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Role of a Lateral Orbital Frontal Cortex-Basolateral Amygdala Circuit in Cue-Induced Cocaine-Seeking Behavior

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Cocaine addiction is a disease characterized by chronic relapse despite long periods of abstinence. The lateral orbitofrontal cortex (lOFC) and basolateral amygdala (BLA) promote cocaine-seeking behavior in response to drug-associated conditioned stimuli (CS) and share dense reciprocal connections. Hence, we hypothesized that monosynaptic projections between these brain regions mediate CS-induced cocaine-seeking behavior. Male Sprague-Dawley rats received bilateral infusions of a Cre-dependent adeno-associated viral (AAV) vector expressing enhanced halorhodopsin 3.0 fused with a reporter protein (NpHR-mCherry) or a control AAV (mCherry) plus optic fiber implants into the lOFC (Experiment 1) or BLA (Experiment 2). The same rats also received bilateral infusions of a retrogradely transported AAV vector expressing Cre recombinase (Retro-Cre-GFP) into the BLA (Experiment 1) or lOFC (Experiment 2). Thus, NpHR-mCherry or mCherry expression was targeted to lOFC neurons that project to the BLA or to BLA neurons that project to the lOFC in different groups. Rats were trained to lever press for cocaine infusions paired with 5-s CS presentations. Responding was then extinguished. At test, response-contingent CS presentation was discretely coupled with optogenetic inhibition (5-s laser activation) or no optogenetic inhibition while lever responding was assessed without cocaine/food reinforcement. Optogenetic inhibition of lOFC to BLA, but not BLA to lOFC, projections in the NpHR-mCherry groups disrupted CS-induced reinstatement of cocaine-seeking behavior relative to (i) no optogenetic inhibition or (ii) manipulations in mCherry control or (iii) NpHR-mCherry food control groups. These findings suggest that the lOFC sends requisite input to the BLA, via monosynaptic connections, to promote CS-induced cocaine-seeking behavior.

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INTRODUCTION

Cocaine addiction is a chronic disease characterized by strong propensity for relapse upon exposure to previously cocaine-associated environmental stimuli even after long periods of abstinence (Ehrman *et al*, 1992). Thus, cue-induced relapse represents an enduring challenge for the successful treatment of cocaine addiction. Several brain regions have requisite roles in cocaine-seeking behavior produced by previously drug-paired conditioned stimuli (CS) in rodent models of cue-induced drug relapse (Bossert *et al*, 2013; Lasseter *et al*, 2010); however, few studies have aimed to identify functionally significant connections between these brain regions. As a result, our understanding of the neural circuitry by which drug-associated cues elicit motivation to seek cocaine is limited.

In the present study, we focused on the lateral orbitofrontal cortex (lOFC) and basolateral amygdala (BLA) given the importance of these brain regions in assessing the motivational significance of drug-associated CSs. In support of this, cocaine-dependent individuals exhibit increased cerebral blood flow in the OFC and BLA upon exposure to images containing drug-related cues (Childress *et al*, 1999; Goldstein and Volkow, 2002). Moreover, $GABA_B$ agonist treatment decreases OFC and BLA activation and self-reported craving in response to drug-related cues (Brebner *et al*, 2002; Young *et al*, 2014). In rats, $GABA_{A/B}$ agonist-induced inhibition of the lOFC (Fuchs *et al*, 2004) or tetrodotoxin-induced inactivation of the BLA (Grimm and See, 2000) decreases CS-induced reinstatement of cocaine-seeking behavior, indicating that these brain regions critically control the retrieval and/or utilization of cocaine-CS associations that guide drug-seeking behavior.

The lOFC and BLA share dense reciprocal connections (Lasseter *et al*, 2014; Price, 2007) and may interact to promote CS-induced reinstatement of drug-seeking behavior. Functional disconnection studies have shown that the lOFC and BLA work together to encode stimulus-outcome associations (Saddoris *et al*, 2005), facilitate flexible decision-making (Zeeb and Winstanley, 2013), and control drug context-induced reinstatement of drug-seeking behavior (Lasseter *et al*, 2011). However, the reciprocal nature of anatomical connections between the lOFC and BLA has limited the interpretability of these functional disconnection

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effects, as the direction of information transfer could not be determined using this methodology. Furthermore, the significance of lOFC–BLA interactions in CS-induced cocaine-seeking behavior, in particular, has not been investigated.

To separately investigate whether CS-induced cocaine-seeking behavior depends on monosynaptic input from the lOFC to the BLA or *vice versa*, we expressed cre-recombinase in adeno-associated viral (AAV) vector serotype 6 (Retro-Cre-GFP), which exhibits retrograde transport in rodents and primates (Salegio *et al*, 2013; San Sebastian *et al*, 2013). To permit optogenetic inhibition of either lOFC neurons that project to the BLA (Experiment 1) or BLA neurons that project to the lOFC (Experiment 2), Retro-Cre-GFP was infused at the terminal region, while a cre-dependent AAV containing enhanced halorhodopsin 3.0 fused to an mCherry reporter protein (NpHR-mCherry) and indwelling optic fibers were introduced at the cell body region of the target circuit. At test, optogenetic inhibition was restricted to the duration of CS-presentations to minimize the influence of manipulations on the drug-paired context, which may regain some of its motivational effects upon CS presentation and contribute to drug-seeking behavior through the activation of neural substrates that promote context-induced reinstatement (Fuchs *et al*, 2008; Remedios *et al*, 2014). Thus, the present study examined the specific contributions of monosynaptic projections between the lOFC and BLA to the motivational effects of a drug-paired CS.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (250–275 g; Harlan Laboratories, Livermore, CA; $N=45$) were individually housed in a climate-controlled vivarium on a reversed light–dark cycle. Rats were maintained on 20–25 g of rat chow per day with water available *ad libitum*. All protocols for rat housing and treatment were approved by the Institutional Animal Care and Use Committee and followed the *Guide for the Care and Use of Laboratory Rats* (Institute of Laboratory Animal Resources on Life Sciences, 2011).

Viral Constructs

To construct AAV6-EF1α-Cre-recombinase-IRES2-GFP (Retro-Cre-GFP, Figure 1a), pCAG-Cre-GFP (Addgene, plasmid #26646, deposited by Dr Anjen Chenn) was overexpressed, purified, and then cloned into an AAV backbone by the University of North Carolina (UNC) BAC Recombineering Core. Key regions of the plasmid were sequenced to confirm correct orientation. The AAV was then packaged into serotype 6 by the UNC Vector Core. All viruses were purchased from the UNC Vector Core (titers: $4.0–6.7\times10^{12}$ vm/ml) and delivered at 0.7 µl/hemisphere (0.1 µl/min). The infusion needles (33-gauge) remained in place for 10 min post infusion. Maximal AAV-mediated gene expression occurs ~2–3 weeks post-infection (Carlezon *et al*, 1997; Penrod *et al*, 2015).

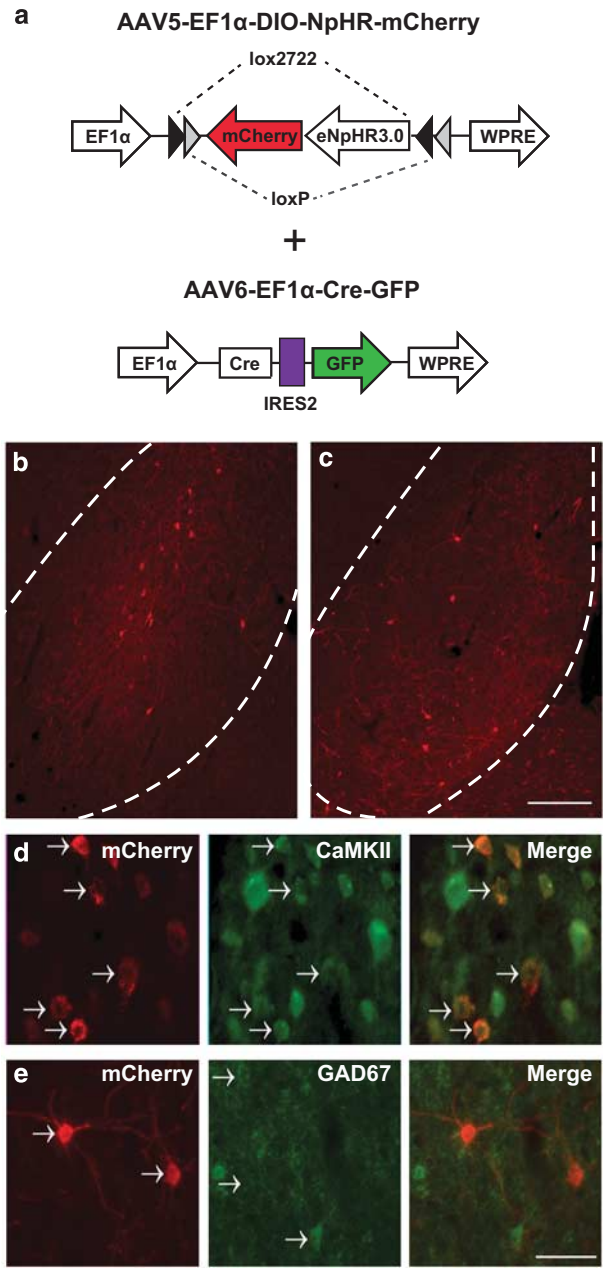

Figure 1 Circuit specific, cre-dependent expression of NpHR-mCherry. (a) Diagrams of the AAV (serotype 5) expressing a cre-dependent, enhanced halorhodopsin 3.0 fused to mCherry (NpHR-mCherry) and AAV (serotype 6) expressing Cre-recombinase-IRES2-GFP (Retro-Cre-GFP) utilized in Experiments 1 and 2. Both AAVs contained transgenes under the control of an EF1α promoter. Representative images showing (b) expression of NpHR-mCherry in the lOFC and (c) in the BLA. Scale bar = 200 µm, × 10 magnification. NpHR-mCherry expression was co-localized with immunoreactivity for (d) the excitatory marker, CaMKII, but not for (e) the GABAergic marker glutamic acid decarboxylase-67 (GAD-67). Scale bar = 50 µm, × 20 magnification.

Surgery

Forty-eight hours after a single food-training session (described in Supplementary Methods), rats were anesthetized with ketamine and xylazine (80.0 mg/kg and 5.0 mg/kg, i.p., respectively). AAV5-EF1α-DIO-eNpHR3.0-mCherry

(NpHR-mCherry) or AAV5-EF1α-DIO-mCherry (mCherry) was infused bilaterally into the lOFC (Experiment 1; +3.5 mm AP, +2.8 mm ML, − 5.0 mm DV from bregma) or the BLA (Experiment 2; − 2.7 mm AP, +5.0 mm ML, − 8.7 mm DV from bregma) (Figure 1a). Retro-Cre-GFP was infused bilaterally into the BLA (Experiment 1) or lOFC (Experiment 2). Optical fibers (200 μm diameter; Thor Labs, Newton, NJ) encased in ceramic ferrules (1.25 mm O.D., 230 μm I.D.; Thor Labs) were assembled in house (Sparta *et al*, 2012) and implanted bilaterally, 0.5 mm dorsal to the NpHR-mCherry or mCherry virus infusion sites. Screws and cranioplastic cement secured the ferrules to the skull. Additional rats received NpHR-mCherry into the lOFC either alone or with Retro-Cre-GFP into the BLA for immunohistochemical verification experiments, or NpHR-mCherry into BLA with Retro-Cre-GFP into the lOFC for slice electrophysiology experiments (see Supplementary Methods). Intravenous (i.v.) catheters were constructed in-house (Fuchs *et al*, 2007) and were inserted into the right jugular vein and threaded subcutaneously to exit on the back. Catheter patency was tested and maintained as described in Supplementary Methods.

Cocaine Self-Administration Training

Drug (Figures 3a and 4a) or food (Supplementary Figure S4A) self-administration training was conducted in sound-attenuated operant conditioning chambers (Coulbourn Instruments, Allentown, PA) during the rats' dark cycle for 2 h each day. Throughout each session, a red house light (0.4 fc brightness) was illuminated on the wall opposite to levers. During the cocaine self-administration session, responses on a designated active lever resulted in 2-s cocaine infusions (cocaine hydrochloride dissolved in sterile saline; 0.15 mg/0.05 ml per infusion, i.v.; NIDA Drug Supply Program, Research Triangle Park, NC) under a fixed ratio 1 (FR1) reinforcement schedule with a 20-s timeout period. During the food self-administration sessions, active lever presses resulted in delivery of a single food pellet under gradually increasing FR schedules with 20-s timeout periods (see Supplementary Methods). Each reinforcer (drug or food) was paired with 5-s presentation of a CS complex (white stimulus light above the active lever plus a 75 dB, 1 kHz tone). Active lever responses during the timeout periods and inactive lever responses during the session had no programmed consequences. Training was terminated when rats reached a criterion of $\geqslant$10 cocaine infusions per session on $\geqslant$10 days. Data collection and reinforcer delivery were controlled using Graphic State Notation software version 4.1.04 (Coulbourn Instruments).

Extinction Training

After the completion of drug or food self-administration training, all rats received daily 2-h extinction training sessions on at least 7 days until they reached the extinction criterion ($\leqslant$25 active lever responses per session on two consecutive days). During the sessions, lever responses had no programmed consequences. During the last three sessions, the rats' indwelling optic fibers were connected bilaterally to mock optical patch cords to acclimatize them to the apparatus.

Testing

At test, all rats' optic fibers were connected bilaterally to mono-fiber optic patch cords (50 μM I.D.; Doric Lenses, Quebec, CAN) encased in spring leashes (Plastics One). The patch cords were connected to one end of an optical commutator (1 × 2 Fiber Optic Rotary Joint; Doric Lenses), to allow for free movement of rats and provide bilateral division of laser light from a single source. The other end of the optical commutator was connected to a solid state laser (Shanghai Laser & Optics Century, Shanghai, China) via an optic patch cable (105 μm, 3 m; Thor Labs). The laser was located outside of the sound-attenuation chamber. Laser light (532 nm) was calibrated to 10–12 mW intensity at the tip of the patch cords immediately before testing (Sparta *et al*, 2012).

During two 1-h test sessions, active lever presses resulted in 5-s presentations of the previously cocaine-paired or food-paired CS either alone (Laser OFF) or discretely coupled with laser activation (Laser ON; continuous for 5 s). Each CS presentation initiated a 20-s timeout period during which active lever presses had no programmed consequences. The order of Laser ON and Laser OFF test sessions was counterbalanced, and the test sessions were separated by additional extinction sessions until active lever responding was $\leqslant$25 per session on two consecutive days.

Histology and Data Analysis

Rats were overdosed using ketamine and xylazine (240 mg/kg and 15 mg/kg, i.v., respectively) and transcardially perfused. Brain tissue was collected for assessment of optic fiber placement, immunohistochemical experiments, and whole-cell electrophysiological recordings (see Supplementary Methods). Rats with insufficient mCherry expression, off-target mCherry or GFP expression, or misplaced optic fibers were excluded from data analysis. All data were analyzed using analyses of variance (ANOVA) or *t*-tests, when appropriate (see Supplementary Methods). *Post-hoc* comparisons were done using Tukey's tests. Alpha was set at 0.05.

RESULTS

Histology

In the virus verification experiments, NpHR-mCherry expression was not observed in the lOFC unless Retro-Cre-GFP was infused into the BLA (Supplementary Figure S1A and B). Expression was robust within both the somal and dendritic compartments of cells within the lOFC and BLA, (Figure 1b and c), with similar numbers of mCherry-immunoreactive cells visible in each target region (Supplementary Figure S1G). NpHR-mCherry expression was exclusive to glutamatergic neurons, as indicated by co-localization with the excitatory marker calcium/calmodulin-dependent protein kinase II (CaMKII, Figure 1d) but no co-localization with the GABAergic marker glutamic acid decarboxylase-67 (GAD-67, Figure 1e). In Experiment 1, optic fiber tracts and NpHR-mCherry/mCherry expression were restricted to the lOFC (Figure 2a) and Retro-Cre-GFP expression was restricted to the BLA (Supplementary

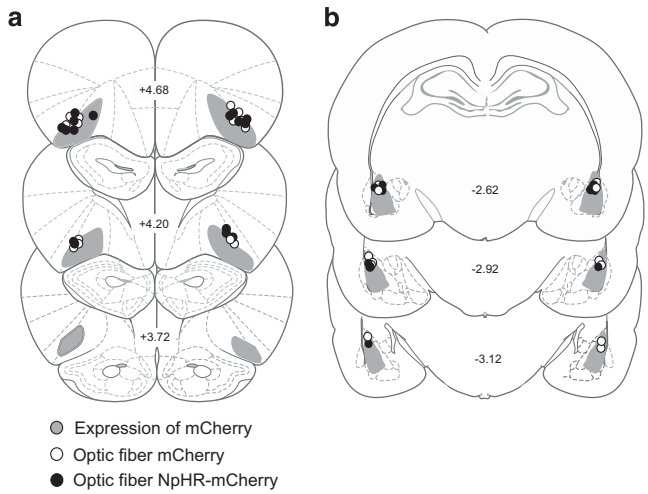

Figure 2 Virus spread and optic fiber placements. Schematics depicting mCherry and NpHR-mCherry viral spread (shaded areas) and the most ventral point of optic fiber tracts for rats that received mCherry (white circles) or NpHR-mCherry (black circles) into the (a) lOFC (mCherry, $n=8$; NpHR-mCherry, $n=8$) or (b) BLA (mCherry, $n=6$; NpHR-mCherry, $n=6$). Numbers denote distance from bregma in mm on the schematics modified from the rat brain atlas of Paxinos and Watson (1997).

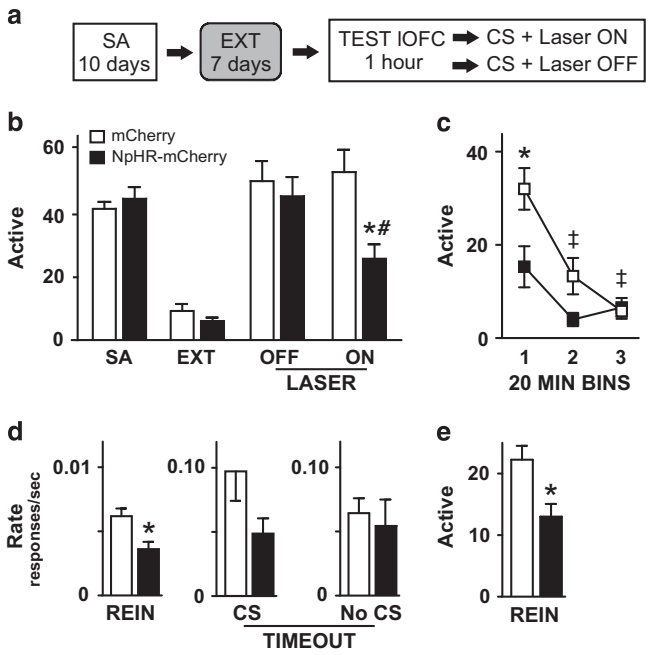

Figure 3 Effect of optogenetic inhibition of lOFC afferents to the BLA on CS-induced reinstatement of cocaine-seeking behavior. (a) Schematic depicting the timeline of the behavioral experiments: cocaine self-administration training (SA), extinction training (EXT), and counterbalanced CS-induced reinstatement tests (1 h each) with or without laser stimulation. (b) Active lever responses (mean ± SEM) during each phase of the experiment for the mCherry ($n=8$) and NpHR-mCherry ($n=8$) groups. (c) Time course of active lever responses (mean ± SEM) during each 20-min bin of the Laser ON test. (d) Response rates (mean responses/second ± SEM) on the active lever during the Laser ON test when active lever presses were reinforced with CS presentation (REIN) or were not reinforced during the 5-s CS presentation (CS) and the remaining 15-s (No CS) of the timeout period. (e) Total number of CS-reinforced active lever responses during the Laser ON test. Symbols represent difference between mCherry and NpHR-mCherry groups (*, b, c: Tukey's test, $P<0.05$; d, e: *t*-test, $P<0.05$), between the Laser OFF and Laser ON test sessions (#, Tukey's test, $P<0.05$), and change from Bin 1 (‡, Tukey's test, $P<0.05$).

Figure S2A) ($n=8$/group). In Experiment 2, optic fiber tracts and NpHR-mCherry/mCherry expression were restricted to BLA (Figure 2b) and Retro-Cre-GFP expression was restricted to the lOFC (Supplementary Figure S2B) ($n=6$/group). mCherry expression was not visible in terminal regions (Supplementary Figure S1C–F).

Behavioral History and Inactive Lever Presses

The groups did not differ in drug or food intake or lever responding during self-administration and extinction training. They also did not differ in inactive lever responding during any phase of the experiment (Supplementary Figure S3). These findings and the results of the food control experiment (Supplementary Figure S4) are reported in Supplementary Results.

lOFC → BLA Circuit Inhibition Disrupts CS-Induced Cocaine-Seeking Behavior

Cocaine-seeking behavior depended on laser manipulation and opsin expression (ie, group: mCherry *vs* NpHR-mCherry; 2 × 2 ANOVA laser × group interaction: $F_{1,14}=4.93$, $P=0.04$; group main effect: $F_{1,14}=5.38$, $P=0.04$; laser main effect: $F_{1,14}=2.75$, $P=0.12$). In the mCherry control group, active lever responding did not differ across the Laser ON and Laser OFF tests (Figure 3b). Conversely, in the NpHR-mCherry group, responding during the Laser ON test was lower than during the Laser OFF test ($P<0.05$) and compared with the mCherry group during the Laser ON test ($P<0.01$). A time course analysis of behavior during the Laser ON test indicated time-dependent group differences (2 × 3 ANOVA time × group interaction: $F_{2,14}=3.87$, $P=0.03$; time main effect: $F_{2,14}=17.64$, $P=0.0001$; group main effect, $F_{1,14}=9.15$, $P=0.01$). Specifically, the NpHR-mCherry group exhibited fewer responses than the mCherry group during the first 20 min of the Laser ON test (Figure 3c; $P<0.01$), after which responding decreased (Bin 1 > Bins 2–3; $P<0.01$).

Additional analyses evaluated possible changes in active lever response rate in relation to the termination of laser stimulation (Figure 3d). The NpHR-mCherry group exhibited lower *CS-reinforced* response rates than the mCherry group ($t_{14}=3.08$, $P=0.008$). *Non-reinforced* response rates did not differ between the groups during CS-coupled laser stimulation ($t_{14}=1.88$, $P=0.08$) or during the remainder of the timeout period ($t_{14}=0.42$, $P=0.68$), suggesting that responding did not rebound after laser stimulation was terminated. Furthermore, the NpHR-mCherry group exhibited fewer CS-reinforced responses than the mCherry group during the Laser ON test (Figure 3e; $t_{14}=3.01$, $P=0.009$).

BLA → lOFC Circuit Inhibition Fails to Alter CS-Induced Cocaine-Seeking Behavior

There were no differences in cocaine-seeking behavior between the groups (Figure 4b; 2 × 2 ANOVA laser × group

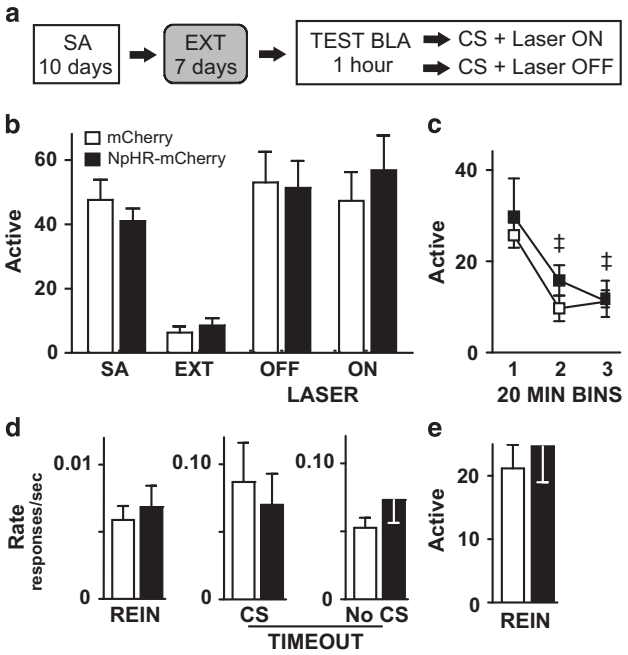

Figure 4 Effect of optogenetic inhibition of BLA afferents to the lOFC on CS-induced reinstatement of cocaine-seeking behavior. (a) Schematic depicting the timeline of the behavioral experiments: cocaine self-administration training (SA), extinction training (EXT), and counterbalanced CS-induced reinstatement tests with or without laser stimulation (1 h each). (b) Active lever responses (mean ± SEM) during each phase of the experiment for the mCherry ($n=6$) and NpHR-mCherry ($n=6$) groups. (c) Time course of active lever responses (mean ± SEM) during each 20-min bin of the Laser ON test. (d) Response rates (mean responses/second ± SEM) on the active lever during the Laser ON test when active lever presses were reinforced with CS presentation (REIN) or were not reinforced during the 5-s CS presentation (CS) and the remaining 15-s (No CS) of the timeout period. (e) Total number of CS-reinforced active lever responses during the Laser ON test. Symbols represent change from Bin 1 (‡, Tukey's test, $P<0.05$).

interaction, $F_{1,10}=0.46$, $P=0.51$, group main effect, $F_{1,\ 10}=0.19$, $P=0.67$; laser main effect, $F_{1,14}=0.003$, $P=0.96$). A time course analysis of behavior during the Laser ON test revealed a time main effect only (Figure 4c; 2×3 ANOVA time main effect, $F_{2,10}=7.26$, $P=0.004$; laser × group interaction, $F_{2,10}=0.98$, $P=0.39$; group main effect, $F_{1,10}=0.78$, $P=0.40$). Thus, active lever responding declined in both groups during the Laser ON test session (Bin 1 > Bin 2–3; $P<0.05$).

In terms of active lever response rates (Figure 4d), no differences were observed between the NpHR-mCherry and mCherry groups in *CS-reinforced* ($t_{10}=0.51$, $P=0.62$) and *non-reinforced* response rates during CS-coupled laser stimulation ($t_{10}=0.46$, $P=0.66$) or during the remainder of the timeout period ($t_{10}=1.11$, $P=0.29$). Thus, responding did not rebound after laser stimulation was terminated. Furthermore, no differences were observed between the groups in the total number of CS-reinforced responses during the Laser ON test (Figure 4e; $t_{10}=0.52$, $P=0.62$) even though BLA neurons exhibited light-induced hyperpolarization (Figure 5a and b) and inhibition of evoked action potential firing *ex vivo* (Figure 5c–e) (see Supplementary Results).

DISCUSSION

The present study examined the direction-specific contribution of monosynaptic projections between the lOFC and BLA in cue-induced cocaine-seeking behavior. To permit the selective inhibition of these circuits, a Retro-Cre-GFP was infused at the terminal region, and a Cre-dependent NpHR-mCherry was infused at the cell body region, of the target circuit. Cre-dependent expression of mCherry was observed in the cell body regions of the lOFC (Experiment 1) and BLA (Experiment 2) (Figure 1). Importantly, optogenetic inhibition of lOFC afferents to the BLA impaired CS-induced reinstatement of cocaine-seeking behavior compared with no optogenetic inhibition (Figure 3) and to manipulations in the mCherry control groups that did not express the opsin (Figure 3) and in the NpHR-mCherry groups in the food control experiment (Supplementary Figure S4). Optogenetic inhibition of BLA afferents to the lOFC did not impair CS-induced cocaine-seeking behavior (Figure 4) despite eliciting light-induced hyperpolarization and inhibition of evoked action potential firing (Figure 5).

Direct Input from the lOFC to the BLA is Necessary for CS-Induced Cocaine-Seeking Behavior

The present study demonstrates that the lOFC and BLA interact in a direction-specific manner to promote cue-induced reinstatement, extending localization studies that focused on the individual contributions of these brain regions to CS-induced reinstatement (Fuchs *et al*, 2004; Grimm and See, 2000) and disconnection studies that showed their interaction in drug context-induced reinstatement (Lasseter *et al*, 2011). In addition to the interpretational difficulty stemming from interhemispheric connections between the BLA and lOFC, results from functional disconnection did not provide insight into the direction of information processing between these brain regions. In contrast, the approach utilized in the present study has allowed us to demonstrate that information transfer from the lOFC to the BLA, in particular, is necessary for CS-induced reinstatement.

Importantly, the decrease in cocaine-seeking behavior following optogenetic inhibition of lOFC afferents to the BLA was not mediated by the inhibition of postsynaptic BLA neurons that project back to the lOFC (ie, via a lOFC → BLA → lOFC functional loop) as inhibition of BLA projections to the lOFC failed to alter this behavior (Figure 4b). In line with previous studies (Calu *et al*, 2013), behavioral impairment was also not due to hypoactivity or nonspecific performance deficits associated with AAV or laser exposure. Consistent with this, optogenetic inhibition of lOFC projections to the BLA failed to alter responding on the inactive lever (Supplementary Figure S3) or on the active lever during the timeout periods (Figure 3d). Furthermore, laser exposure failed to alter responding on the active lever in the mCherry group in Experiment 1 (Figure 3b) or in the NpHR-mCherry group that received optogenetic inhibition of BLA projections to the lOFC in Experiment 2 (Figure 4b). Finally, it is unlikely that lOFC → BLA circuit inhibition produced a nonspecific impairment in reversal learning (Schoenbaum *et al*, 2007) or in the processing of conditioned reinforcement (Roberts *et al*, 2007) because optogenetic manipulation failed

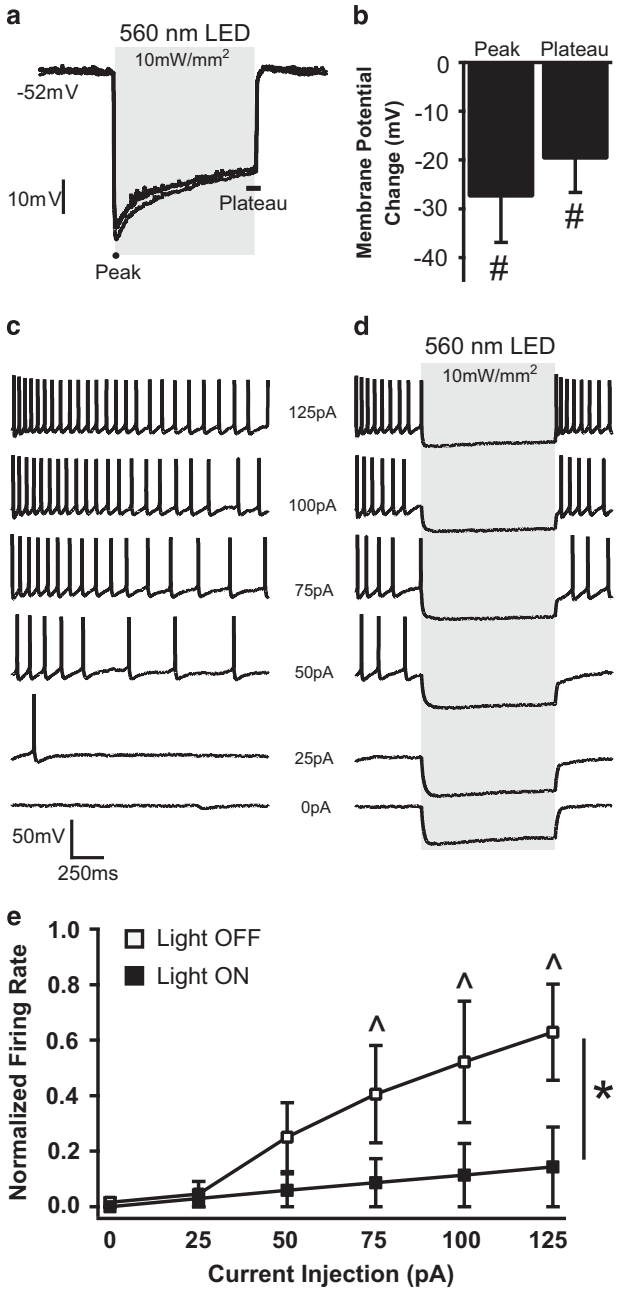

Figure 5 Effects of optogenetic manipulation on neuronal membrane potential and evoked action potential firing in BLA afferents to the lOFC. (a) Overlay of current-clamp recording of BLA neuronal membrane potential in response to three consecutive light pulses (560 nm, 5 s, shaded area). (b) Mean change in membrane potential (±SEM) in NpHR-mCherry-expressing neurons ($n=5$) at the peak and plateau of the light response. (c, d) Representative recordings of action potential firing evoked by current injection steps in the absence (c) and presence (d) of a light pulse (560 nm, 1 s, shaded area). (e) Current injection-evoked firing rates (mean ± SEM, normalized to maximum rate in 'Light OFF' condition) in NpHR-mCherry-expressing BLA neurons in the presence and absence of a light pulse (560 nm, 1 s). Symbols represent difference relative to baseline membrane potential (#, *t*-tests, $P<0.05$), relative to the Light OFF condition (*, ANOVA light main effect, $P<0.05$), and relative to current steps 0–75 pA (^, Tukey's tests, $P<0.05$).

to alter food CS-induced food-seeking behavior (Supplementary Figure S4). Therefore, inhibition of lOFC afferents to the BLA selectively altered the motivational effects of the cocaine-associated CS.

As lOFC projection neurons are glutamatergic, our findings seem inconsistent with an earlier report that glutamate receptor antagonism in the BLA failed to inhibit CS-induced cocaine-seeking behavior (See *et al*, 2001). Null effects in that pharmacological study may have resulted from insufficient dosing in the BLA. Alternatively, CS-induced reinstatement may require metabotropic glutamate receptor stimulation within the BLA, similar to cocaine-conditioned place preference (Krishnan *et al*, 2011). Overall, the present findings suggest that the lOFC and BLA interact, and specifically the lOFC provides critical input to the BLA via monosynaptic projections, to stimulate CS-induced cocaine-seeking behavior.

The Role of the BLA in CS-Induced Cocaine-Seeking Behavior

The importance of the BLA in CS-induced motivated behavior is well recognized, yet optogenetic inhibition of BLA afferents to the lOFC failed to alter CS-induced cocaine-seeking behavior in the present study (Figure 4b). This unexpected finding may have resulted from possible off-target effects of our optogenetic manipulation (see review by Otchy *et al*, 2015). Mitigating this possibility, previous localization studies that utilized NMDA lesions, or tetrodotoxin-induced or lidocaine-induced neural inactivation, targeted BLA neurons in general (Grimm and See, 2000; Kantak *et al*, 2002; Meil and See, 1997,Whitelaw *et al*, 1996). Some of these manipulations inhibited fibers of passage and axon terminals, including lOFC terminals within the BLA, which would have impaired CS-induced cocaine-seeking behavior based on the findings reported here. Furthermore, earlier optogenetic studies targeted BLA neuronal populations that project to the nucleus accumbens core (NACc) or prelimbic cortex (PrL) and have requisite roles in CS-induced reinstatement (Stefanik and Kalivas, 2013). One population of BLA neurons that projects to the PrL and/or NACc also sends collaterals to the agranular insular cortex (Shinonaga *et al*, 1994), a region adjacent to the lOFC. In comparison, it is unclear whether BLA neurons that project to the lOFC possess direct connections with the PrL or NACc as we did not observe mCherry expression in the lOFC or these putative terminal regions (Supplementary Figure S1E and F) likely because mCherry expression in terminal regions requires more than 4–5 weeks to develop. However, our findings indicate that the activity of the BLA → lOFC circuit is not functionally required for CS-induced reinstatement, independent of whether component BLA neurons are anatomically connected with the PrL or NAc.

Further supporting this conclusion, the null effect observed following optogenetic inhibition of BLA afferents to the lOFC was not due to insufficient opsin expression as the number of mCherry expressing cells was similar in the BLA and lOFC (Supplementary Figure S2G). Furthermore, halorhodopsin-expressing cell bodies in the BLA exhibited reliable light-induced hyperpolarization (Figure 5a and b) and inhibition of evoked action potential firing (Figure 5c–e). Termination of optogenetic inhibition did not

generally induce rebound excitation (Figure 5a; Raimondo *et al*, 2012). Finally, our behavioral findings conclusively demonstrated that the NpHR-mCherry and mCherry groups did not differ in response rate immediately after CS-coupled laser stimulation was discontinued (Figure 4d). Accordingly, rebound in neural activity was not responsible for the null effect in cocaine-seeking behavior. Based on these considerations, we conclude that requisite monosynaptic communication between the lOFC and BLA is unidirectional, and the contribution of the BLA → lOFC circuit to CS-induced reinstatement is not critical. This does not rule out that the BLA → lOFC circuit may make nonessential contributions that are masked by intact information transfer from the BLA to the lOFC through multi-synaptic, parallel circuits. Overall, direct BLA input to brain regions *other than the lOFC* is sufficient (present study) and necessary (eg, NACc and PrL; Stefanik and Kalivas, 2013) for CS-induced reinstatement.

Selective Optogenetic Inhibition of CS-Induced Motivated Behavior

A unique methodological feature of the present study was optogenetic inhibition coupled with discrete CS presentation. In comparison, pharmacological manipulations inhibit neural activity for extended time periods, and thus possibly disrupt the cognitive representation or processing of the drug-paired CS and context alike. While contextual background stimuli acquire more modest motivational effects than CSs due to weaker temporal contiguity with the effects of cocaine, contextual stimuli facilitate drug-seeking behavior (Fuchs *et al*, 2008). For instance, response-contingent presentation of an alcohol-associated CS elicits more drug-seeking behavior in an alcohol-associated context than in a control context (Remedios *et al*, 2014). Following extinction training, such motivational effects of the context may be renewed by CS presentation. In the present study, optogenetic inhibition did not alter cocaine-paired context-induced motivation as the NpHR-mCherry and mCherry groups did not differ in responding during the timeout period, when CS presentations were unavailable, and re-obtained the extinction criterion at similar rates after a Laser ON test (see Supplementary Results). Thus, the robust effect of CS-coupled optogenetic inhibition highlights the degree to which lOFC afferents to the BLA control the motivational effects of cocaine-paired CSs in particular. To further explore the putative contributions of this neural circuit to the motivational effects of the drug-paired context during CS-induced reinstatement, a control group receiving laser stimulation exclusively unpaired from CS presentation will be a useful addition in future studies.

Combinatorial Viral Approach

Another novel methodological component of the present study was the use of a combinatorial viral approach. Retro-Cre-GFP was packaged in AAV serotype 6 which exhibits efficient retrograde transport via mechanisms that are likely dependent on dynein and kinesin-2 proteins (Murlidharan *et al*, 2014; Rothermel *et al*, 2013; Salegio *et al*, 2013). Similar studies have used recombinant canine adenovirus 2 (CAV2) or AAV serotype 5 to manipulate neural circuits (Beyeler *et al*, 2016; Marchant *et al*, 2015). Most commonly, studies have utilized methodology that involves optogenetic inhibition at the virus infusion site (to target discrete brain nuclei) or at the terminal region of the target circuit following virus injection at the cell body region, even though these approaches are more prone to affect fibers of passage (Beyeler *et al*, 2016; Stamatakis and Stuber, 2012). In particular, the combinatorial viral approach is essential (a) to manipulate proteins that are not expressed at the terminal region of target circuits and (b) to mitigate the current scarcity of Cre-driver lines and unavailability of Cre-dependent opsins packaged in retrogradely transported viruses (Saunders *et al*, 2015; Witten *et al*, 2011; Warden *et al*, 2014). One caveat is that retrograde transport efficiency in various AAV serotypes appears to vary across species (Aschauer *et al*, 2013; Rothermel *et al*, 2013). The current viral approach is challenging as it requires co-infection of neurons by two viruses, but it can also be employed to selectively manipulate subpopulations of neurons using unique promoter combinations. Therefore, this combinatorial viral approach is an important tool for circuit-specific manipulations.

Conclusions: The Position of the lOFC and BLA in the CS-induced Relapse Circuitry

The present findings refine our understanding of the neural circuitry of cocaine-seeking behavior. According to previous studies, optogenetic inhibition of PrL to NACc, BLA to PrL, or BLA to NACc projections disrupts CS-induced reinstatement (Stefanik and Kalivas, 2013; Stefanik *et al*, 2013b), while optogenetic inhibition of NACc to dorsolateral ventral pallidum (dlVP) or VTA to NACc projections impairs CS +cocaine-primed reinstatement of cocaine-seeking behavior (Stefanik *et al*, 2013a). Other studies indicate that muscarinic cholinergic and dopamine receptor stimulation in the BLA mediates CS-induced reinstatement (See *et al*, 2001, 2003). Together these and the present findings are consistent with a circuitry model in which the BLA assesses the motivational significance of cocaine-paired CSs at the time of testing (Fuchs *et al*, 2002) based on input from the lOFC and VTA (See *et al*, 2001). The BLA may transmit the results of these computations to the PrL, which holds executive function (Lasseter *et al*, 2010) and integrates this information with input from the VTA (Ciccocioppo *et al*, 2001). Subsequently, the PrL mediates response selection and initiation via the NACc-dlVP circuit (Kalivas and McFarland, 2003). However, response execution at the level of the NACc occurs with additional requisite input from the BLA and VTA, and this may permit more nuanced control over behavior in complex situations where multiple reinforcers and response opportunities exist.

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