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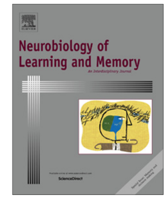

Memory-enhancing intra-basolateral amygdala clenbuterol infusion reduces post-burst afterhyperpolarizations in hippocampal CA1 pyramidal neurons following inhibitory avoidance learning

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ABSTRACT

Activation of the basolateral amygdala can modulate the strength of fear memories, including those in single-trial inhibitory avoidance (IA) tasks. Memory retention, measured by the latency to re-enter a dark-compartment paired 24 h earlier with a footshock, varies with intensity of this aversive stimulus. When higher intensity footshocks were used, hippocampal CA1 pyramidal neurons exhibited reduced afterhyperpolarizations (AHPs) 24 h post-trial, an effect blocked by immediate post-trial inactivation of the basolateral complex of the amygdala (BLA). Similar AHP reductions in CA1 have been observed in a number of learning tasks, with time courses appropriate to support memory consolidation. When less intense footshocks were used for IA training of Sprague-Dawley rats, immediate post-trial infusion of the β-adrenergic agonist clenbuterol into BLA was required to enhance hippocampal Arc protein expression 45 min later and to enhance memory retention tested 48 h later. Here, using Long-Evans rats and low-intensity footshocks, we confirmed that bilateral immediate post-trial infusion of 15 ng/0.5 μl of the β-adrenergic agonist clenbuterol into BLA significantly enhances memory for an IA task. Next, clenbuterol was infused into one BLA immediately post-training, with vehicle infused into the contralateral BLA, then hippocampal CA1 neuron AHPs were assessed 24 h later. Only CA1 neurons from hemispheres ipsilateral to post-trial clenbuterol infusion showed learning-dependent AHP reductions. Excitability of CA1 neurons from the same trained rats, but from the vehicle-infused hemispheres, was identical to that from untrained rats receiving unilateral clenbuterol or vehicle infusions. Peak AHPs, medium and slow AHPs, and accommodation were reduced only with the combination of IA training and unilateral BLA β-receptor activation. Similar to previous observations of BLA adrenergic memory-related enhancement of Arc protein expression in hippocampus, increased CA1 neuronal excitability in the fear-modulated IA task was activated by immediate post-trial β-receptor activation of the ipsilateral BLA.

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1. Introduction

Emotional arousal can enhance memory formation, with stressful emotional stimuli creating strong, lasting memories (McIntyre, Power, Roozendaal, & McGaugh, 2003). Stress from fear activates the basolateral complex of the amygdala (BLA), which in turn modulates consolidation of memory via the hippocampus and other synaptically-connected brain regions (McIntyre et al., 2003). Bilateral infusions of D-amphetamine into the amygdala immediately following training on a hippocampal-dependent task enhance learning compared to vehicle infusions (Packard, Cahill, & McGaugh, 1994). The basal amygdalar adrenergic system has been found to be important in the process of modulating memory (Ferry & McGaugh, 1999; McIntyre, Hatfield, & McGaugh, 2002; McIntyre et al., 2005). More specifically, β-adrenergic modulation of the BLA during memory consolidation can enhance or impair the formation of strong emotional memories (McIntyre et al., 2005). Norepinephrine (NE) release in the BLA immediately post-training on an emotionally arousing single-trial inhibitory avoidance (IA) task is positively correlated with subsequent retention performance (McIntyre et al., 2002). Additionally, post-trial infusion of the β-adrenergic agonist clenbuterol into the BLA dose-dependently enhanced memory (i.e. increased the latency of rats to avoid entry into the dark-compartment of an IA chamber paired in a single trial with a low-intensity footshock) (Ferry & McGaugh, 1999).

Arc protein, an immediate early gene product signaling a short-term increase in neuronal activity, is transiently expressed in the hippocampus following acquisition of a number of tasks

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(McIntyre et al., 2005). After a single IA training trial using a low-intensity aversive footshock, Arc expression was enhanced in CA1 of hippocampus 45 min after immediate post-trial clenbuterol infusions into the BLA; using a higher-intensity footshock, immediate post-trial lidocaine infusions into the BLA reduced CA1 Arc expression 45 min post-trial (McIntyre et al., 2005). Similarly, 24 h after acquisition of an IA task, in which a high-intensity footshock was paired with the dark compartment, hippocampal CA1 post-burst afterhyperpolarizations (AHPs) were significantly reduced, i.e. intrinsic excitability was enhanced (Farmer & Thompson, 2012), while immediate post-trial BLA lidocaine infusions blocked this learning-dependent plasticity in CA1 AHPs. Learning-dependent reductions in CA1 neuron AHPs are a reliable and highly replicable cellular mechanism expressed in hippocampus, with a time-course appropriate to support memory consolidation (Disterhoft & Oh, 2006; Farmer & Thompson, 2012; McKay, Mathews, Oliveira, & Disterhoft, 2009; Moyer, Thompson, & Disterhoft, 1996; Oh, Kuo, Wu, Sametsky, & Disterhoft, 2003; Oh, Oliveira, & Disterhoft, 2010; Thompson, Moyer, & Disterhoft, 1996).

After learning new tasks, CA1 pyramidal neurons exhibit transient reductions in AHPs (Farmer & Thompson, 2012; Moyer et al., 1996; Oh et al., 2010). Consistent with a hypothesis that AHP reductions serve as a cellular learning mechanism in hippocampal pyramidal neurons, drugs that reduce CA1 AHP amplitudes/durations improve acquisition in several different memory tasks (Disterhoft & Oh, 2006; Donzis & Thompson, 2014; Moyer, Thompson, Black, & Disterhoft, 1992). Normal aging, which is accompanied by enhanced AHPs (reduced excitability) in CA1 neurons, impairs learning (Disterhoft & Oh, 2006; Moyer et al., 1992; Oh et al., 2010). After acquisition of new spatial (Disterhoft & Oh, 2006; Oh et al., 2003) or trace eyeblink (Moyer et al., 1996; Thompson et al., 1996) learning tasks, reductions in the amplitude and area or duration of AHPs are seen in CA1 pyramidal neurons prepared *in vitro* up to 72 h post-learning. AHP reductions are observed 1–24 h after IA learning in CA1 and CA3 pyramidal neurons (Farmer & Thompson, 2012). AHPs can be further segregated into fast (generated by BK channels), medium (generated by apamin-sensitive SK channels), and slow (generated by apamin-insensitive, currently unknown channels) AHPs (Farmer & Thompson, 2012; Oh et al., 2010; Sah & Faber, 2002). Time-course analyses of AHPs show fast (peak), medium (mAHP) and slow (sAHP) AHPs are all transiently reduced after learning an IA task with a high-intensity footshock used as an aversive stimulus (Farmer & Thompson, 2012). Learning-dependent reduction of AHPs can also reduce accommodation to a sustained stimulus, an effect observed in several tasks, including trace eyeblink conditioning, fear conditioning, and single-trial IA training (Disterhoft & Oh, 2006; Farmer & Thompson, 2012; McKay et al., 2009; Moyer et al., 1996; Thompson et al., 1996).

In the current study, after rats underwent a single IA training trial, they were immediately infused (bilaterally) with clenbuterol into the BLA, and dose-dependent effects on memory retention (latency to enter the compartment of the apparatus paired with an aversive footshock) were assessed 24 h later. In a second experiment, 24 h after post-trial BLA clenbuterol infusion, intrinsic excitability (measures of AHPs and accommodation) were assessed in hippocampal CA1 neurons *in vitro*. Intrinsic excitability of CA1 neurons from IA-trained BLA clenbuterol-infused hemispheres was compared to that of neurons from contralateral control (vehicle-infused) hemispheres, as well as to neurons from untrained litter-mate control rats which also received respective unilateral BLA clenbuterol and BLA vehicle infusions.

2. Materials and methods

2.1. Subjects

Experiments were performed using male Long-Evans rats (2–3 mo). Rats were locally bred, with litter-mates maintained in our animal facility under conditions approved by the UT Dallas Institutional Animal Care and Use Committee on a 12/12 h light/dark schedule prior to testing. Rats were handled daily for 5 min for 5 d prior to experimental use. All behavioral testing took place in low ambient light conditions. Rats were trained on an IA task (Experiments 1 and 2), or served as litter-mate untrained controls prior to subsequent brain slice recordings (Experiment 2). The experimenter was blind to experimental conditions for all dose-response testing and data analysis (Experiment 1). While data in Experiment 2 was not collected blindly (the same researcher infused drugs, prepared slices, and recorded from them), data analysis was carried out in a blind fashion. Only one emotionally-arousing experience and one class of assay (behavioral or neurophysiological) was carried out per animal.

2.2. Cannula implantation

Rats were anesthetized with isoflurane, and stereotaxically implanted bilaterally with cannula (15 mm, 23 ga.) into the BLA (−2.7 mm AP, ±5.1 mm ML, −7.4 mm DV) 5 d prior to training and subsequent *in vitro* recordings. 1 mg/kg, i.m., of antibiotic (enrofloxacin) was given post-surgery. Fig. 1 illustrates the timeline of the methods used in the two following experiments.

2.3. Histology

Following behavioral or neurophysiological experiments, cannula placement was verified (see Fig. 2). In experiment 1, rats were anesthetized with isoflurane, decapitated and brains were rapidly

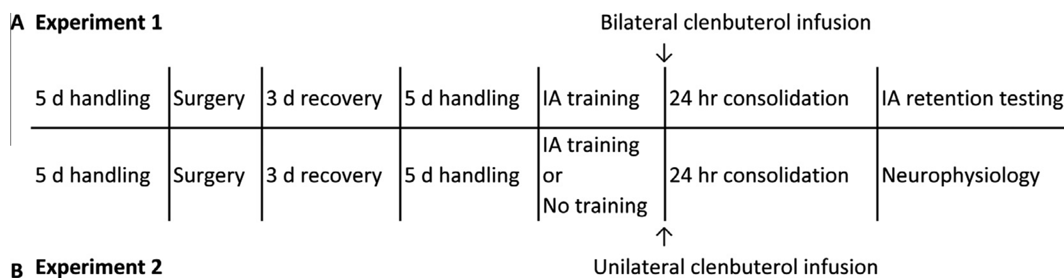

Fig. 1. Timelines for behavioral and neurophysiological assessment of BLA clenbuterol infusions. (A) The timeline used to determine a bilateral memory-enhancing dose of clenbuterol delivered immediately after a single-trial inhibitory avoidance (IA) task. Cannula implanted rats were trained on the IA task with a low-intensity aversive footshock. Immediately post-trial, they were infused bilaterally into the BLA with vehicle or doses of clenbuterol. Memory retention was tested 24 h later. (B) The timeline used to assess the effects of BLA infusion of clenbuterol (into one hemisphere) or vehicle (contralateral hemisphere) on CA1 neuron intrinsic excitability in IA trained and untrained control rats.

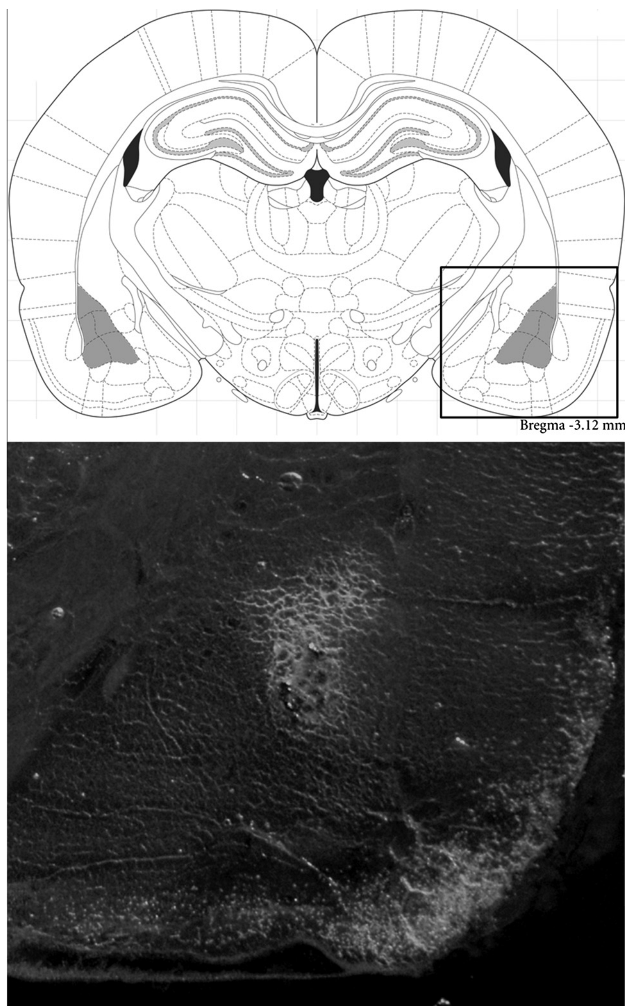

Fig. 2. Top: Schematic diagram shows the BLA and surrounding brain regions. Bottom: Photomicrograph showing representative cannula tracks and drug infusion sites in the basolateral complex of the amygdala (BLA). In Experiment 1, infusions were identical in each hemisphere (i.e. bilateral); in Experiment 2, unilateral infusions of clenbuterol were made into one BLA, and of vehicle into the contralateral BLA, so that each rat served as its own control for modulatory effects on intrinsic excitability of the ipsilateral hippocampus.

removed and flash-frozen by submersion in 2-methylbutane (−40 °C, 1 min). 40 μm coronal sections were taken of all cannula tracks using a cryostat and dry mounted. Sections were analyzed by light microscopy, and only data from rats with bilateral cannula tip placement in the BLA were used for analysis. In experiment 2, a series of 400 μm horizontal slices including the amygdala and overlying tissue were saved for localization of cannula tracks and tips. Slices were fixed in paraformaldehyde (4%, 1 wk) and wet-mounted. Slices were analyzed using light microscopy, and only neurons from rats with bilateral BLA-placed cannula tips were used for analysis.

2.4. Experiment 1 – Behavioral dose-response curves

2.4.1. IA behavioral training

In a single training trial, each of 39 rats were placed into the brightly lit compartment of a rectangular Plexiglas shuttle box (90 cm long × 15 cm deep × 23 cm wide; light compartment: 30 cm long, dark compartment 60 cm long). The rat was allowed to cross to the dark compartment (which had a 2-piece metal floor), and a guillotine door slid shut to lock the animal in the dark compartment. Latency to cross fully into the dark compartment was measured. When a rat reached the end of the dark compartment and turned around, a single low-intensity (0.42 mA, 1 s) footshock was given. The rat remained in the dark compartment for an additional 15 s following the footshock to ensure association of the aversive stimulus with this context.

2.4.2. Drug infusion

Immediately following the single IA training trial, pseudo-randomly assigned rats were infused bilaterally with either 5, 10, or 15 ng of clenbuterol (0.5 μl/hemisphere dissolved in 0.1 M PBS; $n = 9$, $n = 8$, and $n = 8$ respectively) or with vehicle (0.5 μl/hemisphere 0.1 M PBS; $n = 14$), with infusion rates of 0.5 μl/60 s, similar to procedures used in other studies (Holloway-Erickson, McReynolds, & McIntyre, 2012; Introini-Collison, Dalmaz, & McGaugh, 1996; Oh et al., 2003; Sah & Faber, 2002) to ensure infusion throughout the entire BLA. Infusion needles remained inserted for 5 min following infusion, after which rats were returned to their home cages.

2.4.3. IA retention testing

For behavioral assessment of memory, retention testing took place 24 h after IA training. Each rat was again placed into the brightly lit compartment of the IA apparatus. Latency to enter the dark compartment was measured, with a maximum of 10 min allowed. Immediately following retention testing, 0.5 μl/hemisphere of fast green dye was infused bilaterally to aid in histological cannula localization and to determine the extent of infusion. Behavioral retention testing was only performed on animals in this dose-response experiment.

2.5. Experiment 2 – Effects of BLA clenbuterol on intrinsic excitability of hippocampal CA1 neurons

2.5.1. IA behavioral training

Eighteen rats underwent IA behavioral training identical to that in experiment 1. Twenty-five rats did not undergo IA training but served as untrained controls. These control rats were brought into the same room as rats that underwent IA training in a carrier, were picked up and handled for 15 s, were placed back into the carrier, 20 s later heard the audible click generated by the footshock delivery equipment, and then 15 s later were picked up and placed back into the carrier. These control methods were designed to account for the additional handling and sensory cues entailed in IA training, without exposing control rats to the IA apparatus or to the emotionally-arousing footshock.

2.5.2. Drug infusion

Immediately following the single IA behavioral training trial, or the control procedures detailed above, rats were infused with clenbuterol (the behaviorally effective dose of 15 ng/0.5 μl as determined in Experiment 1, dissolved in 0.1 M PBS) into one BLA and vehicle (0.1 M PBS) into the contralateral (control) BLA. Again, infusion rates of 0.5 μl over 60 s were used. Infusion needles remained inserted for 5 min following infusion, after which rats were returned to their home cages.

2.5.3. Brain slice preparation

24 h after BLA infusions (the same time point at which memory retention was assessed in Experiment 1), rats were anesthetized with isoflurane and decapitated. The brain was rapidly removed, hemisected and immersed in cooled (0–1 °C) oxygenated (95% O_2: 5% CO_2) sucrose aCSF [in mM: 124 sucrose; 3 KCl; 1.3 $MgSO_4$; 1.24 NaH_2PO_4; 2.4 $CaCl_2$; 26 $NaHCO_3$; 10 D-glucose; pH 7.4]. After chilling for 3–4 min, the brain was blocked and 400 μm ventral brain slices that included the CA1 region were cut using

vibratomes. Tissue from each hemisphere was maintained separately in room temperature (23 °C) aCSF [in mM: 124 NaCl; 3 KCl; 1.3 $MgSO_4$; 1.24 NaH_2PO_4; 2.4 $CaCl_2$; 26 $NaHCO_3$; 10 D-glucose; pH 7.4]. Slices were continuously oxygenated (95% O_2: 5% CO_2). Sharp electrodes were prepared from thin-walled borosilicate glass (3 M KCl; 30–80 MΩ), and intracellular recordings were made using AxoClamp 2b amplifiers and National Instruments LabView interfaces from submerged slices (31 °C).

2.5.4. Recording protocols

CA1 neurons were recorded in a pseudo-randomized order from both BLA clenbuterol-infused and BLA vehicle-infused (control) hemisphere slices for comparison within subjects. Pyramidal neurons and other cell types were identified on the basis of neurophysiological parameters, and only data from pyramidal neurons are reported. Data was collected at a 20 kHz sampling rate. *AHP recording protocol:* after collecting a 100 ms baseline, AHPs were elicited by a 100 ms depolarizing current sufficient to elicit 4 and only 4 action potentials (APs), with AHPs recorded for 15 s post-burst. AHP amplitude at peak, 250 ms, 500 ms, 750 ms, 1 s, and 2 s post-burst, and AHP duration were also measured. *Accommodation protocol:* following a 100 ms baseline, accommodation was recorded using the same depolarizing current intensity as in the AHP protocol, but sustained for 800 ms, again verifying that 4 and only 4 APs were elicited in the first 100 ms of depolarizing current injection. The number of spikes generated within this 800 ms period was used as a measure of accommodation. *I/V protocol:* To determine current/voltage (*I*/*V*) relations, following a 100 ms baseline, 400 ms current injections (−1.0 to 0.2 nA) elicited membrane voltage responses. Input resistance (IR) was determined from the line of best fit generated from the linear portion of this I/V curve. *Sag* was calculated from voltage-responses to hyperpolarizing −1.0 nA current injections, as the difference between the most negative voltage within the first 80 ms and the average voltage of the last 75 ms of the hyperpolarizing response, as an indirect measure of I_H (Farmer & Thompson, 2012; Moyer et al., 1992, 1996). *Resting membrane potential* (RMP) was measured as the difference in potential measured from the intracellular recording pipet inside to that after withdrawal from the neuron.

2.5.5. Inclusion criteria

All neurons were held for at least 5 min prior to measurements, and those included for analysis had a RMP −68 ± 4 mV, IR > 30 MΩ, AP amplitude > 80 mV, and exhibited no spontaneous action potential firing.

2.6. Data analysis

All analyses were performed using StatView (SAS Inst.), Igor Pro (WaveMetrics), and Excel (Microsoft). Alpha was set at 0.05 for rejection of the null hypothesis. All data are presented as means ± SEM.

2.6.1. Experiment 1

Behavioral data was analyzed based on drug infusion group. Non-parametric Wilcoxon signed rank tests were performed for intra-group comparisons. A Kruskal-Wallis test was performed on retention scores for between group comparisons, using post-hoc Bonferroni corrected Mann-Whitney *U*-tests to identify individual group differences.

2.6.2. Experiment 2

Sharp electrode recordings were analyzed comparing data from vehicle control vs. clenbuterol-infused hemispheres from the same trained or untrained animals, as well as between behavioral conditions, using ANOVA. Post-hoc Scheffe's tests were performed to evaluate between-group differences.

3. Results

3.1. Experiment 1 – Dose-response studies to determine a memory-enhancing dose of clenbuterol

Single-trial pairing of a low-intensity footshock with the context of the dark compartment significantly increased the latency to enter the dark compartment 24 h later (*Wilcoxon signed rank test*: 0 ng: $z = -3.17$, $p = 0.0015$, 5 ng: $z = -2.666$, $p = 0.0077$, 10 ng: $z = -2.521$, $p = 0.0117$, 15 ng: $z = -2.521$, $p = 0.0117$, Fig. 2), indicating significant memory for the learning had occurred. While bilateral infusion of clenbuterol into the BLA enhanced memory retention compared to vehicle infusion at all doses tested, Bonferroni-corrected analysis of individual differences showed that only the dose of 15 ng/BLA was effective at significantly enhancing the latency to enter the dark compartment (*Kruskal-Wallis*: $X^2 = 9.003$, $p = 0.0292$; *Mann-Whitney U tests*: 0 ng vs. 5 ng: $U = 29$, $p = 0.0322$; 0 ng vs. 10 ng: $U = 25$, $p = 0.0344$; 0 ng vs. 15 ng: $U = 20$, $p = 0.014$; Bonferroni corrected $p < 0.016$ required for significance at $\alpha = 0.05$; Fig. 2). The bilateral 15 ng dose was subsequently used in Experiment 2 as the dose to modulate AHPs 24 h after unilateral BLA infusions. While behavioral effects of unilateral clenbuterol infusion were not explicitly tested, an identical research design was used in earlier studies (Holloway-Erickson et al., 2012; McIntyre et al., 2005) to effectively demonstrate that memory-enhancing bilaterally-effective doses of clenbuterol, when delivered ispsilaterally, enhanced rostral anterior cingulate cortical and hippocampal Arc expression in IA trained rats in the BLA-treated hemisphere, compared to expression in contralateral vehicle-infused control hemispheres.

3.2. Experiment 2 – Effects of BLA clenbuterol infusion on CA1 neuron intrinsic excitability

Unilateral immediate post-trial infusion of clenbuterol (15 ng/0.5 μl) into the BLA of IA trained rats enhanced intrinsic excitability (assessed 24 h later) of CA1 pyramidal neurons only in slices ipsilateral to the infusion, reducing AHP peak amplitudes compared to those of neurons from the contralateral vehicle-infused hemispheres from these same trained rats, or to neurons from vehicle- or clenbuterol-infused hemispheres of untrained control rats (*ANOVA*: $F(3) = 10.432$, $p < 0.0001$; *Scheffe's test*: IA trained clenbuterol vs. IA trained vehicle: $p = 0.0003$; IA trained clenbuterol vs. untrained vehicle: $p = 0.002$; IA trained clenbuterol vs. untrained clenbuterol: $p < 0.0001$; Fig. 3c). CA1 neurons from IA trained rats' vehicle-infused hemispheres or from untrained rats' clenbuterol-infused hemispheres did not exhibit significantly reduced AHP amplitudes compared to neurons from untrained rats' vehicle-infused control hemispheres ($p > 0.35$; Fig. 3c).

Unilateral immediate post-trial infusion of clenbuterol into the BLA of IA trained rats also reduced AHP integrated areas measured from CA1 pyramidal neurons in slices ipsilateral to the infusion compared to those of neurons from the contralateral vehicle-infused hemispheres from these same trained rats, or to neurons from vehicle- or clenbuterol-infused hemispheres of untrained control rats (*ANOVA*: $F(3) = 6.623$ $p = 0.0003$; *Scheffe's test*: IA trained clenbuterol vs. IA trained vehicle: $p = 0.007$; IA trained clenbuterol vs. untrained vehicle: $p = 0.0241$; IA trained clenbuterol vs. untrained clenbuterol: $p = 0.0009$; Fig. 3d). No significant reductions in the total duration of AHPs were observed in any of the groups tested (data not shown).

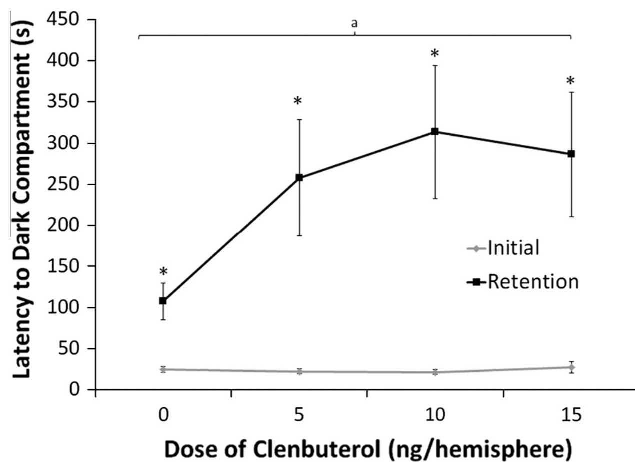

Fig. 3. Initial latency and retention latency to enter the dark (shock-paired) compartment 24 h after different immediate post-trial doses of clenbuterol. All rats showed increased latency to enter the dark compartment during retention testing (■) compared to initial training latency (♦) (*=$p < 0.02$), indicating all learned to fear the dark compartment. When retention latencies were compared to controls (0 ng), all doses of clenbuterol enhanced memory (increased latency). However, only the 15 ng dose was found to significantly increase latency to enter the dark compartment compared to controls ([a]=$p < 0.015$, 0 ng: $n = 14$, 5 ng: $n = 9$, 10 ng: $n = 8$, 15 ng: $n = 8$). This dose of 15 ng clenbuterol was used in experiment 2 to assess unilateral changes in hippocampal intrinsic excitability.

Further analyses showed that unilateral immediate post-trial infusion of clenbuterol into the BLA of IA trained rats also reduced the amplitudes of mAHPs and sAHPs of CA1 pyramidal neurons in slices ipsilateral to the infusion compared to those of neurons from the contralateral vehicle-infused hemispheres from these same trained rats, or to neurons from vehicle- or clenbuterol-infused hemispheres of untrained control rats. Significant reductions of mAHPs were observed 250 ms post-burst only in neurons from hemispheres subjected to both IA training and ipsilateral BLA clenbuterol infusion (*ANOVA*: $F(3) = 10.221$, $p < 0.0001$; *Scheffe's test*: IA trained clenbuterol vs. IA trained vehicle: $p = 0.0004$; IA trained clenbuterol vs. untrained vehicle: $p = 0.0057$; IA trained clenbuterol vs. untrained clenbuterol: $p < 0.0001$). Significant reductions of sAHPs were observed 500 ms, 750 ms, and 1 s post-burst only in neurons from hemispheres subjected to both IA training and ipsilateral post-trial BLA clenbuterol infusion (500 ms: *ANOVA*: $F(3) = 9.412$, $p < 0.0001$; *Scheffe's test*: IA trained clenbuterol vs. IA trained vehicle: $p = 0.0008$; IA trained clenbuterol vs. untrained vehicle: $p = 0.002$; IA trained clenbuterol vs. untrained clenbuterol: $p < 0.0001$; 750 ms: *ANOVA*: $F(3) = 10.244$, $p < 0.0001$; *Scheffe's test*: IA trained clenbuterol vs. IA trained vehicle: $p = 0.0002$; IA trained clenbuterol vs. untrained vehicle: $p = 0.024$, IA trained clenbuterol vs. untrained clenbuterol: $p < 0.0001$; 1s: *ANOVA*: $F(3) = 7.196$, $p = 0.0001$; *Scheffe's test*: IA trained clenbuterol vs. IA trained vehicle: $p = 0.0019$; IA trained clenbuterol vs. untrained vehicle: $p = 0.0401$; IA trained clenbuterol vs. untrained clenbuterol: $p = 0.0008$; Fig. 3b).

Additionally, unilateral immediate post-trial infusion of clenbuterol into the BLA of IA trained rats also reduced accommodation of CA1 pyramidal neurons in slices ipsilateral to the infusion compared to those of neurons from the contralateral vehicle-infused hemispheres from these same trained rats, or to neurons from vehicle- or clenbuterol-infused hemispheres of untrained control rats (*ANOVA*: $F(3) = 6.444$, $p = 0.0003$; *Scheffe's test*: IA trained clenbuterol vs. IA trained vehicle: $p = 0.0443$; IA trained clenbuterol vs. untrained clenbuterol: $p = 0.0009$; Fig. 5). Other neurophysiological measures which have not previously exhibited learning-dependent plasticity, including resting membrane potential, input resistance, or sag (Farmer & Thompson, 2012; Moyer et al., 1992, 1996; Thompson et al., 1996), showed no significant drug- or training-dependent plasticity (Table 1).

4. Discussion

Dose-response experiments demonstrated that immediate post-trial bilateral infusions of 15 ng/BLA of clenbuterol significantly enhanced memory retention for a single-trial IA task (Fig. 2) in male Long-Evans rats. While this dose is in the range shown to be effective in previous studies (Ferry & McGaugh, 1999; Holloway-Erickson et al., 2012; Introini-Collison, Miyazaki, & McGaugh, 1991; Introini-Collison et al., 1996; McIntyre et al., 2005), variability in methodology, effective doses, and strains of rats used necessitated that we carry out an independent dose-response study using a low footshock-intensity, to determine a dose in LE rats where ceiling effects would not mask memory-enhancing effects of the β-agonist clenbuterol. While one prior study found a 3 ng dose of clenbuterol was memory-enhancing, a 10 ng dose was ineffective (Introini-Collison et al., 1991). Another study found a 10 ng dose was memory-enhancing, while a 30 ng dose was ineffective (Introini-Collison et al., 1996). Yet another study found a 10 ng dose of clenbuterol was memory-enhancing, but doses orders of magnitudes higher were ineffective (Ferry & McGaugh, 1999). Two other studies (Holloway-Erickson et al., 2012; McIntyre et al., 2005) infused unilateral 4 ng doses of clenbuterol into the BLA to enhance Arc expression in the ipsilateral hemisphere of IA trained rats, while contralateral vehicle-infusions had no effect on Arc expression in hippocampal tissue from the vehicle-infused hemisphere. A low-intensity footshock (0.42 mA, 1 s) was paired with the dark compartment in the present study, to reduce the salience of the aversive stimulus in the absence of clenbuterol infusion. Our laboratory previously observed that when a more intense (0.5 mA, 1 s) aversive footshock was paired with the dark compartment, CA1 neuron intrinsic excitability was significantly enhanced (pyramidal neuron AHPs recorded *in vitro* 24 h post-trial were significantly reduced) (Farmer & Thompson, 2012); here, after the less-intense footshock, no AHP reductions were observed in vehicle-infused hemispheres, but only in hemispheres ipsilateral to post-trial BLA clenbuterol infusion. These findings are also consistent with earlier observations that NE release in the BLA *in vivo*, acting through adrenoreceptors, including the β-receptors manipulated in the current study, is strongly correlated with intensity of emotional arousal and with memory retention (Introini-Collison et al., 1991), and that the BLA modulates memory consolidation in IA tasks (Wilensky et al., 2000).

The same experimental design as used here, i.e. a single low-intensity aversive footshock training trial paired with unilateral infusion of the β-agonist clenbuterol [with a dose previously demonstrated when bilaterally infused to enhance memory under the same experimental conditions], was used in two earlier studies to demonstrate that enhanced Arc protein expression in CA1 (McIntyre et al., 2005) or in rostral anterior cingulate cortex (Holloway-Erickson et al., 2012) occurred only when IA training was combined with ipsilateral clenbuterol infusion, while ipsilateral clenbuterol-infusion alone (without training) or IA training alone (paired with vehicle-infusion) was ineffective at enhancing Arc expression in either brain region downstream from the BLA. Both enhanced Arc protein expression (Holloway-Erickson et al., 2012; Guzowski et al., 2006; McIntyre et al., 2005; McReynolds et al., 2010) and reduced AHPs (Disterhoft & Oh, 2006; Farmer & Thompson, 2012; McKay et al., 2009; Moyer et al., 1996; Oh et al., 2003, 2010; Thompson et al., 1996) have been strongly linked to memory consolidation. Recently, our laboratory also observed that a single systemic injection of 6 mg/kg (a nootropic dose) of

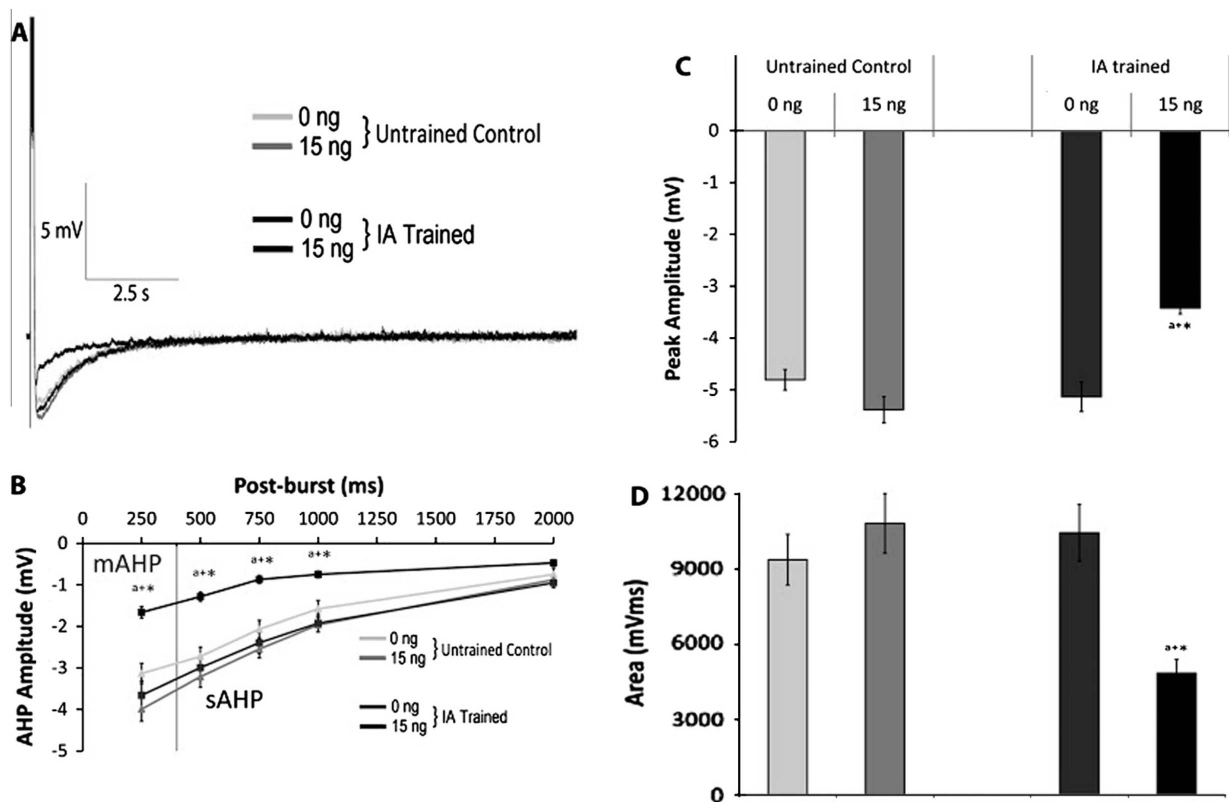

Fig. 4. Measures of post-burst AHPs from IA trained and from untrained control rats 24 h after post-trial unilateral infusions of clenbuterol (15 ng; IA trained neurons $n = 16$, untrained controls: $n = 21$) or vehicle (0 ng; IA trained neurons $n = 12$, untrained controls: $n = 16$). (A) Average AHP waveforms from untrained controls and IA trained animals from both the vehicle- and clenbuterol-infused hemispheres. AHPs were significantly reduced only by the combination of IA training + clenbuterol infusion. (B) Medium and slow components of AHPs. The amplitudes of mAHPs, measured 250 ms post-burst, and of sAHPs, measured 500 ms, 750 ms, and 1 s post-burst, were significantly reduced by BLA clenbuterol infusion immediately post-trial in neurons from IA trained rats compared to neurons from the contralateral hemisphere which also underwent IA training but received only vehicle infusion ($^{*}=p < 0.002$), as well as to neurons from untrained control rats receiving either unilateral vehicle infusion ($^{a}=p < 0.05$) or clenbuterol infusion ($^{+}=p < 0.0009$). Neither BLA clenbuterol alone nor training alone caused significant changes in medium or slow AHPs in CA1. (C) Peak AHP amplitudes. Clenbuterol infusion into the BLA reduced subsequent peak AHP amplitudes of CA1 pyramidal neurons 24 h after IA training compared to neurons from the contralateral hemisphere which also underwent IA training but received only vehicle infusion ($^{*}=p < 0.0004$), as well as to neurons form untrained control rats receiving either unilateral vehicle infusion ($^{a}=p < 0.003$) or clenbuterol infusion ($^{+}=p < 0.0002$). Again, neither BLA clenbuterol infusion alone nor training alone caused significant changes in CA1 neuron AHP peak amplitudes. (D) AHP area. The integrated area of the AHP was reduced 24 later in CA1 neurons only by the combination of BLA clenbuterol + IA training, compared to IA trained vehicle infusion ($^{*}=p < 0.008$), as well as to AHPs of neurons from untrained control rats receiving unilateral BLA vehicle infusion ($^{a}=p < 0.03$) or clenbuterol infusion ($^{+}=p < 0.001$).

Table 1
Membrane properties of CA1 pyramidal neurons from BLA-infused untrained vehicle, untrained clenbuterol, IA trained vehicle, and IA trained clenbuterol hemispheres.

	Rats (*n*)	Neurons (*n*)	Input resistance (MΩ)	Sag (mV)	Resting potential (mV)	Spike half-width (ms)	AP amplitude (mV)
Untrained							
Vehicle	15	16	45.0 ± 1.8	10.3 ± 0.5	−67.2 ± 0.5	1.03 ± 0.02	88.8 ± 1.0
Clenbuterol	18	21	41.5 ± 1.8	10.0 ± 0.3	−68.3 ± 0.3	1.05 ± 0.01	89.5 ± 0.9
IA trained							
Vehicle	12	12	47.5 ± 2.5	10.3 ± 0.4	−67.4 ± 0.4	1.01 ± 0.02	87.8 ± 1.3
Clenbuterol	13	16	42.5 ± 3.1	9.2 ± 0.6	−68.3 ± 0.5	1.02 ± 0.02	86.1 ± 1.3

No significant differences in these membrane properties of CA1 pyramidal neurons were observed between untrained controls and inhibitory avoidance trained rats independent of BLA infusion given. All data are presented as means ± SEM.

D-cycloserine significantly reduced CA1 AHPs and enhanced CA1 Arc protein expression 1 h post-treatment in young male LE rats (Donzis & Thompson, 2014), further supporting a hypothesis that the same mechanisms can modulate both learning-dependent up-regulation of Arc expression and learning-dependent reductions of AHPs in hippocampal neurons. Further experimental work should address these parallel outcome measures, utilizing a wider variety of behavioral tests.

The present study found that bilateral β-adrenergic activation of the BLA immediately following single-trial IA training with a weak aversive stimulus was memory-enhancing, and also that the same dose of the β-adrenergic agonist infused unilaterally immediately post-trial enhanced hippocampal CA1 neuron excitability assessed 24 h after this ipsilateral activation. Another measure of intrinsic excitability, spike-frequency accommodation, was also altered in a manner consistent with findings from other studies of learning-dependent plasticity in the hippocampus (Disterhoft & Oh, 2006; Farmer & Thompson, 2012; McKay et al., 2009; Moyer et al., 1996). In this IA task, unilateral activation of BLA adrenergic receptors was sufficient to enhance the emotionally-arousing signal value of the low-intensity footshock to significantly reduce post-burst AHP amplitudes in CA1 pyramidal cells from that same hemisphere, a form of post-training plasticity that was not observed in the contralateral (vehicle control) hemisphere nor in

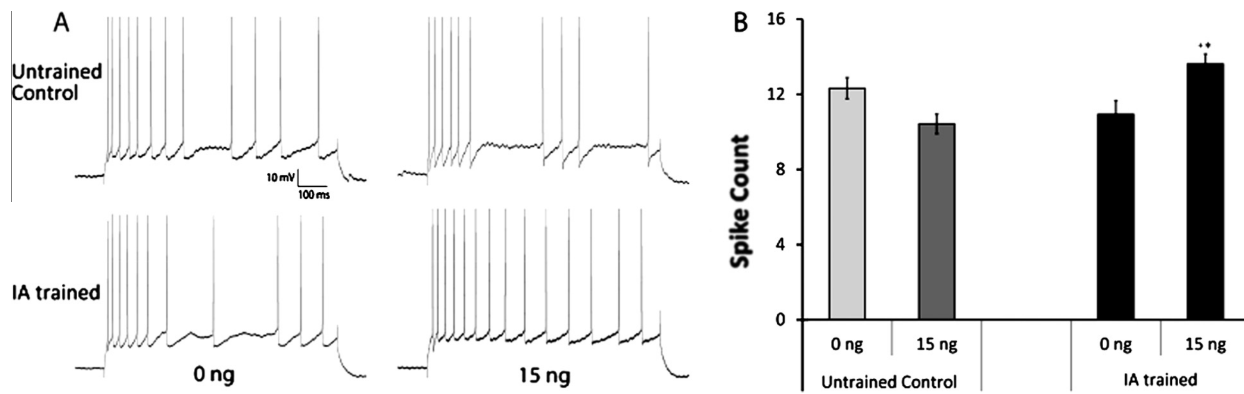

Fig. 5. Neuronal excitability, measured by spike-frequency accommodation of neurons from IA trained or untrained control rats 24 h after immediate post-trial unilateral infusions of clenbuterol (15 ng) or vehicle (0 ng) [data taken from the same set of neurons shown in Fig. 4]. (A) Representative accommodation waveforms from neurons of untrained control and IA trained rats from both the clenbuterol-infused and the contralateral vehicle-infused hemispheres. Accommodation was reduced only in neurons recorded in hemispheres 24 h after IA training + clenbuterol-infusion. (B) Action potential firing during an 800 ms sustained depolarization was significantly increased only in neurons from hemispheres recorded 24 h after clenbuterol-infusion + IA training compared to vehicle-infused + IA trained hemispheres (*=p < 0.05) or to neurons from clenbuterol-infused untrained control hemispheres (⁺=p < 0.001).

the other control conditions tested. Under these paired clenbuterol + training conditions, AHP reductions were observed in CA1 neurons in all major components of the post-burst AHP, including peak amplitude, the medium AHP, and the slow AHP (Fig. 3), and were accompanied by reduced spike-frequency accommodation (Fig. 4), similar to previous research findings (Farmer & Thompson, 2012; McKay et al., 2009) of transient learning-dependent increases in intrinsic excitability in CA1 during the time course of consolidation of memories of intense aversive footshock experiences (see Fig. 5).

Effects of unilateral clenbuterol infusions on memory retention were not explicitly assessed in experiment 2, for several reasons, although determining such effects could be of interest in future studies. If the rats in Experiment 2 had been tested for retention, this testing itself would have constituted a single trial of extinction, and extinction constitutes new learning (Bouton, 2004), since experiencing the environment context previously associated with the aversive footshock without the footshock occurring is a new experience, and thus would have necessitated additional groups be used beyond the scope of the current work. In fact, our laboratory has shown previously that the novelty of simple exposure of rats to the IA apparatus alone, with no emotionally arousing aversive footshock paired with the dark compartment, is sufficient to induce very transient AHP reductions, lasting only 1 h post-exposure (Farmer & Thompson, 2012). It should be noted that the effects of unilateral lesions of the amygdala on fear conditioning have produced mixed results, so considerable additional study may be required to fully explicate unilateral effects of BLA clenbuterol on memory. One study (Labar & LeDoux, 1996) reported that, although fear memory (freezing to a conditioned tone) still occurred after unilateral electrolytic ablation of lateral amydala and portions of BLA and the central nucleus, freezing was significantly attenuated compared to that seen in sham controls. An earlier study (Swartzwelder, 1991), however, found no reductions in freezing in mice with unilateral damage to the amygdala. A more recent study (Goosens & Maren, 2001) observed reductions in freezing only after combined complete unilateral ablation of one amygdala combined with contralateral ablation of lateral, basolateral, or central nuclei of the amygdala, illustrating the complexity of the issue, well beyond the scope of the present study's aims.

As described above, the same experimental design used in the current study was previously used to demonstrate up-regulation of Arc protein expression (Holloway-Erickson et al., 2012; McIntyre et al., 2005) after pairing unilateral clenbuterol + IA training. When a more intense aversive footshock stimulus was used for training, no exogenous modulation of BLA β-receptors was required to produce reductions of AHPs from 1 to 24 h after IA training (Farmer & Thompson, 2012). Reductions of CA1 neuron AHPs (increasing intrinsic excitability) systematically enhance information flow through the hippocampal network for multiple seconds after each neuronal firing event, providing a temporal window for enhancement of synaptic plasticity and memory consolidation.

In our previous study (Farmer & Thompson, 2012), when Long-Evans rats were trained in a single trial with a higher-intensity, more aversive 0.5 mA footshock to induce a robust memory, reductions were seen in all of the AHP and accommodation measures also observed here, with no exogenous increase in β-receptor activation of the BLA required. Conversely, in this earlier study, transient immediate post-trial inactivation of the BLA with lidocaine blocked both memory retention and excitability enhancements assessed 24 h post-trial (Farmer & Thompson, 2012). In the current study, utilizing a lower-intensity (0.42 mA) footshock to induce less emotional arousal, retention latencies averaged ~107 s after bilateral vehicle infusion alone. In the earlier study from our laboratory (Farmer & Thompson, 2012) using a higher-intensity footshock stimulus, retention latencies averaged ~220 s for rats infused with vehicle into the BLA. In the present experiment, when the lower-intensity footshock was paired with a bilateral 15 ng dose of clenbuterol infused into the BLA, retention latencies were significantly increased to ~286 s, i.e. longer than the retention achieved previously with the higher-intensity footshock stimulus. Again, our results parallel those obtained in an earlier study using Sprague-Dawley rats trained on an IA task (McIntyre et al., 2005), in which a low-intensity (0.32 mA) footshock alone led to only low levels of expression of the immediate early gene product Arc in vehicle-infused hemispheres, while pairing of the same aversive stimulus with a unilateral clenbuterol-infusion significantly enhanced Arc expression only in the clenbuterol-infused hemisphere. We observed significant reductions of all components of CA1 post-burst AHPs along with reduced accommodation only in neurons from the hemispheres of rats trained 24 h earlier with a weakly aversive stimulus paired with immediate post-trial BLA infusions of clenbuterol.

Reductions in AHPs and in accommodation (i.e. enhancement of excitability) of CA1 pyramidal cells even after a strongly emotionally-arousing inhibitory avoidance training trial are blocked by post-trial inactivation of the BLA (Farmer & Thompson, 2012), and, as shown here using a less intense aversive stimulus, are enabled by BLA beta-receptor activation. These same conditions are shared with the other learning-dependent mechanism

described above in CA1, transient enhanced Arc protein expression (McIntyre et al., 2005). These results and many others support the hypothesis that the basolateral amygdala can modulate the intensity or strength of emotional memories encoded in a network of other brain regions, in this example contributing to the transient enhancement of hippocampal CA1 neuron excitability that has repeatedly been associated with memory consolidation (Disterhoft & Oh, 2006; Farmer & Thompson, 2012; Kaczorowski & Disterhoft, 2009; McKay et al., 2009; Moyer et al., 1992, 1996; Oh et al., 2003, 2010; Thompson et al., 1996).

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