcloned into an HSV vector backbone that coexpresses GFP as a fluorescent reporter (HSV-p1005 [Russo et al., 2009]). Transgene expression using this viral system typically peaks at 3 days, and dissipates within 7–12 days, following microinjection (Barrot et al., 2002; Cole et al., 2012; Josselyn et al., 2001; Vetere et al., 2011). The average titer of the virus stocks was 4.0×10^7 infectious units/ml. See Supplemental Experimental Procedures for details.

Surgery

Mice were pretreated (atropine sulfate 0.1 mg/kg, i.p.), anesthetized (chloral hydrate, 400 mg/kg, i.p.), and viral vectors were infused (1.5 μl/side, 0.1 μl/min) into the LA (AP = −1.45, ML = ± 3.45, V = −5.0 mm from bregma), CeA (AP = −1.35, ML = ± 3.3, V = −5.3 mm from bregma), or BA (AP = −1.4, ML = ± 3.45, V = −5.5 mm) (Paxinos and Franklin, 2001). For the optogenetic experiment, mice were similarly microinjected with HSV encoding ChR2, and bilateral optical fibers were implanted slightly above each LA (Sparta et al., 2012; Stuber et al., 2011).

Electrophysiology

Hippocampal Cultured Neurons

Low-density cultures of dissociated mouse hippocampal neurons were prepared as previously described (Acton et al., 2012). After 10–15 days in culture, neurons were transfected with DNA constructs (GFP, CREB, dnKCNQ2, Kir2.1, CREB+Kir2.1). Electrophysiological recordings were performed on both transfected and untransfected (control) neurons from the same culture dish; transfected neurons were identified by GFP fluorescence. Neuronal excitability was determined in current-clamp mode by injecting current in 10 pA steps from −50 pA to 150 pA.

To verify that hM3Dq expression increased neuronal excitability following CNO, and that ChR2 expression increased excitability upon blue light application, we estimated the change in the baseline membrane potential for neurons transfected with these constructs (as well as untransfected controls) both before and after CNO/light, respectively.

Acute Ex Vivo LA Slices

Mice (~4 weeks of age) were microinjected with viral vectors (GFP, CREB, dnKCNQ2) into the LA, as above. After 1 day, brains were removed and placed into chilled modified aCSF. Coronal slices (350 μM) containing the LA were prepared. Recordings were obtained from infected neurons (identified by GFP fluorescence in the LA) and their uninfected neighbors.

Stereological Cell Counting

To determine the number of neurons infected by our viral microinjections, we used unbiased stereological principles and systematic sampling techniques to estimate the percentage of the total number of LA neurons infected by our viral manipulations. See Supplemental Experimental Procedures for details.

cFos Immunostaining

To further verify that the DREADD hM3Dq increases neuronal activity in vivo only following CNO administration, we examined cFos levels (as a marker of neuronal activity) in infected cells. Mice were microinjected with hM3Dq or GFP vector, and 2 days later these homecage mice were administered VEH or CNO systemically. After 90 min, mice were perfused transcardially with 4% PFA. Brains were sliced coronally (50 μm) and incubated with antibodies directed against cFos (Rabbit, K0306; 1:1,000, Calbiochem) and GFP (Chicken, 1:1,000, Millipore). The number of infected neurons that were also positive for cFos was assessed by two experimenters blind to the treatment condition. See Supplemental Experimental Procedures for details.

Auditory Fear Training and Testing

Auditory Fear Conditioning

Fear conditioning training consisted of placing mice in a conditioning chamber and 2 min later presenting a tone (2,800 Hz, 85 dB, 30 s) that coterminated with a shock (2 s, 0.3 mA = weak training; 2 s, 0.6 mA = strong training; 0.5 mA for optogenetic experiment; Figure 7B). For the optogenetic experiment, the implanted optrodes were tethered to a laser source (473 nm; Laserglow) through a split optic fiber (Precision Fiber Products) during conditioning. Light stimulation, given for 30 s immediately preceding the tone, consisted of a 20 Hz train of 5 ms pulses, and the average output light power was calibrated to 1 mW at each fiber end.

Auditory Fear Testing

Testing for auditory fear conditioning occurred 24 hr after conditioning (except where explicitly stated). Mice were placed in a novel chamber, and 2 min later the tone CS was presented (for 1 min). The amount of freezing (defined as an immobilized, crouched position, with an absence of any movement except respiration [Blanchard and Blanchard, 1969; Bolles and Fanselow, 1982]) during tone was assessed.

Statistical Analysis

Amount of freezing spent during the tone was compared across groups by ANOVA. Where appropriate, significant effects were further analyzed using Newman-Keuls or Fisher's Least Square Difference (LSD).

Assessing Anxiety-like Behavior

Open Field

Mice were placed in an open field for 10 min, and total distance and amount of time spent in the periphery were examined.

Elevated plus Maze

Mice were placed in the center of the maze, and behavior was monitored for 5 min. We analyzed time in open versus closed arms, crossings in open versus walled (closed) arms, and total distance traveled. A decrease in open arm time and/or open arm crossings is taken to reflect an anxiety-like phenotype.

catFISH or *arc* Fluorescent In Situ Hybridization for the Activity-Dependent Gene *arc*

We identified neurons specifically activated by the fear memory test (and presumably part of the fear memory trace), and we used the cellular localization of *arc* mRNA as a marker of neuronal activity, a technique that takes advantage of the unique transcriptional time course of the activity-dependent gene *arc* (Guzowski et al., 1999, 2001), as previously described (Han et al., 2007, 2009).

CNO

CNO (Toronto Research Chemicals [TRC]) was made in a stock solution of 10 mg/ml in DMSO and then diluted in saline to desired concentration. CNO was injected at a dose of 2.0 mg/kg i.p., 15 min and 3 hr before training. For the after-training (Figure 6B) and before-testing (Figure 6D) experiments, we injected the same dose of drug.

Description of the experimental procedures is included in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2014.07.017.

AUTHOR CONTRIBUTIONS

S.A.J. and P.W.F. designed, directed, and coordinated the study along with S.A.K. In addition, A.P.Y. and H.L.H. conducted behavioral experiments and performed the surgeries with help from M.M.T., and V.M. designed and performed the cell culture and immunohistochemistry experiments. C.Y. performed the in situ hybridization. A.J.R. designed the viral constructs and generated viral vectors. B.R., J.P., V.M., and M.A.W. designed and performed the electrophysiological experiments in the M.A.W. lab. A.P.Y. performed statistical analysis. S.A.J. and P.W.F. wrote the manuscript, with help from all authors.

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