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ARTICLE

The medial entorhinal cortex mediates basolateral amygdala effects on spatial memory and downstream activity-regulated cytoskeletal-associated protein expression

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The basolateral amygdala (BLA) modulates the consolidation of dorsal hippocampus (DH)-dependent spatial and dorsolateral striatum (DLS)-dependent cued-response memories, often in competition with one another. Evidence suggests that a critical mechanism for BLA influences on memory consolidation is via effects on activity-regulated cytoskeletal-associated protein (ARC) in downstream brain regions. However, the circuitry by which the BLA modulates ARC in multiple competing memory systems remains unclear. Prior evidence indicates that optogenetic stimulation of BLA projections to the medial entorhinal cortex (mEC) enhances the consolidation of spatial learning and impairs the consolidation of cued-response learning, suggesting this pathway provides a circuit for favoring one system over another. Therefore, we hypothesized the BLA-mEC pathway mediates effects on downstream ARC-based synaptic plasticity related to these competing memory systems. To address this, male and female Sprague–Dawley rats underwent spatial or cued-response Barnes maze training and, 45 min later, were sacrificed for ARC analysis in synaptoneurosomes from the DH and DLS. Initial experiments found that spatial training alone increased ARC levels in the DH above those observed in control rats and rats that underwent a cued-response version of the task. Postspatial training optogenetic stimulation of the BLA–mEC pathway altered the balance of ARC expression in the DH vs. DLS, specifically shifting the balance in favor of the DH-based spatial memory system, although the precise region of ARC changes differed by sex. These findings suggest that BLA–mEC pathway influences on ARC in downstream regions are a mechanism by which the BLA can favor one memory system over another.

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INTRODUCTION

Decades of research suggest the existence of multiple memory systems responsible for processing different types or attributes of learning [1–5]. Notably, the dorsal hippocampus (DH) and dorsolateral striatum (DLS) systems mediate spatial and cued-response memory, respectively, often in competition with one another [1, 2, 6]. In contrast, the basolateral complex of the amygdala (BLA) plays a unique role in memory consolidation, as it modulates many different kinds of memories [7–13]. Indeed, post-training BLA manipulations alter the retention of spatial and cued-response memories, even as BLA lesions do not disrupt the memories themselves [6]. These findings suggest that the BLA interacts with other memory systems to promote the consolidation of memories of places, cue associations, and other types of information, likely during times of emotional arousal [14]. This provides an obvious adaptive advantage, as mammals cannot afford to learn about potentially dangerous situations through many repeated trials.

Prior work suggests that a critical mechanism for BLA influences on memory consolidation is via effects on activity-regulated cytoskeletal-associated protein (ARC) in downstream brain regions [15, 16]. ARC is a plasticity-associated protein that has been widely implicated in learning and memory as a marker and effector of synaptic plasticity [15, 17, 18]. Arc mRNA is targeted to stimulated regions along dendrites, where it can be locally translated and interact with structural proteins to influence synaptic plasticity [19, 20]. Evidence suggests that BLA activity influences hippocampal ARC protein but not mRNA expression, indicating that a post-transcriptional influence of the BLA on ARC protein expression is a likely mechanism by which the BLA modulates memory consolidation [12, 15, 17]. Indeed, studies suggest that memory-enhancing and memory-impairing drug infusions into the BLA alter ARC protein expression in synaptic fractions taken from downstream regions, such as the DH and anterior cingulate cortex [15, 16]. Furthermore, inhibiting ARC activity in both downstream regions impairs memory consolidation [18, 21]. These data indicate that BLA activity influences ARC protein expression elsewhere in the brain in a learning-dependent manner and that changes in ARC in those regions are related to observed changes in memory. However, the circuitry by which the BLA modulates ARC in multiple competing memory systems, particularly when they may not have monosynaptic connections with the BLA, remains unclear. Although the BLA does not directly project to the DH in rats [22], it innervates the medial entorhinal cortex (mEC) [23], a region that processes spatial information [24–28] that is then communicated to the DH [29, 30]. The BLA does not project

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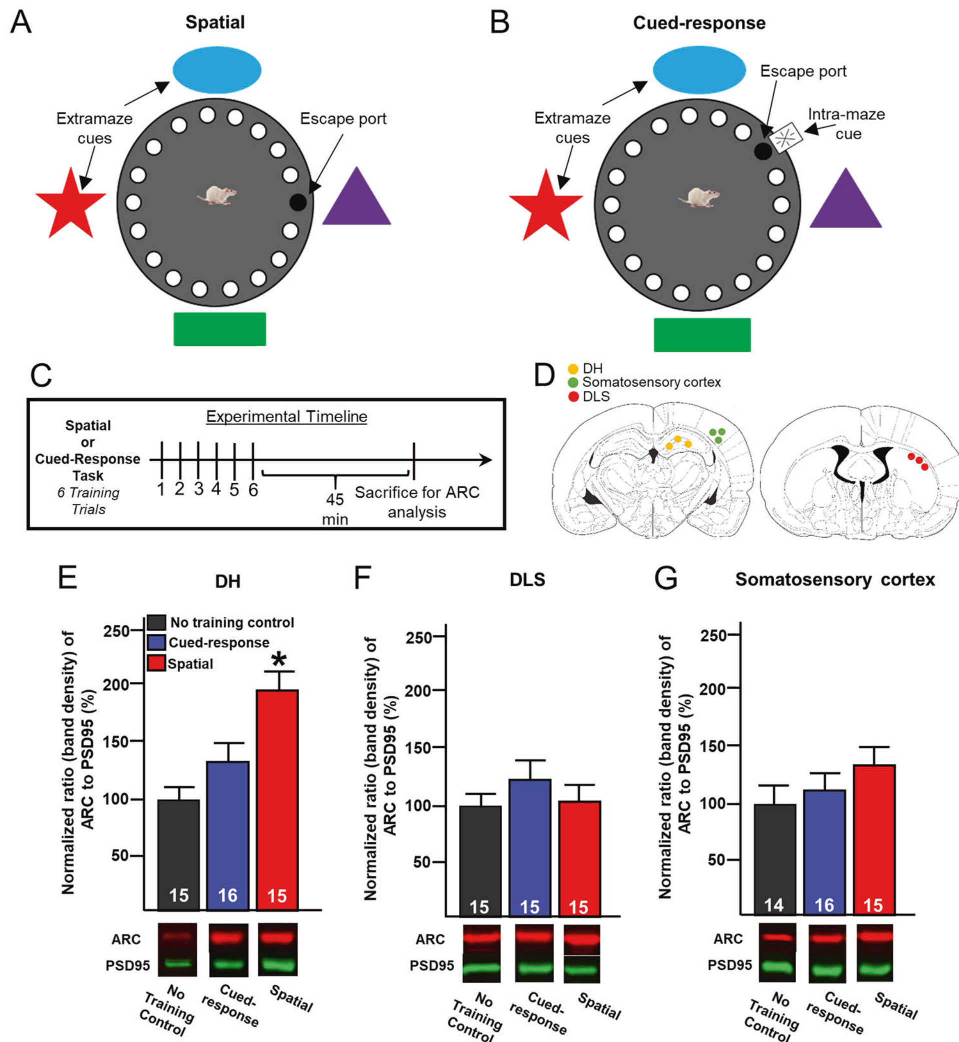


Fig. 1 ARC protein expression effects of spatial and cued-response Barnes maze training (Experiment 1). a, **b** Illustrations of the Barnes maze for spatial and cued-response training, respectively. For spatial training, the escape port remained in the same location on every trial, enabling rats to use a spatial strategy to find the port. For cued-response training, a distinct intramaze cue was attached directly to the escape port. The escape port and cue were randomly shifted to a different cardinal direction for each training trial. **c** Experimental timeline for spatial and cued-response training. **d** Schematic diagram illustrating the tissue punch sites in the DH, somatosensory cortex, and DLS where ARC was analyzed. **e**–**g** ARC protein expression (mean ± SEM) (top) and representative immunoblots (bottom) for synaptoneurosomes from the DH (**e**), DLS (**f**), and somatosensory cortex (**g**) for those rats that received no training, cued-response training, or spatial training. Rats that received spatial training had significantly higher ARC levels in the DH compared to no training-control rats and those rats that received cued-response training. There were no significant differences in ARC protein expression in the DLS or somatosensory cortex. $*p < .05$ compared to no training-control and cued-response values. ARC was normalized to PSD95 by calculating the ratio band density of ARC to that of PSD95 and then expressed as a percentage of normalized control values.

in a widespread manner throughout the dorsal striatum, though it innervates the more posterior regions [6]. Our prior work found no effect of stimulating BLA terminals in the posterior dorsal striatum on cued-response learning [31], indicating that the circuit by which the BLA influences DLS activity with regard to cued learning is more complex than previously believed [32–34].

Recent work from this laboratory found that optogenetic stimulation of BLA projections to the mEC selectively enhances the consolidation of DH-dependent spatial/context learning and impairs the consolidation of DLS-dependent cued-response learning [31]. Thus, in the present study, we hypothesized that the BLA–mEC pathway mediates effects on downstream ARC-based synaptic plasticity related to these competing memory systems. To address this, several experiments were conducted to examine changes in ARC protein expression in relationship to learning and stimulation of the BLA–mEC pathway. Rats received optogenetic stimulation of this pathway immediately following spatial training in a Barnes maze, as we have done previously [31], and were subsequently sacrificed for ARC protein analysis. Overall, the results reveal that BLA–mEC stimulation shifts the balance of downstream ARC expression in the DH and DLS in favor of spatial learning systems, providing a circuit-based mechanism for how the BLA may direct the influence of one memory system over another.

MATERIALS AND METHODS

For all sections in "Materials and methods", additional details are provided in the Supplement.

Subjects and surgery

Male and female Sprague–Dawley rats (185–200 g and 150–175 g, respectively, at time of first surgery; Envigo; $n = 189$) were used for this study. All rats were single-housed. All procedures were in

compliance with the National Institutes of Health guidelines for care of laboratory animals and were approved by the University of Iowa Institutional Animal Care and Use Committee. Rats (in Experiments 2–4) received virus microinjections (rAAV5-CaMKIIα-hChR2(E123A)-eYFP or rAAV5-CaMKIIα-eYFP) into the BLA. Four weeks later rats underwent a second surgery in which optical probes were aimed bilaterally at the mEC.

Optical manipulations

When used, illumination was provided bilaterally via a DPSS laser (300 mW, 473 nm), connected via fiber optic cables and set to provide 10 mW of light at the fiber tip [31, 35–38]. The illumination was provided to rats in a separate holding chamber (30 cm × 30 cm × 30 cm). In all cases, the comparison control was a CaMKIIα-eYFP group used to examine the effects of illumination (and, thus, possible heating) alone. Optogenetic manipulations occurred 5 weeks after virus microinjections.

Behavioral training

A Barnes maze was used to investigate the consolidation of spatial and cued-response learning. The Barnes maze consisted of an exposed and elevated, brightly lit circular platform with evenly spaced holes along the perimeter, one of which led to an escape port (Fig. 1a). Extramaze cues consisted of specific symbols on the walls around the maze as well as the general layout of equipment in the room. NOLDUS Ethovision recording software was used to record the time to find the escape port (latency) and the time spent in each quadrant of the maze (duration). For spatial training, the escape port of the Barnes maze was maintained in the same location relative to the extramaze cues on each trial (Fig. 1a). For cued-response training, a distinct intramaze cue was attached directly to the escape port. The escape port and cue were randomly shifted to a different cardinal direction for each training trial (Fig. 1b).

For the 3 days prior to training, rats were handled individually for 1 min/day and placed in the holding chamber (used for optogenetic manipulations) for 1 min to habituate the rats to the holding chamber, with the exception of the last day of handling where rats were placed in the holding chamber for 9 min.

For both kinds of training, rats underwent multiple trials on the training day (Day 1). For each trial, the rat was placed in the center of the Barnes maze and allowed to freely explore the entire apparatus for 60 s to find the escape port and enter. If a rat entered the escape port prior to the 60 s mark, it was permitted to remain in the escape port for 30 s. If the rat did not enter the escape port within 60 s, it was placed in the escape port and permitted to remain there for 30 s. After each trial, the rat was removed from the escape port and placed in its home cage for 1 min while the maze was wiped with 20% EtOH to remove olfactory cues. This process was repeated for six consecutive trials (Experiment 1 spatial and cued-response strategy training) or three consecutive trials (Experiment 2 spatial strategy training). The number of training trials was extended for Experiment 1 to ensure sufficient training for ARC protein expression.

In a subset of experiments (Experiment 2), retention was tested 2 days later (Day 3) when rats again were placed on the center of the Barnes maze and allowed to freely explore for 180 s. For this spatial version of the task, the escape port was oriented in the same direction as it had been during training on Day 1. Latency to enter the escape port and the duration spent in the target quadrant were used as the indices of retention.

Tissue preparation and Western blotting

For ARC analysis, rats were euthanized 45 min [15, 39, 40] after the completion of spatial or cued-response Barnes maze training (Experiment 1) or 45 min after the completion of optical stimulation (Experiments 2–4). To examine BLA influences on multiple competing memory systems, ARC expression was analyzed in the DH and DLS for all experiments as well as a control region (somatosensory cortex). The DH, DLS, and somatosensory cortex tissue from the left hemisphere was homogenized with its respective tissue from the right hemisphere, using a sonicator in homogenization buffer solution [17]. An enriched synaptic fraction (synaptoneurosomal preparation) was created, as demonstrated by an increase in PSD95 expression and an absence of nuclear proteins such as acetyl-H3 [41]. Western blotting was then conducted for ARC and PSD95, and average density of each ARC band was normalized to PSD95 and then expressed as a percentage of normalized control values.

Experimental design

Experiment 1. Male and female rats received either spatial (Fig. 1a) or cued-response (Fig. 1b) training or were part of a no training-control group in which they were removed from their home cage and placed in the holding chamber (same as the illumination holding chamber used for optogenetic manipulations in Experiment 2–4) for 15 min. Forty-five minutes after training, rats were euthanized for ARC protein analysis.

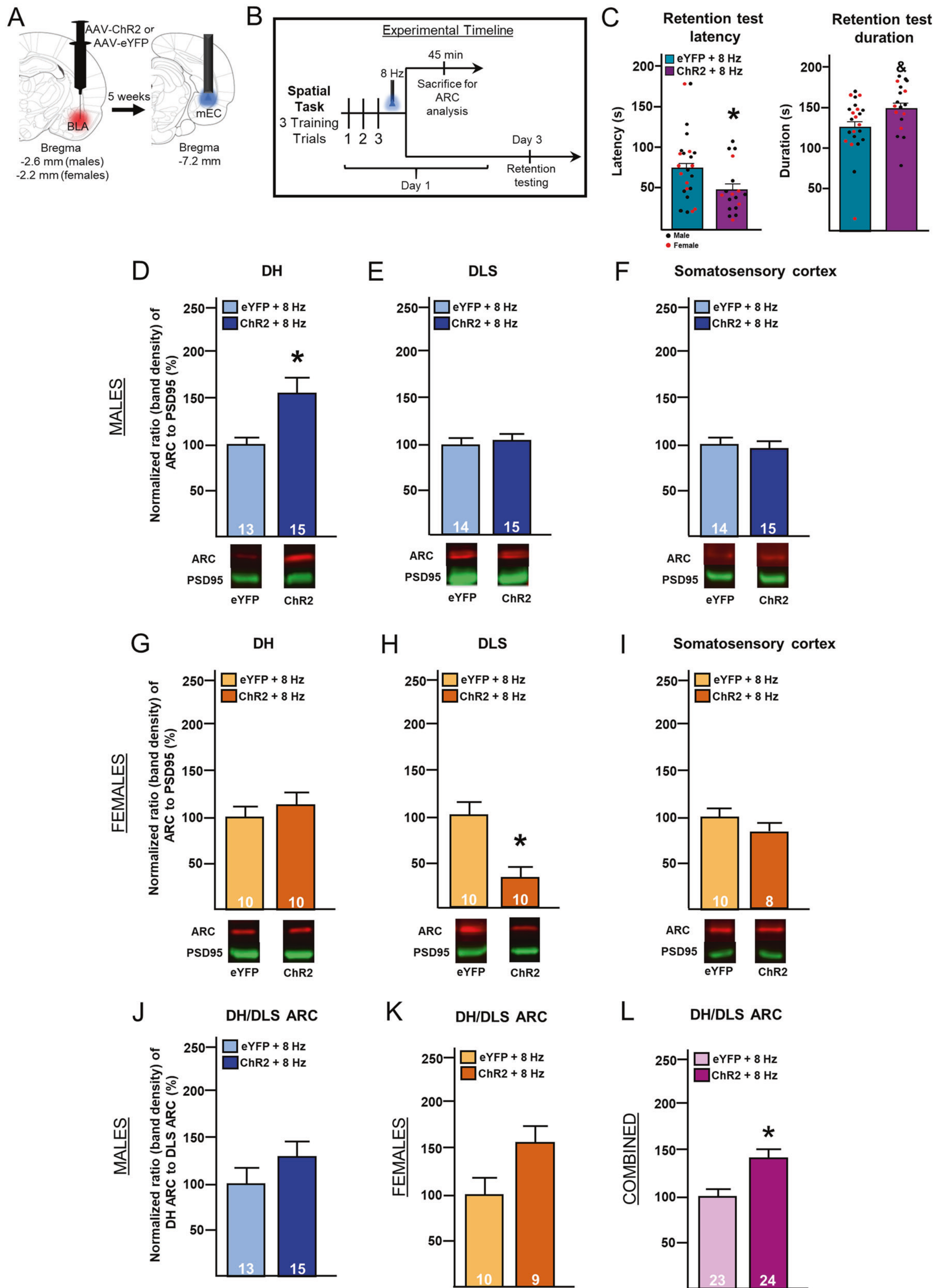
Experiment 2. Experiment 2 examined whether the BLA–mEC pathway mediates effects on downstream ARC-based synaptic plasticity. For this experiment, male and female rats received illumination of either ChR2 or eYFP-control-transduced BLA fibers in the mEC (Fig. 2a) immediately following the final spatial training trial (Fig. 2b), using the following illumination parameters (as previously used): 15 min of 2 strains of 8 Hz light pulses (pulse duration = 5 ms), given every 10 s [31]. Three training trials were used in Experiment 2 to prevent ceiling effects in order to observe any enhancement in learning. Half of the animals were sacrificed following post-training stimulation to assess the effects of 8 Hz stimulation on ARC protein expression. Additionally, each rat was given a ratio score, which reflected the ratio of ARC protein expression in DH vs. DLS [42]. The other half of the animals were given a single retention test 2 days after spatial training to verify our previously published work [31].

Experiment 3. Experiment 3 examined whether stimulating the BLA–mEC pathway in the absence of behavioral training alters ARC protein expression [15]. Male and female rats received optical illumination of the mEC to provide illumination of either ChR2 or eYFP-control-transduced BLA fibers in the absence of spatial training, using the same parameters as Experiment 2. Because exploration of novel environments alone engages the hippocampus and increases hippocampal ARC expression [39, 40, 43], the stimulation-alone groups were not exposed to the Barnes maze. Rather, on the day of stimulation, rats were removed from their homecage and placed directly into the holding chamber, to which they had previously been exposed, for the duration of optical stimulation.

Experiment 4. Based on the results with 8 Hz stimulation in male rats in Experiment 3, Experiment 4 examined whether stimulating the pathway with a different (higher) frequency would alter ARC expression. For this experiment, male rats received optical illumination of the mEC to provide illumination of either ChR2 or eYFP-control-transduced BLA fibers in the absence of spatial training, using the following illumination parameters: 15 min of 2-s-trains of 40 Hz light pulses (pulse duration = 5 ms), given every 10 s [31].

Histology and statistical analysis

Opsin expression and mEC optic probe placement was verified for Experiments 2–4 via immunohistochemistry and Nissl staining, respectively (see Supplement Fig. 1). GraphPad Prism 8 was used for all statistical analyses in Experiments 1–4. For behavioral analysis, training latencies and time in target quadrant (duration)



during training for Barnes maze experiments were analyzed using two-way ANOVAs. Retention latencies and durations in target quadrant for all behavioral experiments were analyzed using *t*-tests. Scatter plots are included to best reflect the numerical spread of the data. For protein analysis, either a *t*-test or a one-way ANOVA with a Holm-Sidak multiple comparisons test was used. Additionally, in Experiment 2, each rat was given a ratio score, which reflected the ratio of ARC protein expression (normalized to PSD95) across the DH and DLS. Ratios were compared across groups using unpaired *t*-tests. The Grubbs method was used to

Fig. 2 Effects of optical stimulation of the BLA–mEC pathway immediately after spatial training on ARC protein expression (Experiment 2). a Schematic diagram of BLA injection site (left), incubation time, and optic probe placement in mEC (right). **b** Experimental timeline for Experiment 2. **c** Two days after training, half of the rats were tested for retention in a single 180 s trial. Left: latencies to locate the escape port during the retention test. Rats that received 8 Hz stimulation of ChR2-transduced BLA fibers in the mEC had significantly decreased latencies to find the escape port compared to their eYFP-control counterparts (eYFP + 8 Hz $n = 24$; ChR2 + 8 Hz $n = 18$). Right: duration spent in the target quadrant during the retention test. Rats that received 8 Hz stimulation of the BLA–mEC pathway showed a trend towards increased time spent in the target quadrant of the maze compared to their eYFP-control counterparts (eYFP + 8 Hz $n = 23$; ChR2 + 8 Hz $n = 18$). **d**–**f** ARC protein expression (mean ± SEM) (top) and representative immunoblots (bottom) for synaptoneurosomes from the DH (**d**), DLS (**e**), and somatosensory cortex (**f**) for male rats that were given spatial training immediately followed by 8 Hz stimulation of the BLA–mEC pathway. Rats that received 8 Hz stimulation of the BLA–mEC pathway had significantly higher levels of ARC protein expression in the DH compared to eYFP-control animals. There were no significant differences in ARC expression in the DLS or somatosensory cortex. **g**–**i** ARC protein expression (mean ± SEM) (top) and representative immunoblots (*bottom*) for synaptoneurosomes from the DH (**g**), DLS (**h**), and somatosensory cortex (**i**) for female rats that were given spatial training immediately followed by 8 Hz stimulation of the BLA–mEC pathway. There were no significant differences in ARC expression in the DH or somatosensory cortex. However, rats that received 8 Hz stimulation of the BLA–mEC pathway had significantly lower levels of ARC protein expression in the DLS compared to eYFP-control animals. **j**–**l**, Ratio of ARC expression (mean ± SEM), DH/DLS, in males (**j**), females (**k**), and males and females combined (**l**) for rats that were given spatial training immediately followed by 8 Hz stimulation of the BLA–mEC pathway. Rats that received 8 Hz stimulation of the BLA–mEC pathway had a significantly higher DH/DLS ARC ratio compared to eYFP-control animals. $^{*}p < .05$ compared to eYFP-control values; $^{\&}p < 0.1$, compared to eYFP-control values. ARC was normalized to PSD95 by calculating the ratio band density of ARC to that of PSD95 and then expressed as a percentage of normalized control values.

identify statistical outliers across all experiments. The α level was set to 0.05. All measures are expressed as mean ± SEM, and each group's n is indicated in the figure below its respective bar.

RESULTS

Experiment 1

Experiment 1 examined whether spatial or cued-response training in the Barnes maze task alters ARC protein expression in synaptic fractions. Figure 1a–d shows illustrations of the spatial and cued-response versions of the Barnes maze (A and B, respectively), the experimental timeline for Experiment 1 (C), and the schematic diagram illustrating the tissue punch sites where ARC was analyzed (D).

Males and females did not show differences during spatial training or cued-response training, and therefore, were combined in each group (see Supplement). Additionally, rats that were trained on the spatial task did not show performance differences during training compared to those rats, that received cued-response training on their respective tasks (see Supplement).

Figure 1e shows ARC protein expression in DH synaptic fractions for the three groups of rats. As males and females showed the same pattern of changes in ARC, males and females were not disaggregated. A one-way ANOVA revealed a statistically significant difference in ARC expression between groups ($F_{(2, 43)} = 7.23$, $p = 0.002$). Posthoc analyses indicated that ARC levels in the DH in rats that underwent spatial training was significantly higher compared to those observed in the no training-control group ($p = 0.0015$), and the cued-response group ($p = 0.045$). However, ARC levels did not significantly differ between those rats given cued-response training and those in the no training-control group ($p = 0.15$). Figure 1f, g show ARC protein expression in DLS and somatosensory cortex synaptic fractions, respectively, for the three groups. One-way ANOVAs revealed no effect in either case ($F_{(2, 42)} = 1.07$, $p = 0.35$; $F_{(2, 42)} = 1.14$, $p = 0.33$, respectively).

Experiment 2

Experiment 2 investigated the influences of BLA projections to the mEC on ARC expression in synaptic fractions in downstream brain regions as a possible circuit by which the BLA alters the balance between multiple memory systems. Figure 2a shows a schematic diagram for the virus injection and optical fiber implantation. Figure 2b shows the experimental timeline.

There were no significant differences during training between males and females that received post-training stimulation of ChR2 or eYFP-control vector-transduced BLA axons in the mEC, respectively, thus males and females were not disaggregated (see Supplement). Likewise, rats that received posttraining stimulation of ChR2-transduced BLA axons in the mEC on Day 1 did not show differences in their training compared to eYFP-control rats prior to optogenetic stimulation on Day 1 (see Supplement).

There were no differences in retention testing between males and females that received post-training illumination of ChR2 or eYFP-control vector-transduced BLA axons in the mEC, and, therefore, male and female rats in each group were not disaggregated (see Supplement). Figure 2c shows retention latencies (left) and duration in target quadrant (right) for rats that were tested on Day 3. An unpaired t-test revealed a significant difference in retention latencies ($t_{(40)} = 2.22$, $p = 0.033$) and a trend toward a significant difference in duration in target quadrant during retention testing ($t_{(39)} = 1.81$, $p = 0.078$). Consistent with our prior work in male rats [31], male and female rats that received 8 Hz posttraining stimulation of BLA axons in the mEC required less time to find the escape port and spent more time in the target quadrant compared to eYFP-control rats.

The results from the analyses on ARC expression from male and female rats, shown in Fig. 2d–i, were disaggregated and the groups fully powered because the male and female rats appeared to differ in terms of the effects of optical stimulation on ARC protein expression. Figure 2d shows ARC protein expression in DH synaptoneurosomes for male rats that were given spatial training immediately followed by 8 Hz stimulation of the BLA–mEC pathway. Male rats that received 8 Hz stimulation had significantly higher ARC protein levels in the DH compared to eYFP-control rats ($t_{(21.90)} = 2.10$, $p = 0.048$). Figure 2e, f show that ARC protein expression in DLS and somatosensory cortex synaptic fractions, respectively, did not differ for male rats ($t_{(27)} = 0.33$, $p = 0.74$; $t_{(27)} = 0.43$, $p = 0.67$, respectively).

Figure 2g, i show that ARC protein expression in DH and somatosensory cortex synaptoneurosomes, respectively, did not differ for female rats that were given spatial training immediately followed by 8 Hz stimulation of the BLA-mEC pathway ($t_{(18)} = 0.89$, $p = 0.38$; $t_{(16)} = 0.68$, $p = 0.50$, respectively). Figure 2h shows ARC protein expression in DLS synaptic fractions for female rats that were given spatial training immediately followed by 8 Hz stimulation of the BLA–mEC pathway. Female rats that received 8 Hz stimulation of ChR2-transduced BLA axons in the mEC had significantly lower levels of ARC protein expression in the DLS compared to eYFP-control rats ($t_{(18)} = 2.38$, $p = 0.029$).

The results from the males and females suggested that, although the region where changes in ARC differed by sex, the overall effect in both groups was a change in the balance of ARC expression between the DH and the DLS. To determine whether

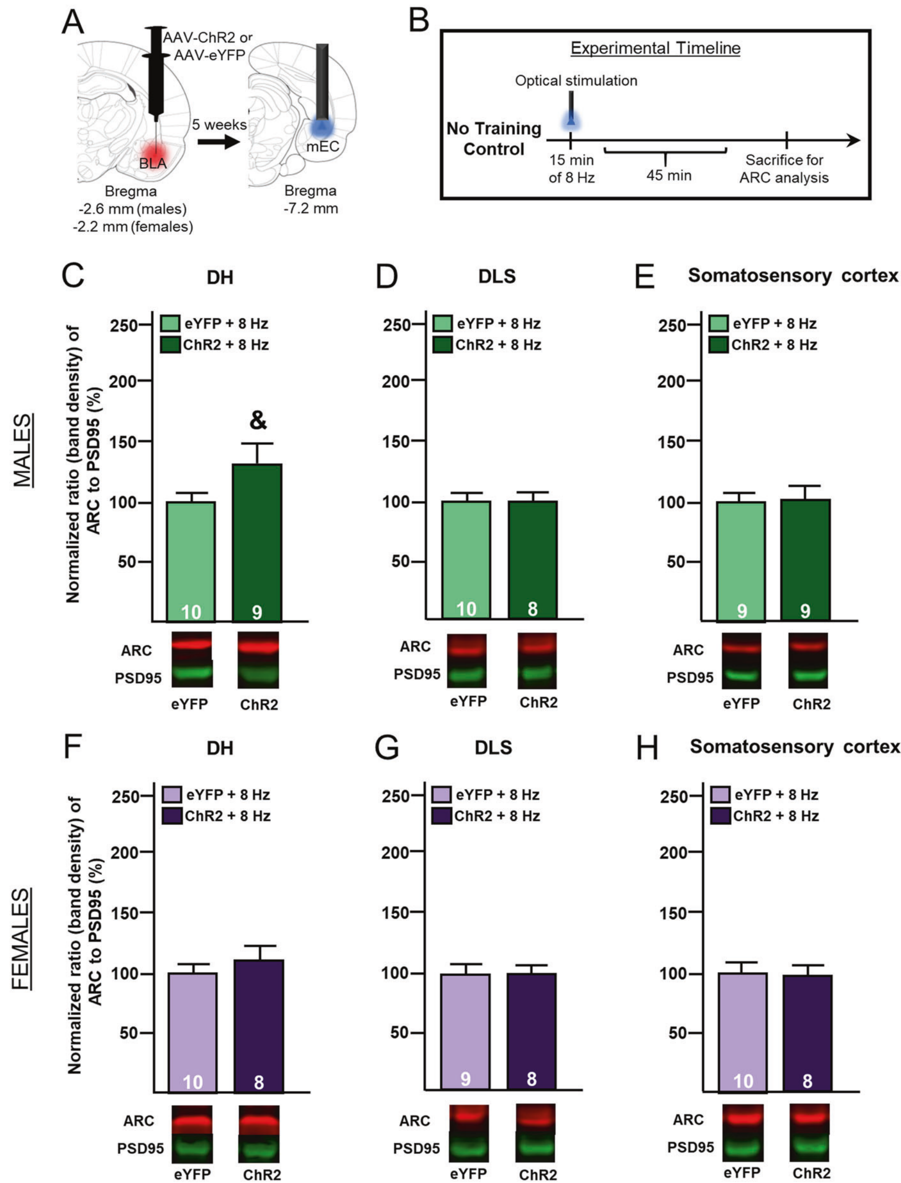


Fig. 3 ARC protein expression effects of 8 Hz optical stimulation of the BLA–mEC pathway in the absence of behavioral training (Experiment 3). a Schematic diagram of BLA injection site (left), incubation time, and optic probe placement in mEC (right). **b** Experimental timeline for Experiment 3. **c**–**e** ARC protein expression (mean ± SEM) (top) and representative immunoblots (bottom) for synaptoneurosomes from the DH (**c**), DLS (**d**), and somatosensory cortex (**e**) for male rats that were given 8 Hz stimulation of the BLA–mEC pathway. Rats that received 8 Hz stimulation of the BLA–mEC pathway had a trend toward significantly higher levels of ARC protein expression in the DH compared to eYFP-control animals. There were no significant differences in ARC expression in the DLS or somatosensory cortex. **f**–**h**, ARC protein expression (mean ± SEM) (top) and representative immunoblots (bottom) for synaptoneurosomes from the DH (**f**), DLS (**g**), and somatosensory cortex (**h**) for female rats that were given 8 Hz stimulation of the BLA–mEC pathway. There were no significant differences between groups in any case. $^{\&}p < 0.1$, compared to eYFP-control values. ARC was normalized to PSD95 by calculating the ratio band density of ARC to that of PSD95 and then expressed as a percentage of normalized control values.

this was the case, a ratio of ARC expression in the two regions (as described in the "Methods" section) was calculated. Figure 2j, k show the ratio of ARC protein expression in the DH/DLS in males and females, respectively, for rats given spatial training immediately followed by 8 Hz stimulation of the BLA–mEC pathway. An unpaired *t*-test revealed no significant difference between groups in either case ($t_{(26)} = 1.21$, $p = 0.24$; $t_{(17)} = 1.73$, $p = 0.10$, respectively). Unpaired *t*-tests revealed no significant difference between males and females that received posttraining illumination of ChR2 or eYFP-control vector-transduced BLA axons in the mEC, respectively, ($t_{(22)} = 0.0084$, $p = 0.99$; $t_{(21)} = 0.60$, $p = 0.55$). Therefore, male and female rats in each group were combined. Figure 2l shows the ratio of ARC protein expression in the DH/DLS in males and females combined, for rats given spatial training immediately followed by 8 Hz stimulation of the BLA–mEC pathway. Rats that received 8 Hz stimulation of ChR2-transduced BLA axons in the mEC had a significantly higher ratio of DH/DLS ARC compared to eYFP-control rats ($t_{(45)} = 2.05$, $p = 0.047$).

Experiment 3

Experiment 3 examined whether 8 Hz stimulation of the BLA–mEC pathway in the absence of spatial training alters downstream ARC protein expression. Figure 3a shows a schematic diagram for the

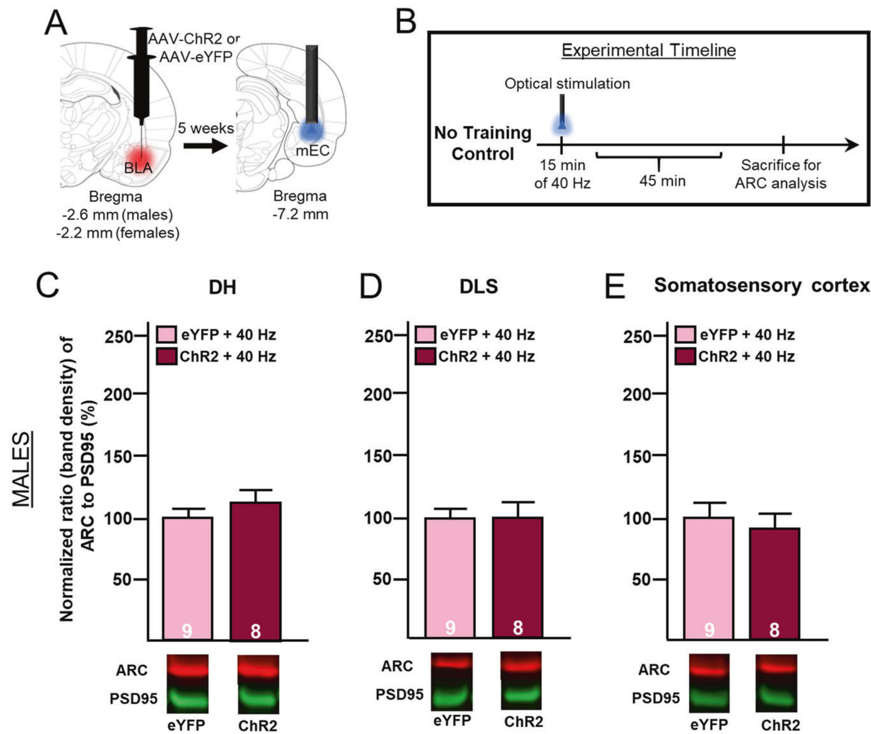


Fig. 4 ARC protein expression effects of 40 Hz optical stimulation of the BLA–mEC pathway in the absence of behavioral training (Experiment 4). a Schematic diagram of BLA injection site (left), incubation time, and optic probe placement in mEC (right). **b** Experimental timeline for Experiment 4. **c**–**e** ARC protein expression (mean ± SEM) (top) and representative immunoblots (bottom) for synaptoneurosomes from the DH (**c**), DLS (**d**), and somatosensory cortex (**e**) for male rats that were given 40 Hz stimulation of the BLA–mEC pathway. There were no significant differences between groups in any case. ARC was normalized to PSD95 by calculating the ratio band density of ARC to that of PSD95 and then expressed as a percentage of normalized control values.

virus injection and optical fiber implantation. Figure 3b shows the experimental timeline.

Figure 3c shows the ARC protein expression in DH synaptic fractions for male rats that were given 8 Hz stimulation of the BLA–mEC pathway in the absence of spatial training. Though not statistically significant, rats that received 8 Hz stimulation of ChR2-transduced BLA axons in the mEC had higher levels of ARC protein expression in the DH compared to eYFP-control rats ($t_{(17)} = 1.99$, $p = 0.063$). Figure 3d, e show that ARC protein expression in DLS and somatosensory cortex synaptic fractions of male rats, respectively, did not differ ($t_{(16)} = 0.13$, $p = 0.90$; $t_{(16)} = 0.35$, $p = 0.73$, respectively).

Figure h show that ARC protein expression in DH, DLS, and somatosensory cortex synaptoneurosomes, respectively, did not differ for female rats ($t_{(16)} = 1.24$, $p = 0.23$; $t_{(15)} = 0.031$, $p = 0.98$; $t_{(16)} = 0.011$, $p = 0.99$, respectively).

Experiment 4

Although not statistically significant, the results of Experiment 3 suggested that optical stimulation alone could alter ARC protein expression in DH of male rats (but not female rats). Therefore, Experiment 4 investigated whether a higher frequency (40 Hz) stimulation of the pathway would elicit a significant increase in ARC expression in the absence of spatial training. Figure 4a shows a schematic diagram for the virus injection and optical fiber implantation. Figure 4b shows the experimental timeline. Figure 4c–e show the ARC protein expression in synaptic fractions from the DH, DLS, and somatosensory cortex, respectively, for male rats that were given 40 Hz stimulation of the BLA–mEC pathway in the absence of spatial training. An unpaired *t*-test revealed no significant difference between groups in all cases ($t_{(15)} = 1.71$, $p = 0.11$; $t_{(15)} = 0.16$, $p = 0.88$; $t_{(15)} = 0.82$, $p = 0.43$, respectively).

Table 1 summarizes the findings from the ARC analyses in Experiments 1–4.

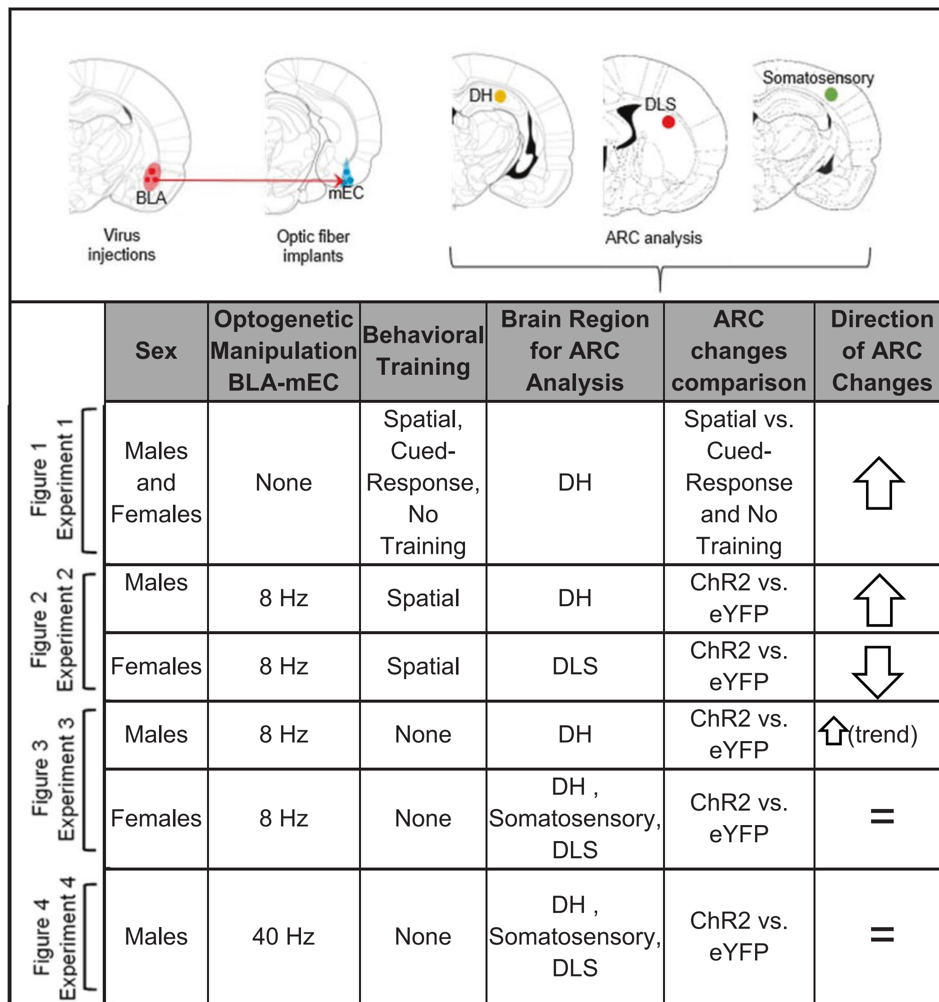
DISCUSSION

The current findings reveal that postspatial training BLA–mEC stimulation shifts the balance of ARC expression between the DH and DLS in favor of the DH-based spatial learning system, pointing toward a circuit-based mechanism by which the BLA modulates multiple competing memory systems. An initial experiment in the present study found that spatial training increased ARC levels in synaptoneurosomes from the DH of both male and female rats significantly above those found with cued-response training or no training. Subsequent experiments found that optogenetic stimulation of the BLA–mEC pathway immediately after spatial training significantly enhanced retention in males and females, confirming previous work using males alone from this laboratory [31]. Such stimulation after training also increased the ARC protein ratio between DH and DLS synaptic fractions, although the precise region where changes in ARC were observed differed by sex. In male rats, stimulation increased ARC protein expression in the DH with no effect on ARC expression in the DLS or somatosensory cortex. In female rats, identical stimulation reduced ARC levels in the DLS without altering ARC levels in the DH or somatosensory cortex. This suggests that the influence of the BLA–mEC pathway on the consolidation of spatial learning occurs through a shift in the balance of ARC-mediated plasticity in the DH and DLS, though the shift differs between sexes.

The BLA–mEC pathway and downstream ARC expression

The present work found that spatial Barnes maze training increased ARC protein levels in synaptoneurosomes from the DH, but not DLS or somatosensory cortex, for male and female

Table 1. Summary of ARC findings from all Experiments.



	Sex	Optogenetic Manipulation BLA-mEC	Behavioral Training	Brain Region for ARC Analysis	ARC changes comparison	Direction of ARC Changes
Experiment 1 Figure 1	Males and Females	None	Spatial, Cued-Response, No Training	DH	Spatial vs. Cued-Response and No Training	⇧
Experiment 2 Figure 2	Males	8 Hz	Spatial	DH	ChR2 vs. eYFP	⇧
	Females	8 Hz	Spatial	DLS	ChR2 vs. eYFP	⇩
Experiment 3 Figure 3	Males	8 Hz	None	DH	ChR2 vs. eYFP	⇧(trend)
	Females	8 Hz	None	DH , Somatosensory, DLS	ChR2 vs. eYFP	=
Experiment 4 Figure 4	Males	40 Hz	None	DH , Somatosensory, DLS	ChR2 vs. eYFP	=

Top: Illustration of the BLA–mEC pathway that was optically manipulated in Experiment 2–4, followed by the brain regions analyzed for ARC protein (DH, DLS, and Somatosensory). Bottom: Summary of ARC expression findings across the four experiments conducted.

rats. This is consistent with prior work implicating ARC in hippocampal-dependent learning and memory [15, 17, 18]. Indeed, evidence suggests that inhibition of ARC expression in the hippocampus impairs spatial memory and synaptic plasticity [15, 18, 39]. Interestingly, the present study did not observe a similar increase in ARC with cued-response training, despite the overall similarity of the external environment. As cued-response and spatial learning involve the DLS and DH, respectively, this suggests that the combination of exposure to the external cues and the type of learning and memory systems engaged was necessary for the increase in ARC in DH synapses. To our knowledge, this finding is the first to show an effect on hippocampal ARC within the context of spatial vs. cued-response learning and memory systems.

Much evidence suggests that ARC is a critical mechanism by which the BLA alters plasticity in other regions, as studies indicate that manipulations of BLA activity alter the expression of ARC protein downstream in the DH and the anterior cingulate cortex [15–17]. However, the circuitry by which manipulations of the BLA alter downstream ARC activity previously remained unknown. The present work first replicated prior work from this laboratory, finding that stimulating the BLA–mEC pathway with 8 Hz stimulation enhanced retention for spatial learning [31], in this case using both male and female rats. Immunoblotting analyses found that such stimulation following spatial training also increased the balance of ARC across the DH and DLS in males and females, suggesting the mEC mediates BLA effects on spatial memory and downstream ARC expression. Critically, in all cases, no changes in ARC expression in the somatosensory cortex were observed, indicating the importance of the observed changes in the DH and DLS in the context of multiple memory systems. One interesting question is whether the DH cells that express ARC receive direct inputs from the mEC. As the mEC provides widespread inputs to the CA1 and CA3 pyramidal cells and those cells are known to express ARC [40, 44–46], it seems likely that this is a monosynaptic connection. However, as the present study focused on the influence of the BLA–mEC pathway on the DH, the current findings do not directly address this level of synaptic precision. Additionally, the ARC expression observed herein is likely involved in the initial consolidation of synapses, although a later wave may contribute to the stabilization of a subset of these synapses [40]. It is unknown whether the optical manipulations that enhance memory and alter initial ARC expression would also affect ARC in the later wave.

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The effects of 8 Hz stimulation on ARC expression is consistent with our prior work indicating that such stimulation, but not 40 Hz stimulation, of the BLA–mEC pathway enhances the consolidation of spatial memories [31]. Moreover, the prior work also found that stimulating the BLA–mEC pathway with 8 Hz pulses increases local field potentials at 8 Hz frequency bands in the DH, whereas similar stimulation with 40 Hz has much lower effects on local field potentials at 40 Hz. Much evidence suggests a critical role for theta rhythm (~8 Hz) activity in the mEC and hippocampus in spatial information processing [47, 48]. Thus, the present findings add to the evidence that stimulating the BLA–mEC pathway at 8 Hz has privileged effects on downstream activity, plasticity, and related memories. Of note, most previous studies indicate that ARC expression is experience-dependent [15, 49–51]. Thus, the nonsignificant trend toward an increase in ARC in the DH of male rats observed with 8 Hz, but not 40 Hz, stimulation in the absence of explicit spatial training may be due to the physiologically relevant stimulation combined with placement into the stimulation context.

Although the current findings suggest that optogenetic stimulation of BLA inputs to the mEC enhances retention for spatial memory and alters ARC expression in downstream brain regions, the present experiments do not rule out the possibility that the observed effects were due, at least in part, to backpropagation of action potentials to the BLA and activation of other BLA efferents. However, evidence suggests that distinct projections are responsible for the ability of the BLA to modulate different types of memories [14, 52] and that the type of information being processed is distinct within each projection-specific BLA neuronal population [53–56]. Previous work from this laboratory found that optical stimulation of the BLA-ventral hippocampus pathway does not alter the consolidation of spatial/contextual memory [31], suggesting that stimulating one BLA output does not replicate the stimulation of another. Moreover, optogenetic inhibition of BLA fibers in the mEC, which would not be expected to produce backpropagation, impaired retention of spatial learning [31]. Thus, it seems unlikely that the present findings were due to nonspecific activation of the BLA and its other efferents.

BLA modulation of multiple memory systems

Evidence suggests multiple memory systems are responsible for processing different types of learning [1–5]. However, the amygdala appears to play a modulatory role across these systems, influencing memory consolidation through interactions with a variety of downstream brain regions [14]. The current work suggests that the ability of the BLA to modulate these multiple memory systems is due, at least in part, to its influence on ARC expression and plasticity in other brain areas. The present experiments found that optogenetic stimulation of the BLA–mEC pathway immediately after spatial training significantly enhanced retention in males and females and elicited the same relative activation of ARC across the DH and DLS in both sexes. Thus, the present results suggest that the BLA modulates multiple memory systems by recruiting which brain region is involved in ARC-related plasticity. Future work investigating BLA efferents that enhance the consolidation of cued-response learning will be critical for providing further support for this idea.

Sex differences in ARC expression

In the present study, spatial training alone increased ARC levels in synaptoneurosomes from the DH of males and females. Furthermore, the results reveal that 8 Hz stimulation of the BLA–mEC pathway enhanced retention for spatial learning while shifting the balance of ARC expression in the DH and DLS for both sexes. However, this shift occurred via increased ARC in the DH in males and decreased ARC in the DLS in females, supporting prior evidence suggesting that sex differentially influences multiple memory systems [57, 58]. These results are surprising and yet, in light of the paucity of previous studies using both males and females, it is difficult to determine how unexpected. Although sparse, there have been reports of sex interactions with ARC expression [59–61], though the differences in ARC expression were of degree, rather than the qualitative differences observed in the present work.

That stimulation of the BLA–mEC pathway after training enhanced retention in both sexes while producing sex-dependent effects on ARC expression raises the possibility, that these consequences on ARC levels represent different mechanisms for accomplishing the same outcome. Evidence suggests that males and females have biological differences yet often behave similarly [62]. This has led to the theory that some sex differences in the brain are compensatory and exist to equate behavior between males and females [63, 64]. Sex convergence occurs when the endpoints are the same but the underlying physiology is different between sexes, an idea that may explain the present results. Namely, the changes in the balance of ARC expression in synaptic fractions from the DH and DLS between males and females may reflect the competition that occurs between the hippocampus-based and basal ganglia-based memory systems [2, 6, 31, 65]. Furthermore, studies indicate that stress and age differentially engage the hippocampus vs. the striatum in males and females [66–68] and that, when given the choice between place and response strategies, female rats in estrous are biased towards response strategies [58]. That post-training stimulation of the BLA–mEC pathway resulted in sex differences in ARC expression, whereas stimulation alone without learning in Experiment 3 showed no effect, suggests differences in learning strategy may be an underlying cause of the observed sex differences.

ARC is implicated in several different kinds of plasticity, including long-term potentiation, long-term depression, and homeostatic scaling of synapses. Like ARC expression, all of these forms of synaptic plasticity are important for long-term storage of memories. Therefore, ARC is considered a “master organizer” of long-term plasticity that underlies long-term memory [69]. Recent findings suggest that ARC interacts with inactive CaMKIIβ in weak synapses to endocytose AMPA receptors in service of a homeostatic scaling process in changing dendrites [70]. This process of strengthening some synapses and weakening others can be directed by spike-timing dependent plasticity, which also influences ARC expression [71]. Spike-timing dependent plasticity may explain the different patterns of ARC expression in males and females. Differences have been observed in hippocampal and parahippocampal theta and high-gamma oscillations in humans performing spatial navigation tasks [72]. Theta-gamma coupling in the entorhinal-hippocampal system is associated with spatial learning [73–75], and may be responsible for the ARC increase in the DH of males. However, sparse connections between the entorhinal-striatal system [76] may result in differential coupling between these regions, causing a shift in the onset of the theta frequency stimulation. We speculate that such a shift in spike timing may drive the observed ARC decrease in the DLS of female rats [77]. Recent work suggests that *Arc* mRNA can be transferred from one neuron to the next via intercellular transfer [78]. ARC can be involved in selectively weakening inactive or competing synapses [70] and a decrease in ARC can be linked to impaired memory performance [15]. Together, this creates the possibility that ARC is trafficked away from DLS synapses in females, reducing the potential for experience-dependent synaptic plasticity in the region, whereas it is increased at DH synapses in males to facilitate plasticity in the hippocampal memory system. Thus, although the underlying changes in ARC expression may differ between sexes, the endpoints of a shift in the balance of ARC expression to the DH and enhanced spatial memory are the same. Therefore, future work must carefully consider how the BLA differentially modulates multiple memory systems in the context of males vs. females.

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AUTHOR CONTRIBUTIONS

KLW substantially contributed to the conception and design of the work, acquired, and analyzed the data, and interpreted the findings. She drafted the work and approved the final version to be published. She agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. AAD contributed to the conception and design of the work. She approved the final version to be published and agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. CKM contributed to the conception and design of the work and interpreted the findings. She drafted the work and approved the final version to be published. She agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. RTL contributed to the conception and design of the work and interpreted the findings. He drafted the work and approved the final version to be published. He agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

ADDITIONAL INFORMATION

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