Published in final edited form as:

Neurobiol Learn Mem. 2016 October ; 134(Pt A): 38–43. doi:10.1016/j.nlm.2016.04.002.

Renewal of Extinguished Fear Activates Ventral Hippocampal Neurons Projecting to the Prelimbic and Infralimbic Cortices in Rats

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Abstract

Anatomical disconnection of the ventral hippocampus (VH) and medial prefrontal cortex (mPFC) impairs the renewal of extinguished fear in rats. Here we examined whether subpopulations of neurons in the VH that project to the mPFC, including the prelimbic cortex (PL) and infralimbic cortex (IL), are selectively or differentially engaged by the renewal of fear to an extinguished auditory conditioned stimulus (CS). Rats were ipsilaterally injected with two distinct fluorescent retrograde tracers into the IL and PL and then underwent fear conditioning, extinction and retrieval in distinct contexts. Ventral hippocampal neurons were found to project to both IL and PL, and a small number of neurons projected to both regions. Fos expression was similarly elevated in each subpopulation of mPFC-projecting neuron in animals tested outside the extinction context relative to those tested in the extinction context or home controls. Interestingly, this pattern of results is not consistent with circuit models suggesting a differential role for VH projections to PL and IL in the bidirectional regulation of fear expression after extinction. Rather, these data suggest that projections from the VH to both PL and IL are uniquely involved in fear renewal, but not the suppression of fear after extinction. VH neurons may drive fear renewal by fostering fear expression by exciting PL while limiting fear suppression by inhibiting IL.

Keywords

Infralimbic; prelimbic; prefrontal cortex; extinction; renewal; fear; rat

Introduction

In recent years, considerable effort has been focused on understanding the neural mechanisms of extinction due to its essential role in clinical interventions, such as exposure

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therapy (Maren, Phan, and Liberzon, 2013; Quirk and Mueller, 2008). During extinction, repeated presentations of the conditioned stimulus (CS) gradually decrease the conditioned fear response (CR), including freezing behavior. As a result, CS no longer produces fear responses at the end of extinction learning. However, substantial evidence suggests that extinction does not erase the fear memory; rather, it generates a new inhibitory memory that competes with original fear memory (Bouton, 1993; 1994). Importantly, the extinction memory is highly context-dependent insofar as it is only expressed in the context where extinction occurred. If animals encounter an extinguished CS outside of the extinction context, fear returns or relapses and this phenomenon is called “renewal” (Bouton and Bolles, 1979). Fear renewal is a major challenge to therapeutic interventions for trauma and stress-related disorders, including post-traumatic stress disorder (PTSD) (Goode and Maren, 2014; Vervliet, Craske, and Hermans, 2013).

Considerable work has revealed that the hippocampus plays a crucial role in the context-dependence of fear memories after extinction (Fanselow, 2010; Gershman, Blei, and Niv, 2010; Komorowski, Eichenbaum, and Manns, 2009; Maren, Phan, and Liberzon, 2013; Redish, Jensen, Johnson, and Kurth-Nelson, 2007). For example, pharmacological inactivation of the hippocampus impairs the renewal of fear indexed both behaviorally and neurally (Corcoran and Maren, 2001; Corcoran and Maren, 2004; Hobin, Ji, and Maren, 2006; Ji and Maren, 2008). Moreover, the medial prefrontal cortex (mPFC) is also critically involved in contextual regulation of fear memory after extinction (Giustino and Maren, 2015; Jin and Maren, 2015b). For instance, disconnection of ventral hippocampal (VH) projections to the mPFC impairs fear renewal (Orsini, Kim, Knapska, and Maren, 2011) and functional tracing studies indicates that VH-mPFC projections are involved in fear expression after extinction (Jin and Maren, 2015a; Knapska, Macias, Mikosz, Nowak, Owczarek, Wawrzyniak, Pieprzyk, Cymerman, Werka, Sheng, Maren, Jaworski, and Kaczmarek, 2012; Orsini et al., 2011). Interestingly, neuroanatomical studies indicate that the VH projects to both the prelimbic region (PL) and infralimbic region (IL) of the mPFC (Hoover and Vertes, 2007). PL is believed to play an important role in the expression of fear memory (Corcoran and Quirk, 2007; Sierra-Mercado, Padilla-Coreano, and Quirk, 2011), whereas IL is preferentially involved in the suppression of conditioned fear responses after extinction (Milad and Quirk, 2012; Zelikowsky, Bissiere, Hast, Bennett, Abdipranoto, Vissel, and Fanselow, 2013). Moreover, neurons in the PL and IL exhibit reciprocal patterns of c-Fos expression during the renewal and suppression of fear, respectively (Knapska and Maren, 2009). Given the fact that the VH has a robust projection to both IL and PL (Hoover and Vertes, 2007), we sought to determine whether VH neurons projecting to these regions are differentially involved in the suppression or renewal of fear to an extinguished CS, respectively. To answer this question, we used fluorescent retrograde tracing together with c-Fos immunohistochemistry to examine the neuronal activity in PL- and IL-projecting VH neurons during memory retrieval. Our results indicate that mPFC projecting neurons in the VH are engaged by the renewal, but not suppression, of extinguished fear and that this effect was similar in IL- and PL-projecting populations.

Materials and Methods

Subjects

Eighteen Long-Evans male adult rats (200–224g, Blue-Spruce) were obtained from Harlan (Indianapolis, IN). The rats were individually housed on a 14/10 h light/dark cycle and had access food and water *ad libitum*. Rats were handled for 5 days before the experiment. All experimental procedures were approved by the Texas A&M University Animal Care and Use Committee.

Behavioral apparatus

All behavioral experiments were carried out in eight identical observation chambers (30 × 24 × 21 cm; MED-Associates, St. Albans, VT). Each observation chamber was constructed of a Plexiglas ceiling and rear wall, two aluminum sidewalls, a Plexiglas door. The floor of each chamber consisted of 19 stainless steel grids wired to a shock source and a solid-state grid scrambler (MED-Associates) to deliver the footshock unconditioned stimulus (US). The auditory conditioned stimulus (CS) was delivered by a speaker mounted outside of the grating in one sidewall of the chamber. A 15-W house light was fixed on the opposite sidewall and a ventilation fan was installed in each chamber. Each chamber was placed in a sound-attenuating cabinet. Three contexts were generated by the manipulation of the combination of sensory stimuli. In Context A, 1% acetic acid was used to wipe the ceiling, sidewalls, rear wall, door and grids of each chamber. The house lights and the fans were turned on. Cabinet doors were left open. White light was on in the behavior room. Rats were transported in white transport boxes. In Context B, the chamber was wiped with 1% ammonium hydroxide. House lights, fans and computer monitor were turned off and cabinet doors were closed. Red room light was turned on in the behavior room. Black transport boxes were used for rat transportation. For Context C, the odor was generated by 70% ethanol. House lights and fans were on. Room light was white and cupboard doors were open. Black Plexiglas floors were placed on the grids. Wood chip bedding was added to white buckets for rat transportation. In all the contexts, a stainless steel pan fill with a thin layer of the respective odor of the contexts was inserted under the grid of each chamber.

Each chamber was seated on a load-cell platform that recorded chamber displacement in response to each rat's motor activity; load-cell activity was digitized and acquired with Threshold Activity software (MED-Associates). Before the experiment, all load-cell amplifiers were calibrated to a fixed chamber displacement. Load-cell amplifier output (–10 to +10 V) from each chamber was digitized (5 Hz) and transformed to a value ranging from 0 to 100. Freezing was quantified by computing the number of observations for each rat that had a value less than the freezing threshold (load-cell activity = 10) for at least 1 sec.

Surgical procedures

Rats were anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and given atropine sulfate (0.4 mg/kg, i.p.). After induction of anesthesia, rats were placed on stereotaxic apparatus (David Kopf Instruments) and 27-gauge injectors were lowered into PL [anteroposterior (AP), +2.9 mm; mediolateral (ML), ±0.45 mm; dorsoventral (DV), –3.3 mm, from dura] and IL (AP, +2.8mm, ML, ±2.8 mm, DV, –4.1mm from dura, with 30° angle

on the coronal plain toward the midline). Each injector was connected to polyethylene tubing, which was attached to a Hamilton syringe (10 μ l) placed on an infusion pump. Alexa Fluor-594 conjugated cholera toxin B (CTb) (Life Technology) was infused into the PL and Alexa Fluor-488 conjugated CTb was infused into the ipsilateral IL at a rate of 0.1 μ l/min for 5 minutes (0.5 μ l each; 5 μ g/ μ l). The injectors remained in the brain for 15 minutes before removal. Rats were placed back to their home cages for post-operative recovery for one week.

Behavioral procedures

Eighteen rats were randomly assigned to three groups: SAME (n=6), DIFF (n=5) and HOME (n=7). We used a three-context renewal procedure (Orsini et al., 2011) ("ABC") for DIFF, in which rats were conditioned in context A, extinguished in context B, and tested in context C. SAME rats were conditioned in context A, extinguished and tested in context C ("ACC"). HOME rats were conditioned in context A, extinguished in either context B or context C, and remained in their home cages during the test of other groups.

After recovery from surgery, rats were conditioned in context A, in which five tone (CS; 10 s, 80 dB, 2 kHz)-footshock (US; 1.0 mA, 2 s) trials were delivered. After 24 hours, rats were extinguished in either context B or C, where they received 45 CS-alone trials (10 s, 80 dB, 2 kHz, 30 s ITIs) for two consecutive days. Before the extinction session, rats were exposed to the alternative context (i.e., they were exposed to context C if they were extinguished in context B) to ensure that the test contexts were equally familiar for all of the rats. The following day, all the rats underwent test in context C, where they received 5 CS-alone trials (10 s, 80 dB, 2 kHz, 30 s ITIs).

Immunohistochemistry

Ninety minutes after the first tone of the retrieval test, rats were euthanized by overdose of sodium pentobarbital (0.5 ml) and were transcardially perfused with ice-cold 0.01 M PBS (pH 7.4) followed by 4% paraformaldehyde (PFA) in 0.1M PBS (pH 7.4). Brains were fixed in 4% paraformaldehyde over night at 4°C then placed in 30% sucrose solution at 4°C until sunken. Coronal brain sections (30 μ m) were collected on a cryostat at -20°C. Sections containing VH were collected every 210 μ m.

Immunohistochemistry was performed on free-floating sections. Brain sections were washed three times in 1 $\times$ Tris-buffered saline with 0.1% Tween 20 (TBST, pH 7.4) for 30 min each. The sections were then incubated in 10% normal donkey serum (NDS) in TBST for 2 h at room temperature followed by two washes in TBST for 5 min each. Then the tissue was incubated in primary antibody in TBST with 3% NDS (goat anti-c-Fos antibody at 1:2000; sc-52-G, Santa Cruz Biotechnology) for 48 h at 4 °C. The sections were washed three times in TBST for 10 min each, and incubated in secondary antibody in TBST with 3% NDS (biotinylated donkey anti-goat antibody at 1:200; sc-2042, Santa Cruz Biotechnology) for 2 h at room temperature. The tissue was washed three times in TBST for 10 min each and then incubated in streptavidin conjugated AlexaFluor 350 in TBST with 3% NDS (Streptavidin-AF350 at 1:500; s-11249, Life Technology) for 1 h at room temperature. The tissue was then

rinsed three times in TBS for 10 min each and mounted onto subbed slides in 0.9% saline and cover slipped with Fluoromount (Sigma-Aldrich).

Image analysis

Three images for the VH (−5.6, −6.3 and −6.8 mm posterior to bregma) were taken for the quantification. All images were taken at $20\times$ magnification with a an Olympus BX53 microscope. Single-, double- and triple-labeled neurons for each fluorophore were counted. Counts for each image was averaged and standardized to counts/mm². For the analysis of Fos expression in PFC-projecting neurons, the number of double- or triple-labeled neurons was normalized to the total number of CTb-positive neurons in each animal. This allowed animals with different degrees of CTb transport and labeling to be compared to one another.

Data analysis

All data were analyzed with analysis of variance (ANOVA). Post-hoc comparisons in the form of Fisher's protected least significant difference (PLSD) tests were performed after a significant overall F ratio. All data are presented as means $\pm$ SEM. One rat failed to extinguish and another two rats were excluded from the neuronal and behavioral analyses due to lack of tracer transport. Hence, the final group sizes were SAME (n=5), DIFF (n=5), and HOME (n=5).

Results

Representative CTb injection sites in the IL and PL are shown in Figure 1A along with a schematic illustration of the injection sites in Figure 1C. IL- and PL-projecting neurons in VH were labeled with different AlexaFluor-CTb conjugates and c-Fos was visualized with AlexaFluor 350 (Figure 1B). IL- and PL-projecting neurons were distributed throughout the ventral hippocampal formation, including hippocampal area CA1 and the ventral subiculum.

Fear conditioning resulted in robust increases in freezing behavior, and this did not differ between the groups (not shown). During extinction training, rats in each group exhibited high levels of freezing to the CS at the beginning of the extinction and similar reductions in conditioned freezing both within and between the two extinction sessions (Figure 2, left panel). This impression was confirmed by a significant main effect of extinction block [$F(3, 56)=45.52$, $p<0.0001$] without a main effect of group or a group $\times$ block interaction ($F_s<1.7$). During the retrieval test, rats exhibited low levels freezing when the CS was presented in the extinction context (SAME), whereas rats tested outside of the extinction context (DIFF) showed fear renewal [Figure 2, right panel; group $\times$ block interaction $F(1, 8)=3.66$, $p<0.01$]. Importantly, differential freezing among the SAME and DIFF groups was not attributable to physical differences in the test contexts because all testing was conducted in an identical context with the same CS.

Ninety minutes after retrieval testing, the rats were perfused with paraformaldehyde and their brains were extracted. IL- and PL-projecting neurons in VH were labeled with different AlexaFluor-CTb conjugates and c-Fos was visualized with AlexaFluor 350 (see Figure 1B). As shown in Figure 3A, CTb injections into the IL labeled significantly more VH neurons than injections into the PL; a small number of neurons projected to both areas [Figure 3A;

main effect of cell type, $F(2, 14) = 19.62$, $p < 0.0001$]. Post-hoc comparisons confirmed that greater numbers of VH neurons projected to IL relative to PL, both of which differed from dual-projecting neurons ($p < 0.05$). Dual-projecting neurons accounted for roughly 3~4% of the total labeled neurons in the ventral hippocampus.

We next examined c-Fos expression in the VH, independent of projection target, as an index of neuronal activity in HOME, SAME or DIFF group. As shown in Figure 3B, the number of c-Fos expressing neurons in the three groups differed [main effect of group, $F(2, 12) = 7.35$, $p < 0.01$]. Post-hoc comparisons revealed that both SAME and DIFF rats exhibited greater level of c-Fos expression than rats in the HOME control ($p < 0.05$; $p < 0.01$), but did not differ from each other. This confirms previous reports showing that presentation of an extinguished CS increases Fos expression in the VH independent of the context in which it is presented (Jin and Maren, 2015; Knapska and Maren, 2009; Orsini et al., 2011).

Of course, of critical interest is the nature of retrieval-induced Fos expression in VH neurons targeting the PL or IL (or both). To this end, we examined the proportion of Fos-positive neurons among CTb-labeled neurons in the VH. As shown in Figure 3C, a greater proportion of PFC projectors in the VH expressed Fos in the DIFF condition relative to animals in the HOME or SAME conditions. This impression was confirmed in a two-way ANOVA with factors of group (SAME, DIFF or HOME) and cell-type (IL-, PL-, or dual-projecting), which revealed only a significant main effect of group [main effect of group, $F(2,12) = 33.7$, $p < 0.0001$]. Post-hoc comparisons ($p < 0.05$) indicated that DIFF rats exhibited a greater proportion of c-Fos-positive CTb-labeled neurons than rats in the SAME and HOME groups, which did not differ from one another. These results indicate that renewal of fear to an extinguished CS similarly increases Fos expression in VH neurons projecting to IL, PL, or both regions. Interestingly, there was a highly significant correlation between the percentage of freezing on the retrieval test and the number of Fos-positive projection neurons (aggregated across PL, IL, and dual-projecting populations) in the VH (Figure 4; Pearson $r = 0.789$ $p < 0.01$). This replicates a previously reported correlation between retrieval-induced Fos expression and freezing behavior after extinction (Jin and Maren, 2015). Collectively, these data suggest that the ventral hippocampus plays a key role in fear renewal through its projections to the medial prefrontal cortex.

Discussion

Consistent with previous work, the present study reveals that the ventral hippocampus sends direct projections to both the prelimbic and infralimbic divisions of the medial prefrontal cortex (Hoover and Vertes, 2007). Interestingly, in the present study VH neurons projecting to the IL outnumbered those projecting to the PL, an observation that has not previously been reported. This might reflect the different distribution of VH efferents along the rostral-caudal extent of the mPFC, although it is possible that there was differential CTb uptake in the two areas. We also found a small number of double-labeled neurons in the VH, suggests that some VH neurons project to both the IL and PL.

As we have previously reported (Jin and Maren, 2015; Knapska et al., 2009; Orsini et al., 2011), ventral hippocampal Fos expression was increased after the presentation of an

extinguished CS in either inside or outside the extinction context. In a previous study, we found that this pattern of retrieval-induced Fos expression was most pronounced in hippocampal area CA1, whereas ventral subicular Fos expression is selectively induced in the renewal context (Jin and Maren, 2015a). It has been suggested that VH neurons projecting to PL and IL might have different roles in the renewal and suppression, respectively, of extinguished fear (Maren, 2011; Maren et al., 2013). However, we now show that both IL- and PL-projecting neurons in the VH exhibit similar increases in c-Fos expression during both the renewal of fear outside of the extinction context (DIFF) as well as the suppression of fear in the extinction context (SAME). Indeed, IL- and PL- projecting VH neurons were preferentially activated in rats in the DIFF condition, suggesting that hippocampal-prefrontal projections have a selective role in increasing the expression of fear (e.g., during renewal) (Adhikari, Topiwala, and Gordon, 2010).

The observation that PL- projecting VH neurons are preferentially engaged during the renewal of extinguished fear corroborates previous reports. In this way, the hippocampus is positioned to drive fear expression through either direct projections to the amygdala (Herry, Ciocchi, Senn, Demmou, Muller, and Luthi, 2008; Knapska et al., 2012; Orsini et al., 2011; Orsini and Maren, 2012) or indirectly via the PL (Corcoran and Quirk, 2007; Sierra-Mercado et al., 2011). However, a surprising outcome was that VH neurons projecting to the IL were also preferentially activated after the renewal of fear outside the extinction context. Given that the IL is involved in the suppression of conditioned fear (Burgos-Robles, Vidal-Gonzalez, and Quirk, 2009; Quirk and Mueller, 2008; Sierra-Mercado et al., 2011), the present results suggest that renewal-related increases in VH neurons projecting to IL might activate an inhibitory microcircuit within IL to attenuate fear inhibition (Lovett-Barron, Turi, Kaifosh, Lee, Bolze, Sun, Nicoud, Zemelman, Sternson, and Losonczy, 2012).

One possibility is that feed-forward inhibition generated by VH projections in IL (Gabbott, Headlam, and Busby, 2002) ultimately dampens neuronal activity in the IL thereby limiting fear suppression and permitting fear relapse. Indeed, if extinction applies an inhibitory “brake” to the expression of conditioned fear, then circumstances that result in a return of fear (such as renewal) must release the brake; VH-mediated inhibition of IL may be involved in this process. Consistent with this, previous work has revealed that electrical stimulation of the VH produces substantial feed-forward inhibition in the mPFC (Tierney, Dégenétais, Thierry, Glowinski, and Gioanni, 2004) and VH-mediated inhibition of mPFC can influence the expression of fear after extinction (Sotres-Bayon, Sierra-Mercado, Pardilla-Delgado, and Quirk, 2012). Therefore, we propose that projections from VH to IL oppose the expression of extinction via feed-forward inhibition by GABAergic interneurons in IL. This proposed feed-forward inhibition model could potentially explain why fear renewal is associated with the activation of IL-projecting neurons in the VH activity and inhibition of neuronal activity in the IL (Knapska and Maren, 2009; Orsini et al., 2011). An interaction between PL and IL during fear renewal might also contribute to the higher activity in IL-projecting neurons (Zelikowsky et al., 2013). Ultimately, the relatively stronger projection of the VH to the IL dictates that feed-forward inhibition of the IL may be greater than that in the PL, thereby yielding a net increase in fear expression when PFC-projecting neurons in the VH are engaged.

In sum, the present results reveal that the presentation of extinguished CSs induces Fos in ventral hippocampal neurons projecting to the medial prefrontal cortex. Importantly, neurons targeting the prelimbic and infralimbic cortices did not differ in their propensity to exhibit renewal-related Fos expression. However, both the substantially greater projection of the VH to IL and the potent feed-forward inhibition in this circuit suggests that the dominant effect of VH activation is an inhibition of IL output. The inhibition of infralimbic output may permit fear renewal by releasing the amygdala from the IL-mediated inhibition that normally contributes to the suppression of fear after extinction. Ultimately, suppressing the activity of inhibitory interneurons in the infralimbic cortex may be a novel strategy for fostering the expression of extinction memories and preventing fear relapse.

Acknowledgments

Supported by grants from the National Institutes of Health (MH045961) and a McKnight Foundation Memory and Cognitive Disorders Award.

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Highlights

- Presentation of the conditioned stimulus outside of the extinction context induced fear renewal.
- Ventral hippocampal (VH) neurons were found to project to both infralimbic cortex (IL) and prelimbic cortex (PL) of the medial prefrontal cortex (mPFC). A small number of neurons projected to both regions.
- Fos expression was similarly elevated in each subpopulation of mPFC-projecting neurons after the renewal of fear to an extinguished CS..

Figure 1.

A. AlexaFluor conjugated cholera toxin B (CTb) infusion sites within the PL and IL. *B.* Representative coronal sections at the level of the VH showing PL- and IL-projecting neurons labeled by the different tracers. *Top left:* HOME, CA1; *Bottom left:* SAME, CA1; *Top right:* SAME, ventral subiculum; *Bottom right:* DIFF, ventral subiculum. *C.* Schematic illustration of the CTb injection sites in the mPFC. Red: AlexaFluor 594-CTb injected in PL; Green: AlexaFluor 488-CTb injected in IL; crosses: injection sites of HOME rats; circles: injection sites of IT rats; dots: injection sites of DIFF rats. PL-projecting neurons are red, IL-projecting neurons are green, and Fos-positive neurons are blue. White arrows: double-labeled neurons; red arrow: triple-labeled neuron.

Figure 2.

Conditioned freezing behavior. *Left:* mean percentage of freezing during the extinction sessions. Freezing was averaged across the early extinction period (E, first five trials) as well as during late extinction trials (L, last five trials). *Right:* mean percentage of freezing during the test session, which consisted of five tone-alone presentations after a baseline (BL) period.

Figure 3.

Quantification of CTb labeling and Fos expression in neurons in the VH after fear renewal.

A. Mean cell counts for CTb-positive neurons in VH. Neurons in VH projected to the infralimbic cortex (IL, empty), prelimbic cortex (PL, black), or both areas (Dual, gray). *B.*

Mean cell counts for Fos-positive neurons in VH among animals tested outside the extinction context (DIFF), inside the extinction context (SAME), or untested animals (HOME). *C.* Mean percentage of Fos-positive projection neurons (IL, PL, or dual-

projecting) in the VH of rats in each of the three behavioral groups; counts were normalized to the total number of CTb neurons in each animal.

Figure 4. Correlation between average freezing behavior during the retrieval test among rats in SAME and DIFF and the average percentage of Fos-positive CTb-labeled cells in the VH.