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ORIGINAL INVESTIGATION

Prefrontal circuits signaling active avoidance retrieval and extinction

Freddyson J. Martínez-Rivera[1] · Christian Bravo-Rivera[2] · Coraly D. Velázquez-Díaz[1] · Marlian Montesinos-Cartagena[1] · Gregory J. Quirk[1]

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Abstract

Objective Neurons in PL and IL project densely to two areas implicated in active avoidance: the basolateral amygdala (BLA) and the ventral striatum (VS). We therefore combined c-Fos immunohistochemistry with retrograde tracers to characterize signaling in platform-mediated active avoidance.

Methods Male rats were infused with retrograde tracers (CTB, FB) into basolateral amygdala and ventral striatum and conditioned to avoid tone-signaled footshocks by stepping onto a nearby platform. In a subsequent test session, rats received either 2 unreinforced tones (avoidance retrieval) or 15 unreinforced tones (avoidance extinction) followed by analysis of c-Fos combined with fluorescent imaging of retrograde tracers.

Results Retrieval of avoidance did not activate IL neurons, but did activate PL neurons projecting to BLA, and to a lesser extent VS. Extinction of avoidance activated IL neurons projecting to both BLA and VS, as well as PL neurons projecting to VS.

Conclusions Our observation that avoidance retrieval is signaled by PL projections to BLA suggests that PL may modulate VS indirectly via BLA, and agrees with other findings implicating BLA in active avoidance. Less expected was the signaling of extinction via PL inputs to VS, which may converge with IL inputs to VS to inhibit expression of avoidance.

Keywords Prelimbic · Infralimbic · Amygdala · Ventral striatum · c-Fos · Retrograde tracers

Introduction

Threats trigger defensive actions, such as active avoidance, that decrease the likelihood of a dangerous encounter. Active avoidance is an increasing focus of aversive conditioning studies (LeDoux et al. 2017), but little is known about the neural circuits mediating the retrieval or extinction of active avoidance. Lesion and inactivation studies have implicated the basolateral amygdala (BLA) and the ventral striatum (VS) in the retrieval of active avoidance (Bravo-Rivera et al. 2014; Choi et al. 2010; Lazaro-Munoz et al. 2010; Ramirez et al. 2015). In addition to BLA and VS, we observed that the prelimbic prefrontal cortex (PL-PFC) is necessary for retrieval of previously learned platform-mediated avoidance (Bravo-Rivera et al. 2014), where rats pressing a bar for sucrose pellets can step onto a nearby platform to avoid a tone-signaled footshock. Furthermore, rats with excessive avoidance (following deficient extinction learning) show elevated activity in PL, BLA, and VS as indicated by c-Fos expression (Bravo-Rivera et al. 2015b). Thus, cortical regulation of avoidance retrieval by PL neurons could occur via direct projections to either BLA or VS (Vertes 2004).

Similarly, extinction of platform-mediated avoidance is impaired by pharmacological inactivation, or BDNF blockade, of infralimbic prefrontal cortex (IL-PFC) (Bravo-Rivera et al. 2014; Rosas-Vidal et al. 2018). Rats unable to extinguish avoidance show reduced c-Fos in IL (Bravo-Rivera et al. 2015b). Similar to PL, neurons in IL project to both BLA and VS (Vertes 2004) and could inhibit avoidance through either of these pathways. To investigate pathways that signal avoidance retrieval and extinction, we combined prefrontal c-Fos immunohistochemistry with retrograde tracers infused

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✉ Freddyson J. Martínez-Rivera
freddyson.martinez@upr.edu

[1] Departments of Psychiatry and Anatomy & Neurobiology, School of Medicine, University of Puerto Rico, P.O. Box 365067, San Juan 00936-5067, Puerto Rico

[2] Present address: Neuroscience Division, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA

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into both BLA and VS. This triple-labeling approach was designed to identify specific projections from PL or IL that signal avoidance retrieval and extinction, respectively.

Methods

Subjects and bar-press training

A total of 51 adult male Sprague–Dawley rats (300–360 g; Envigo Laboratories, Indianapolis, IN, USA) were used in this study. Rats were restricted to 18 g/day of standard laboratory chow and trained to press a bar for sucrose pellets on a variable interval schedule of reinforcement averaging 30 s (VI—30 s). Rats were trained until they reached a criterion of > 10 presses/min. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Puerto Rico School of Medicine, and the Association for Assessment and Accreditation of Laboratory Animal Care.

Surgery

Prior to avoidance conditioning, rats were anesthetized with isoflurane inhalant gas (5%) in an induction chamber and positioned in a stereotaxic frame. Isoflurane (1–2%) was delivered through a face mask for anesthesia maintenance. Rats were stereotaxically infused in the right hemisphere with retrograde tracers in BLA and VS (Paxinos and Watson 2007). Cholera Toxin B (CTB, 594 nm, Thermofisher) was infused in BLA (0.25 μL; − 2.76 mm AP; ± 4.8 mm ML; − 8.9 mm DV) and Fast Blue (FB, 420 nm, Polysciences) was infused in VS (0.2 μL; + 1.68 mm AP; ± 1.10 mm ML; − 7.2 mm DV). Tracers were infused at a rate of 0.025 (BLA) and 0.020 (VS) μL/min over 10 min, and the injectors were left in place for additional 10 min to allow the tracers to diffuse. Afterwards, rats recovered for 1 week prior to behavioral experiments.

Platform-mediated avoidance task

The platform-mediated avoidance task was performed as previously described (Bravo-Rivera et al. 2014, 2015b). Briefly, rats were conditioned with a pure tone (30 s, 4 kHz, 75 dB) coterminating with a shock delivered through a grid floor (2 s, 0.4 mA). Rats received 9 tone–shock pairings each day for 10 days, with a variable inter-trial interval averaging 3 min. An acrylic square platform (14.0 cm each side, 0.33 cm tall) was located in the opposite corner of the sucrose-delivering bar and permitted rats to avoid the shocks. The platform was present during bar-press training prior to conditioning to reduce novelty. On day 11, rats were separated in two groups receiving either 2 unreinforced tones to retrieve the avoidance memory (retrieval) or 15 unreinforced tones to extinguish avoidance (extinction). Avoidance was expressed as a percentage of the tone presentation spent on the platform. Behavioral data was acquired using the Any-Maze tracking system (Stoelting Co).

Immunohistochemistry

c-Fos immunohistochemistry (IHC) was performed as previously described (Bravo-Rivera et al. 2014; Martinez-Rivera et al. 2016) with some modifications. One hour after the end of the behavioral test, rats were anesthetized with sodium pentobarbital (450 mg/kg, i.p.) and transcardially perfused with 250 mL of 0.9% saline followed by 500 mL of cold fresh 4% paraformaldehyde (PFA) in 0.1 M potassium phosphate buffer (KPBS) at pH 7.4. Brains were removed and transferred to 20% and 30% sucrose in 0.1 M KPBS for 48–72 h, for cryoprotection. Using a cryostat (CM 1850; Leica), frozen sections (40 μm) were prepared from the prefrontal cortex, striatum, and amygdala. Sections were initially washed (KPBS) and blocked in a solution of 2% normal goat serum (NGS, Vector Laboratories) and 0.1% tween (Tween-20, Sigma-Aldrich) in 0.1 M KPBS (pH 7.4) for 1 h. The sections were then incubated overnight at room temperature with rabbit anti-c-Fos (Ab-5, Oncogene Science) at a concentration of 1:20,000. Sections were then incubated for 2 h at room temperature in a solution of fluorescent secondary antibody (Alexa Fluor 488, 1:500; Life Technologies). Sections were cover slipped with anti-fading mounting media (Vectashield, Vector Laboratories).

Immunoreactivity and tracer labeling

c-Fos and tracer-labeled neurons were automatically counted at × 20 magnification with an Olympus microscope (Model BX51) equipped with a digital camera. Micrographs were generated for prelimbic and infralimbic regions (+ 3.72 to + 2.52 AP) (Paxinos and Watson 2007). Neuron counts were averaged for the right hemisphere (infused side) across two to three different sections for each structure (Metamorph software version 6.1). Sides showing correct infusion sites and sufficient retrogradely labeling in PFC (> 10 counts/0.1 mm^2 for both CTB and FB) were processed for c-Fos IHC. From a total of 41 PL sides, 18 were excluded due to a lack of transport (7, CTB; 11, FB). Similarly, from a total of 41 IL sides, 17 were excluded due to a lack of transport (4, CTB; 13, FB). Also, one rat was excluded due to a lack of both CTB and FB transport. Density was calculated by dividing the number of positively labeled neurons by the total area of each region (counts/0.1 mm^2). Co-labeled neurons (c-Fos/CTB or c-Fos/FB) were automatically counted and expressed as a percentage of the total number of tracer-labeled neurons.

Data analysis

Student's *t* test, and repeated measures and one-way ANOVAs were performed to determine statistical differences in behavioral and immunocytochemistry experiments. Tukey post-hoc analyses were used for post-hoc tests. Data is presented as mean ± standard error of the mean (SEM) and statistical significance was established as $*p < 0.05$. All statistical analyses were performed using Statistica software (6.0, Statsoft®, Tulsa).

Results

Active avoidance behavior

After infusing retrograde tracers CTB (into BLA) and FB (into VS), rats were conditioned to avoid tone-signaled shocks by stepping onto a nearby platform (Bravo-Rivera et al. 2014) (Fig. 1a). Following 10 days of conditioning, rats were separated into two groups receiving either 2 unreinforced tones to retrieve the avoidance memory (retrieval; Retr, $N = 22$) or 15 unreinforced tones to extinguish avoidance (extinction; Ext, $N = 21$). Retrieval and extinction groups remained in the cage for the same amount of time (55 mins). A control group (Ctl, $N = 8$) received the same number of tones throughout the 10 days of conditioning and 2-tone retrieval test, but was never shocked. Both retrieval and extinction groups exhibited significant avoidance compared with control rats, as indicated by the percent time (of 30-s tone) spent on the platform (Retr, 82%; Ext, 85%; Ctl, 8.3%; main effect of group; $F_{(2,\ 48)} = 48.83$, $p < 0.001$, post hoc: control vs. tone 1 or tone 2, both $p < 0.001$; see Fig. 1b). The extinction group showed significant extinction learning as indicated by a significant reduction in percent time on platform at tone 15 compared to tone 1 (Fig. 1b; 87 to 36%, $F_{(2,\ 60)} = 20.53$, $p < 0.001$, post hoc: tone 1 vs. tone 15, both $p < 0.001$).

Retrograde labeling of PL and IL neurons

Sixty minutes following the test session, rats were perfused and the brains were collected for histological

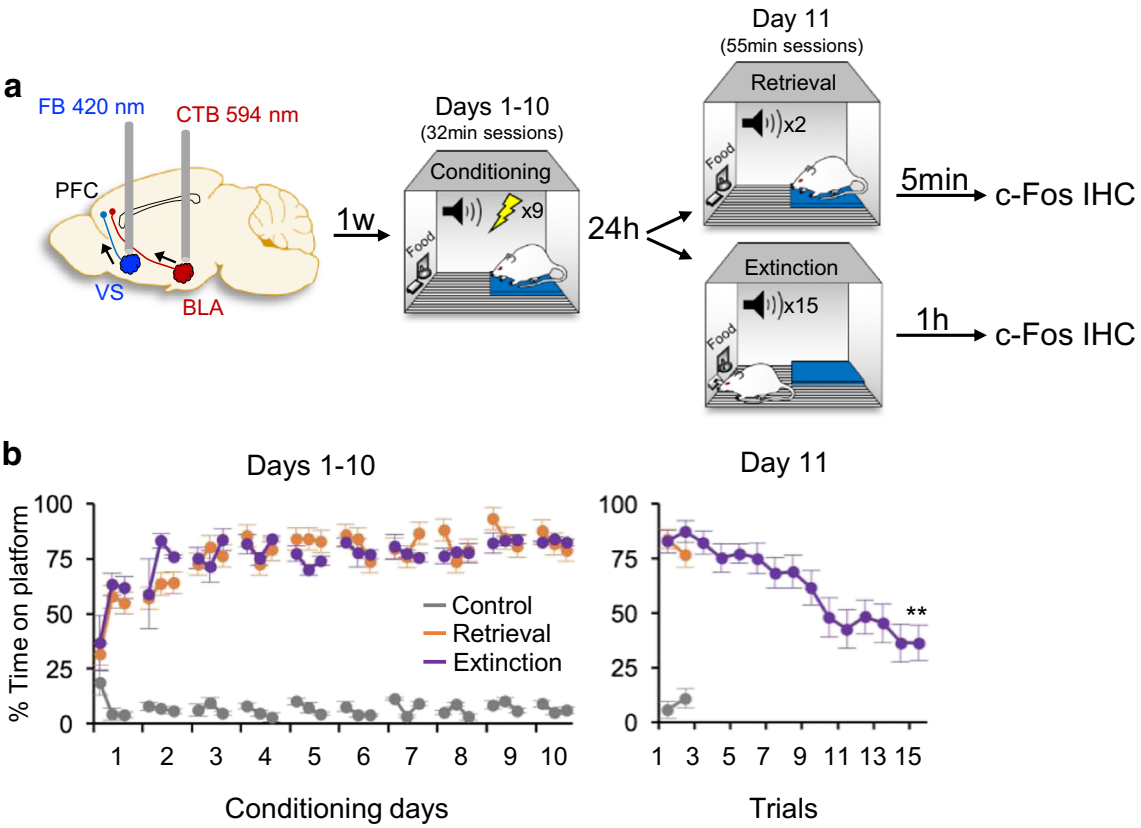

Fig. 1 Retrieval and extinction of platform-mediated avoidance. **a** Rats were first infused with retrograde tracers in BLA and VS and then conditioned in platform-mediated avoidance for 10 days. Twenty-four hours later, rats were tested for avoidance retrieval (2 unreinforced tones, Retr) or avoidance extinction (15 unreinforced tone, Ext), followed 60 min later by collection of brains and c-Fos immunohistochemistry. Unconditioned controls (Ctl) received tones but were never shocked. **b** Right panel: all conditioned rats showed high levels of avoidance (% time on platform; blocks of three) when compared with unconditioned controls. Left panel: subsequently, in rats receiving extinction training, a decrease in avoidance was observed by the end of extinction. Ctl: $N = 8$; Retr: $N = 22$; Ext: $N = 21$. Data shown as mean ± SEM. Repeated measures ANOVA. $**p < 0.01$

analyses. Brains were sectioned at the level of PFC, VS, and BLA and analyzed for corroboration of the infusion sites and retrograde label. Brains showing correct infusion sites and sufficient retrograde labeling in PFC (> 10 counts/0.1 mm^2 for both CTB and FB) were processed for c-Fos IHC (Fig. 2a–c). As expected, there were no significant differences in the quantity of CTB- or FB-labeled cells across experimental groups for PL (Fig. 2d; ANOVA, all p's > 0.05), or for IL (Fig. 2e; ANOVA, all p's > 0.05). However, projections to VS were significantly more pronounced than to BLA for both PL ($F_{(1,\ 60)} = 24.87$, $p < 0.001$) and IL ($F_{(1,\ 61)} = 18.86$, $p < 0.001$), consistent with anatomical studies (Gabbott et al. 2005; Murugan et al. 2017). PL neurons projecting to BLA were located mainly in superficial layers, whereas VS projecting neurons were located mainly in deeper layers, consistent with recent studies (Cheriyan et al. 2016; Gabbott et al. 2005; Murugan et al. 2017).

Retrieval of avoidance activates PL neurons projecting to BLA, but not to VS

As shown in Fig. 3, retrieval of avoidance induced significant c-Fos activity in PL neurons (Fig. 3a upper panel; $F_{(2,\ 38)} = 4.055$, $p = 0.025$, post hoc $p = 0.037$) but not in IL neurons (Fig. 3b upper panel; $F_{(2,\ 38)} = 6.816$, $p = 0.003$, post hoc $p = 0.389$). c-Fos/tracer co-labeling showed that retrieval significantly increased the number of activated PL (c-Fos^+) neurons projecting to BLA (see Fig. 3a lower panels, BLA: $F_{(2,\ 32)} = 6.106$, $p = 0.006$, post hoc $p = 0.015$) but not those projecting to VS ($F_{(2,\ 28)} = 5.111$, $p = 0.013$, post hoc $p = 0.586$). As expected, IL showed no significant retrieval-related co-labeling in projections to either BLA or VS (BLA: $F_{(2,\ 35)} = 10.36$, $p = 0.001$, post hoc $p = 0.718$; VS: $F_{(2,\ 26)} = 6.827$, $p = 0.004$, post hoc $p = 0.750$). Together, these data suggest that retrieval of avoidance recruits predominantly PL neurons projecting to BLA.

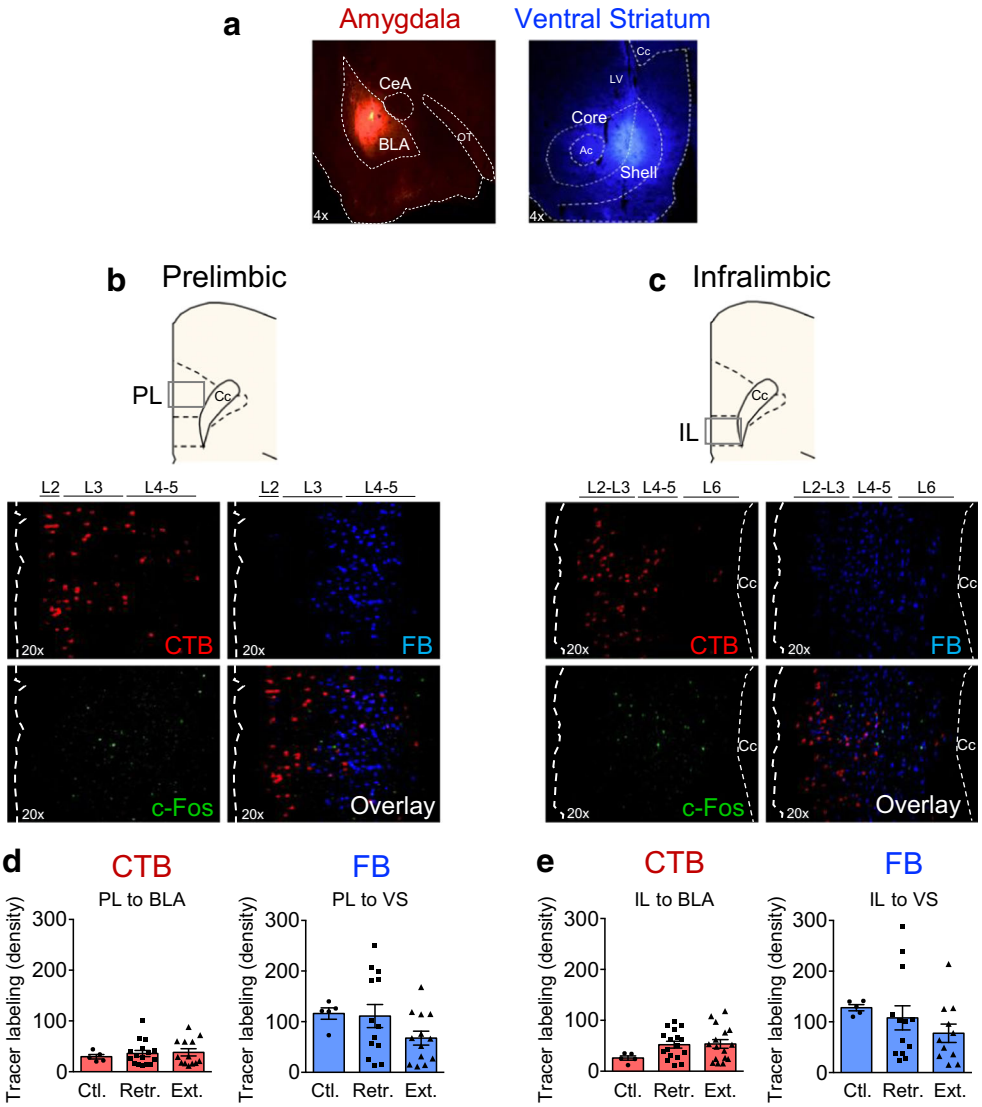

Fig. 2 Retrograde labeling and c-Fos immunohistochemistry of prefrontal neurons projecting to either BLA or VS. **a** Infusion sites of retrograde tracers in BLA and VS. **b**, **c** Upper panels show drawing of prelimbic and infralimbic cortices. Lower panels show representative images of retrogradely labeled, c-Fos-positive, and co-labeled neurons in PL and IL. **d**, **e** Density of retrogradely labeled neurons in different experimental groups. CTB PL: Ctl: $N = 5$; Retr: $N = 16$; Ext: $N = 14$. CTB IL: Ctl: $N = 5$; Retr: $N = 17$; Ext: $N = 16$. FB PL: Ctl: $N = 5$; Retr: $N = 13$; Ext: $N = 13$. FB IL: Ctl: $N = 5$; Retr: $N = 13$; Ext: $N = 11$. Ac, anterior commissure; BLA, basolateral amygdala; Cc corpus callosum; CeA, central amygdala; Core, nucleus accumbens core; LV, lateral ventricle; OT, optic track; Shell, nucleus accumbens shell. Data shown as mean ± SEM

Extinction of avoidance activates IL projections to both BLA and VS, as well as PL projections to VS

Compared with controls, extinction training increased c-Fos activity in both PL and IL (see Fig. 3a, b upper panels, PL: post hoc $p = 0.022$; IL: post hoc $p = 0.008$). Projections from activated IL neurons to both BLA and VS were significantly elevated by extinction training (see Fig. 3b lower panels, IL-BLA: $F_{(2,\ 35)} = 10.36$, $p = 0.001$, post hoc $p = 0.003$; IL-VS: $F_{(2,\ 26)} = 6.827$, $p = 0.004$, post hoc $p = 0.013$). Extinction was also associated with increased activity in PL projections to both BLA and VS (see Fig. 3a lower panels, PL-BLA: $F_{(2,\ 32)} = 6.106$, $p = 0.006$, post hoc $p = 0.004$; PL-VS: $F_{(2,\ 28)} = 5.111$, $p = 0.013$, post hoc $p = 0.023$). However, because c-Fos expression has been shown to last 3–4 h after stimuli exposure (Barros et al. 2015; Cullinan et al. 1995; Dragunow and Faull 1989), c-Fos expression in the PL-BLA projection likely reflects persistence of retrieval-induced activation. This was not the case for the PL projection to VS, which was significantly activated only during extinction (retrieval: $p = 0.586$). However, the difference between extinction and retrieval in the PL-VS projection did not reach statistical significance ($p = 0.052$), leaving open the possibility that some of the labeling in the extinction group may be due to accumulated activation from retrieval.

Discussion

Building on lesion and inactivation studies implicating PFC, BLA, and VS in active avoidance, we combined retrograde tracers with c-Fos immunohistochemistry to identify prefrontal projections activated during avoidance. We found that avoidance retrieval activated PL projections to BLA, whereas avoidance extinction activated IL projections to both BLA and VS. Interestingly, avoidance extinction also activated PL projections to VS.

Retrieval of avoidance

Prior studies have indicated that VS is necessary for avoidance (Bravo-Rivera et al. 2014; Martinez et al. 2013; Ramirez et al. 2015). PL projects to VS (Gabbott et al. 2005; Vertes 2004), and it has been suggested that this projection could mediate active avoidance (Bravo-Rivera et al. 2014). However, our c-Fos data suggest that PL neurons activated by avoidance project to BLA rather than to VS. We have recently observed that retrieval of platform-mediated avoidance is correlated with excitatory responses of PL neurons at platform entry (Diehl et al. 2018). Thus, PL may be in direct communication with BLA to express avoidance memories, similar to Pavlovian fear conditioning (Cho et al. 2013). Projections from basal amygdala to VS have been shown to mediate active avoidance (Ramirez et al. 2015),

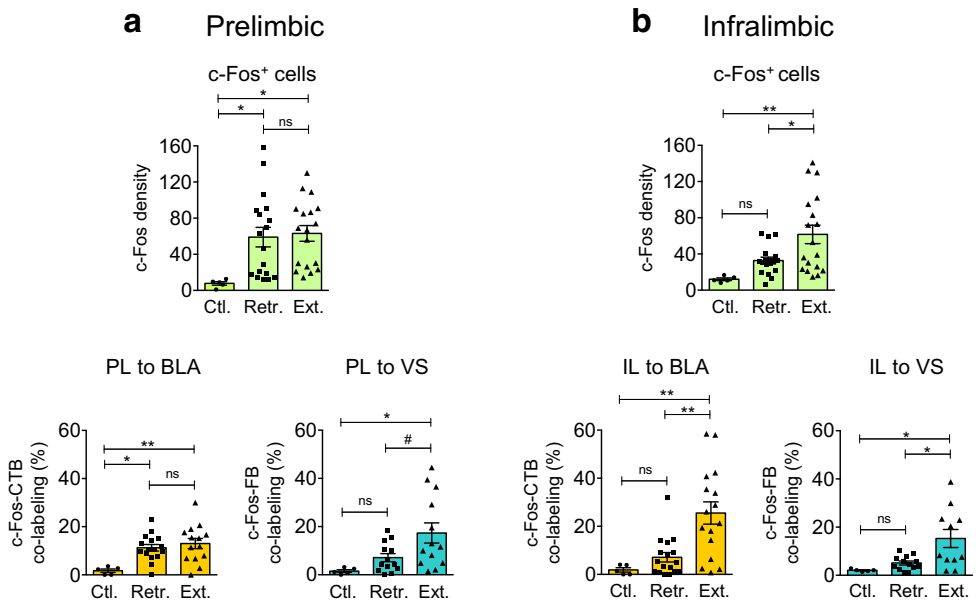

Fig. 3 Density of c-Fos/tracer co-labeling. **a** Both retrieval and extinction of avoidance increased c-Fos immunohistochemistry in PL (upper panel), but retrieval only significantly increased activity in BLA-projecting neurons of PL (lower panels). Extinction resulted in significant activation of PL neurons projecting to VS. **b** IL was significantly activated only during avoidance extinction (upper panel), by neurons projecting to both BLA and VS (lower panels). c-Fos PL: Ctl: $N = 5$; Retr: $N = 18$; Ext: $N = 18$. c-Fos IL: Ctl: $N = 5$; Retr: $N = 18$; Ext: $N = 18$. c-Fos/CTB PL: Ctl: $N = 5$; Retr: $N = 16$; Ext: $N = 14$. c-Fos/CTB IL: Ctl: $N = 5$; Retr: $N = 17$; Ext: $N = 16$. c-Fos/FB PL: Ctl: $N = 5$; Retr: $N = 13$; Ext: $N = 13$. c-Fos/FB IL: Ctl: $N = 5$; Retr: $N = 13$; Ext: $N = 11$. Data shown as mean ± SEM. One-way ANOVAs. $^{**}p < 0.01$, $^{*}p < 0.05$, $^{\#}p = 0.052$, and ns = non-significant

supporting the idea that BLA is a central hub for defensive reactions (LeDoux et al. 2017). What then might be the function of PL inputs to BLA? Recent findings show that pharmacological inactivation of PL delays platform entry during the 30-s tone (Diehl et al. 2018), suggesting that PL outputs may facilitate avoidance when there is less urgency. It is important to keep in mind that c-Fos levels are poor at signaling reductions in activity, and avoidance-induced reduction in PL activity could impact VS, BLA, or other targets. In support of this, we have recently observed that avoidance in this task is also associated with inhibitory responses in PL neurons (Diehl et al. 2018). Thus, retrieval of avoidance memories may integrate PL within the BLA-VS circuit (Fig. 4 upper panel).

In contrast to PL, IL showed no significant retrieval-related activation. This agrees with our prior observation that pharmacological inactivation of IL did not impair retrieval of avoidance in this task, although freezing was increased (Bravo-Rivera et al. 2014). In the shuttle box task, lesions of IL increase freezing but also impair avoidance (Moscarello and LeDoux 2013), consistent with the increased sensitivity of avoidance to freezing levels in this task (Bravo-Rivera et al. 2014; Martinez et al. 2013).

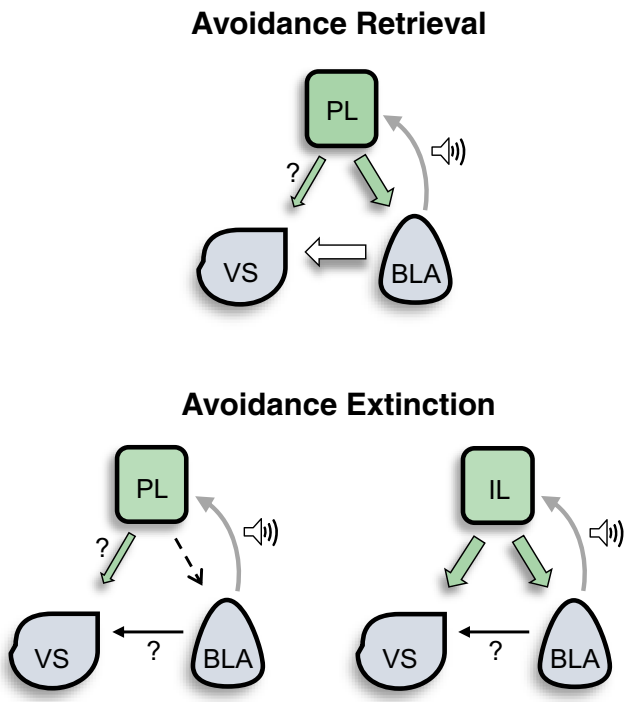

Fig. 4 Neuronal circuits signaling avoidance retrieval and extinction. Based on the present data together with other avoidance studies, the proposed model suggests that retrieval activates PL neurons projecting mainly to BLA (upper panel), whereas avoidance extinction activates IL neurons projecting to both BLA and VS (lower panel). Our data are also consistent with a possible role of PL projections to VS in both retrieval and extinction

Extinction of avoidance

Impairing IL function has been shown to impair extinction of Pavlovian fear conditioning (Do-Monte et al. 2015; Rosas-Vidal et al. 2014; Sierra-Mercado et al. 2011) and more recently extinction of platform-mediated avoidance (Bravo-Rivera et al. 2014; Rosas-Vidal et al. 2018). Furthermore, deficient extinction in this task was associated with decreased c-Fos levels in IL (Bravo-Rivera et al. 2015b). Accordingly, we observed that extinction training increased activity in IL neurons projecting to BLA. In Pavlovian fear conditioning, extinction training increases the intrinsic excitability of IL neurons (Arruda-Carvalho and Clem 2015; Santini et al. 2008), including those that project to BLA (Bloodgood et al. 2018), and photoinhibition of IL terminals in BLA during extinction training impairs the subsequent retrieval of extinction memory (Bukalo et al. 2015). Thus, this IL-BLA circuit may be common to extinction of both fear and avoidance. BLA neurons have been shown to innervate VS interneurons which inhibit medium spiny neurons (Yu et al. 2017). Thus, IL inputs could excite these BLA neurons to reduce avoidance behaviors.

Additionally, avoidance extinction activated IL neurons projecting to VS. This projection may mediate the extinction of the instrumental response of avoidance, as observed in other goal-directed tasks (Augur et al. 2016; Peters and De Vries 2013; Peters et al. 2009, 2008). In fear conditioning, extinction was shown to activate IL projections to VS (Bloodgood et al. 2018). IL could inhibit avoidance by activating local interneurons in VS, or via long-range GABAergic projections reported for this pathway (Bravo-Rivera et al. 2015a; Lee et al. 2014). Therefore, IL projections to both BLA and VS may be necessary for avoidance extinction (Fig. 4 lower panel).

Unexpectedly, extinction training activated PL neurons projecting to VS. While not associated with extinction of conditioned fear (Rosas-Vidal et al. 2014; Sierra-Mercado et al. 2011), PL has recently been implicated in extinction of avoidance. PL lesions have been shown to impair extinction of lever-press avoidance (Fragale et al. 2016), and blocking BDNF in PL impairs retrieval of extinction in this avoidance task (Rosas-Vidal et al. 2018). The involvement of PL in extinction of avoidance, but not extinction of fear conditioning, suggests that PL may regulate strategy switching and behavioral flexibility, especially when avoidance decisions compete with food-seeking (Dalley et al. 2004; Floresco et al. 2008; Kesner and Churchwell 2011; Oualian and Gisquet-Verrier 2010; Rich and Shapiro 2009). However, considering that c-Fos expression lasts several hours after stimuli exposure (Barros et al. 2015; Cullinan et al. 1995; Dragunow and Faull 1989) and the lack of significant difference between extinction and retrieval c-Fos levels ($p = 0.052$), c-Fos expression in the PL-VS projection during extinction learning may also reflect persistence of retrieval-induced activation. Thus,

other techniques with better temporal-spatial resolution such as unit recording or other activity makers with faster dynamics should be considered. Despite this, our results agree with a recent report that PL activity enhances extinction of conditioned fear (Marek et al. 2018). Further studies are needed to determine the role of PL to VS projections during avoidance extinction.

There are several candidate inputs to PFC that could regulate PL and IL in active avoidance. A recent optogenetic study showed that BLA inputs to PL and IL drive expression and extinction of conditioned fear, respectively (Senn et al. 2014). Responses of PL and IL neurons in fear conditioning are regulated by noradrenergic and dopaminergic inputs (Mueller et al. 2010, 2008; Pendyam et al. 2013). Photoactivation of projections from ventral tegmental area to PL evokes anxiety-like behavior and place aversion (Gunaydin et al. 2014), and similar effects were observed with chemoactivation of projections from locus coeruleus to PFC (Hirschberg et al. 2017). Inputs from ventral hippocampus to PL and IL have also been implicated in retrieval and extinction of both fear conditioning (Rosas-Vidal et al. 2014; Sotres-Bayon et al. 2012) and active avoidance (Rosas-Vidal et al. 2018). Future experiments employing optogenetic techniques will be needed to characterize the roles of these prefrontal inputs in the retrieval and extinction of active avoidance.

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Compliance with ethical standards

All procedures were approved by the Institutional Animal Care and Use Committee of the University of Puerto Rico School of Medicine, and the Association for Assessment and Accreditation of Laboratory Animal Care.

Conflict of interest The authors declare that they have no conflict of interest.

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