Biological Psychiatry

Archival Report

The Basolateral Amygdala to Nucleus Accumbens Core Circuit Mediates the Conditioned Reinforcing Effects of Cocaine-Paired Cues on Cocaine Seeking

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ABSTRACT

BACKGROUND: Individuals addicted to cocaine spend much of their time foraging for the drug. Pavlovian drug-associated conditioned stimuli exert a major influence on the initiation and maintenance of drug seeking often long into abstinence, especially when presented response-contingently, acting as conditioned reinforcers that bridge delays to drug use. The acquisition of cue-controlled cocaine seeking has been shown to depend on functional interactions between the basolateral amygdala (BLA) and the nucleus accumbens core (NAcC). However, the precise neuronal circuits underlying the acquisition of cue-controlled cocaine-seeking behavior have not been elucidated.

METHODS: Here, we used a projection-specific Cre-dependent DREADD (designer receptor exclusively activated by designer drugs)-mediated causal approach to test the hypothesis that the direct projections from the BLA to the NAcC are required for the acquisition of cue-controlled cocaine-seeking behavior.

RESULTS: In Sprague Dawley rats with Cre-mediated expression of the inhibitory DREADD hM4D(Gi) in the NAcC-projecting BLA neurons, treatment with clozapine N-oxide, but not vehicle, selectively prevented the impact of cocaine-associated conditioned reinforcers on cocaine seeking under a second-order schedule of reinforcement. This effect was attributable to the chemogenetic inhibition of the NAcC-projecting BLA neurons, as it was reversible, and it was absent in clozapine N-oxide–treated rats expressing an empty control virus. In contrast, chemogenetic inhibition of the anterior insula, which receives collateral projections from NAcC-projecting BLA neurons, was without effect.

CONCLUSIONS: These data demonstrate that the acquisition of cue-controlled cocaine seeking that depends on the conditioned reinforcing effects of cocaine cues requires activity in the direct projections from the BLA to the NAcC.

https://doi.org/10.1016/j.biopsych.2020.07.022

Individuals with severe substance use disorder do not simply take drugs; they also spend a considerable amount of their time seeking and obtaining them. Over a prolonged history of drug use, this drug seeking becomes compulsive, persisting despite adverse personal as well as social consequences (1). It is therefore important to understand the neural basis of both drug-seeking and drug-taking behavior, which are mediated by dissociable psychological processes (2–4).

Instrumental drug-seeking behavior is greatly influenced by drug-associated pavlovian conditioned stimuli (CSs) (5). When presented unexpectedly and noncontingently, these drug cues capture attention (6), elicit approach behavior (7,8), and invigorate instrumental behavior through the process of pavlovian-instrumental transfer (9–11). However, it is when CSs are response-produced, acting as conditioned reinforcers (CRfs), that they exert their most powerful effects on drug-seeking behavior, maintaining it over extended periods of time (12–15), precipitating relapse after abstinence (16–22), and increasing in impact the longer the period of abstinence [incubation of craving (23)].

At the neural systems level, the effects of self-administered cocaine that reinforce taking responses depend on activity in the mesolimbic dopamine system (3,24), and especially dopaminergic transmission in the shell of the nucleus accumbens (NAc) (13). In marked contrast, the acquisition of cocaine seeking, in which responding maintained over protracted time periods is strongly enhanced by the presentation of cocaine-associated CRfs (12–15,25,26), requires the functional integrity of the basolateral amygdala (BLA), the NAc core (NAcC), and putative circuit interactions between these structures (27,28).

Studies using excitotoxic lesions or pharmacological manipulations have shown that the acquisition of cue-controlled cocaine-seeking behavior, as well as seeking responses for CRfs associated with food (29–31) and sex reward (32), depends on the BLA (33), which mediates the motivational representation of CS–

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 ISSN: 0006-3223

unconditioned stimulus associations (9), and the NAcC, but not the NAc shell (27). Functional disconnection studies further revealed that cue-controlled cocaine-seeking behavior depends on dopamine-dependent interactions between the BLA and the NAcC (28), because unilateral blockade of dopamine receptors in the BLA combined with blockade of AMPA receptors in the contralateral NAcC, which thereby functionally disconnects these structures, impaired cue-controlled cocaine-seeking behavior to the same extent as bilateral manipulations of either structure alone (28).

While these studies suggest an important function of the BLA and the NAcC and their functional interaction in the acquisition of cue-controlled cocaine seeking, they do not precisely identify the circuit involved. However, glutamatergic BLA neurons exert robust physiological control over the activity of NAcC medium spiny neurons associated with reward-seeking behavior (34) and undergo synaptic plasticity following cocaine exposure (35), thereby suggesting that direct BLA→NAcC projections comprise the circuit that is required for the acquisition of cue-controlled cocaine seeking.

To test this hypothesis, we used a Cre-dependent, pathway-specific DREADD (designer receptor exclusively activated by designer drugs)-mediated approach to causally investigate the role of the BLA→NAcC circuit in the acquisition of cue-controlled cocaine-seeking behavior in Sprague Dawley rats.

The results show that the direct BLA→NAcC circuit mediates the acquisition of cue-controlled cocaine seeking measured under a second-order schedule of reinforcement (SOR) (26). Clozapine-N-oxide (CNO) administration prevented the ability of a cocaine-associated CRf to potentiate instrumental seeking responses in rats expressing the inhibitory hM4D(Gi) DREADD, but not an empty control virus, in the BLA→NAcC neurons. This inhibition of cocaine seeking was reversible and was not observed after chemogenetic inhibition of the anterior insula, to which the NAcC-projecting BLA neurons send collateral afferents (36).

METHODS AND MATERIALS

Animals

Experiments were performed on 59 male Sprague Dawley rats as previously described (37) (see the Supplement for more details) and conducted in accordance with the UK 1986 Animals (Scientific Procedures) Act following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body under the project license number 70/8072.

Procedures

A schematic of the timeline of the experiments detailed in the Supplement is presented Figure 1A, C. Key resources are listed in the dedicated Key Resources Table.

Drugs

Cocaine hydrochloride (National Institute on Drug Abuse Drug Supply Program, Rockville, MD) was dissolved in sterile 0.9% NaCl. CNO (National Institute on Drug Abuse Drug Supply Program) was dissolved first in 5% DMSO (Sigma-Aldrich, Poole, United Kingdom) and then in sterile 0.9% NaCl (Henry Schein Ltd, Gillingham, United Kingdom). A vehicle (Veh) solution was prepared with 5% DMSO in sterile 0.9% saline as a control for CNO.

Viral Vectors

Cre-dependent expression of hM4D(Gi) was mediated by coadministration of a transsynaptic CAV2-Cre virus (Plateforme de Vectorologie de Montpellier, Montpellier, France) and a pAAV8-hSyn-DIO-hM4D(Gi)-mCherry virus (plasmid #44362; Addgene, Watertown, MA) while that of the mCherry reporter alone was mediated by coadministration of the CAV2-Cre virus and a pAAV8-hSyn-DIO-mCherry virus (plasmid #50459; Addgene) (referred to throughout the article as “empty”). Non–Cre-dependent expression of hM4D(Gi) was mediated by administration of a pAAV8-CaMKIIa-hM4D(Gi)-mCherry virus (plasmid #50477; Addgene), while expression of a GFP (green fluorescent protein) reporter was achieved by administration of an AAV5-CaMKII-GFP virus (Boyden; UNC Vector Core, Chapel Hill, North Carolina), which is also referred to as “empty” throughout.

Stereotaxic Surgery and Viral Infusions

Viral infusions (design illustrated in Figure 1B) were performed as described in the Supplement.

Intrajugular Catheterization Surgery

Rats were implanted with a laboratory-fabricated indwelling catheter into their right jugular vein, as previously described (38) and detailed in the Supplement.

Self-administration

Experiments were conducted in 24 standard operant chambers, as previously described (39) and detailed in the Supplement.

Rats were trained to self-administer cocaine (0.25 mg/100 μL/5.7 s/infusion) under a fixed ratio 1 (FR1) schedule of reinforcement over 7 daily 2-hour sessions, as previously described (40) and detailed in the Supplement.

The daily schedule of reinforcement was then changed to fixed intervals, increasing across daily training sessions from 1 minute (fixed interval 1 minute [FI1]) to FI2, FI4, FI8, FI10, and eventually FI15 (41), as detailed in the Supplement. As previously described (25,40–42), under a FI15 schedule of reinforcement, each day instrumental responding was maintained over the 15-minute interval in the absence of the drug, but in anticipation of the eventual, contingently delivered intravenous infusion of cocaine.

Note that drug-seeking behavior measured in this procedure differs in psychological terms from that measured in cued reinstatement of drug-seeking paradigms (23,43–45), in which rats with a history of drug self-admininstration (usually under low ratio schedules) are required to respond under extinction conditions (i.e., no drug is delivered). Because instrumental responding never leads to a drug infusion under these conditions, it decreases sometimes even during a single session, reflecting the learning of a new response–no unconditioned stimulus association, which is often conflated with the introduction of a new response–CS contingency (42).

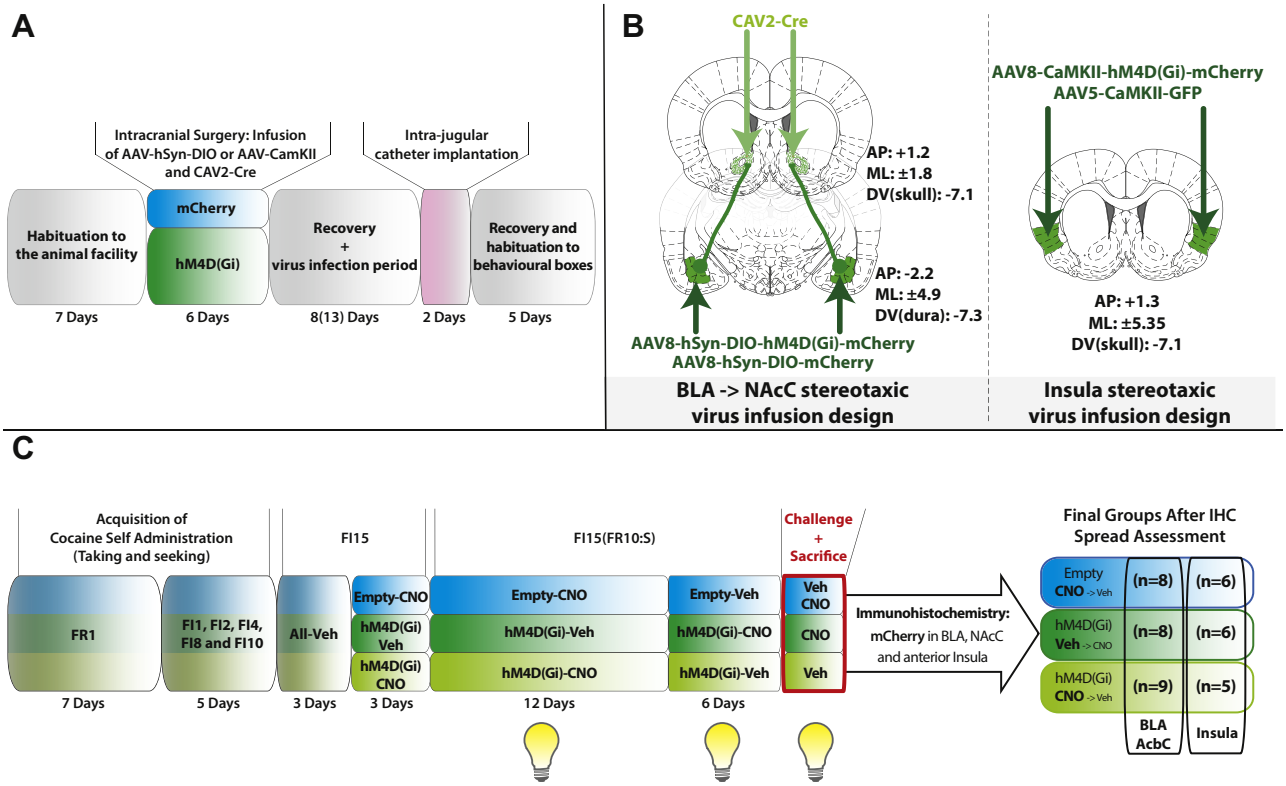


Figure 1. Timeline and experimental design. After a week of habituation to the vivarium **(A)** rats underwent intracranial surgeries, during which they received virus infusions **(B)** enabling a projection-specific expression of empty control or hM4D(Gi) DREADD in the NAcC-projecting BLA neurons or in the anterior insula, to which NAcC-projecting BLA neurons send massive collateral projections. **(C)** After 8–13 days, postsurgery rats were implanted with an indwelling catheter into their right jugular veins 5 days prior to the initiation of self-administration training. The various experimental groups, shown here in a color code used throughout, were initially trained to acquire cocaine self-administration, under continuous reinforcement (FR1) for 7 days. Rats were then progressively trained to respond under FI schedules of reinforcement, from 1 to 10 minutes, over 5 sessions. They were then trained to seek cocaine for 3 days under an FI15 schedule of reinforcement prior to receiving either Veh or CNO treatment daily, for 3 additional FI15 sessions and 12 days of responding under an FI15(FR10:S) second-order schedule of reinforcement, in which cocaine-seeking responses were reinforced every 10th lever press by the contingent presentation of the cocaine-paired cue acting as a conditioned reinforcer. Treatment was subsequently reversed for 6 additional days of training under FI15(FR10:S), after which rats were deeply anesthetized, and perfused brains harvested for immunohistochemical assessments. AP, anteroposterior; BLA, basolateral amygdala; CNO, clozapine N-oxide; DREADD, designer receptor exclusively activated by designer drugs; DV, dorsoventral; FI, fixed interval; FR, fixed ratio; IHC, immunohistochemistry; ML, mediolateral; NAcC, nucleus accumbens core; Veh, vehicle.

After completing three FI15 sessions, before which rats had been habituated to daily intraperitoneal injections of Veh (1 mL/kg), they were then tested for their drug-seeking behavior under FI15 over 3 daily sessions following administration of either Veh or CNO (5 mg/kg) delivered intraperitoneally ~30 minutes before the beginning of the session (46–49), thereby ensuring that peak blood concentration was reached when rats engaged in instrumental seeking behavior (50).

Having tested the influence of chemogenetic inhibition of the BLA→NAcC pathway on responding for cocaine under FI15, the role of the pathway on the impact of conditioned reinforcement of the cocaine-associated CS was measured over 12 daily sessions. Thus, each 10th lever press resulted in the contingent presentation of the CS for 1 second, while cocaine (and the associated CS) was delivered on the first 10th lever press after a 15-minute interval had elapsed; formally this is an SOR of the type FI15(FR10:S). CNO or Veh were administered prior to each daily session as described above, and, after 12 SOR sessions, the reversibility of the CNO-induced inhibition of the BLA→NAcC pathway in hM4D(Gi)-expressing rats was tested over 6 additional sessions during which those expressing hM4D(Gi) that had previously received CNO received Veh and vice versa.

Immunohistochemistry

As detailed in the Supplement, 35-μm coronal brain sections were incubated with a primary antibody (rabbit anti-mCherry; 1:1000; ab167453; Abcam, Cambridge, United Kingdom) in 2% bovine serum albumin and 0.1% Triton X-100 overnight (18 hours) at 4°C after blocking in 5% bovine serum albumin (A7906; Sigma-Aldrich). Sections were then washed and incubated with a secondary antibody (goat Alexa Anti-Rabbit 488, 1:1000; #A-11008; Thermo Fisher Scientific, Waltham, MA) at room temperature. Sections were washed and mounted onto glass slides (Fisherbrand Superfrost Microscope Slides; Thermo Fisher Scientific), allowed to dry, and covered with a

coverslip and fluoroshield mounting medium (ab104135; Abcam). Images were acquired with a Zeiss Axio Imager M2 equipped with an AxioCam MRm camera (Zeiss, Oberkochen, Germany), using Visiopharm software, version 2017.2(4.3387) (Medicon Valley, Copenhagen, Denmark), at magnification 5× and tiled to create the whole slices images, or at magnification 10× for the regions of interest.

Data and Statistical Analyses

Data, analyzed using STATISCA 10 (StatSoft, Palo Alto, CA), are presented as mean ± SEM or boxplots (median ± 25% [percentiles] and minimum/maximum as whiskers).

The experimental design relied on one experimental group, namely that with chemogenetic inhibition of the targeted neurons (hM4D(Gi)-CNO group), and two control groups, one to control for any nonspecific effects of CNO (the empty-CNO group) and one to control for any nonspecific effects of the viral infection/expression of the transgene (the hM4D(Gi)-Veh group). While the two control groups were designed to control for different experimental variables, it was expected that they would not differ functionally.

Data were analyzed with 1-, 2-, or 3-way analyses of variance (ANOVAs). When assumptions for parametric analyses were violated, data were log transformed. However, for the sake of transparency, both nontransformed and transformed data are presented.

Lever presses during acquisition of both cocaine self-administration and cocaine seeking across the increasing duration of the FI schedules of reinforcement were analyzed using repeated-measures ANOVAs with lever (active and inactive) and session as within-subjects factors (Figures S1 and S2) and group (hM4D(Gi)-CNO, hM4D(Gi)-Veh, and empty-CNO) as between-subjects factor.

As detailed in the Supplement, data pertaining to the acquisition of cue-controlled cocaine seeking were analyzed using 2-way ANOVAs with 3-day blocks as within-subjects factors and group as between-subjects factors.

For the analysis of the potentiation of responding by the conditioned reinforcing properties of the CS, data were normalized for each individual to the average of their group during the baseline, pretreatment FI15 block with the equation potentiation ratio = [(Xr – Xt)/Xt] × 100, where Xr is individual performance at reversal and Xt is individual performance prior to reversal, and they were subjected to the same analysis as described above. The data shown as boxplots were analyzed using a 1-way ANOVA with blocks as within-subjects factors. As detailed in the Supplement, significant interactions were analyzed further using ANOVAs, Duncan's post hoc analyses, and/or hypothesis-driven planned comparisons wherever appropriate. For all analyses, significance was set at $\alpha = .05$. Effect sizes are reported as η_p^2.

RESULTS

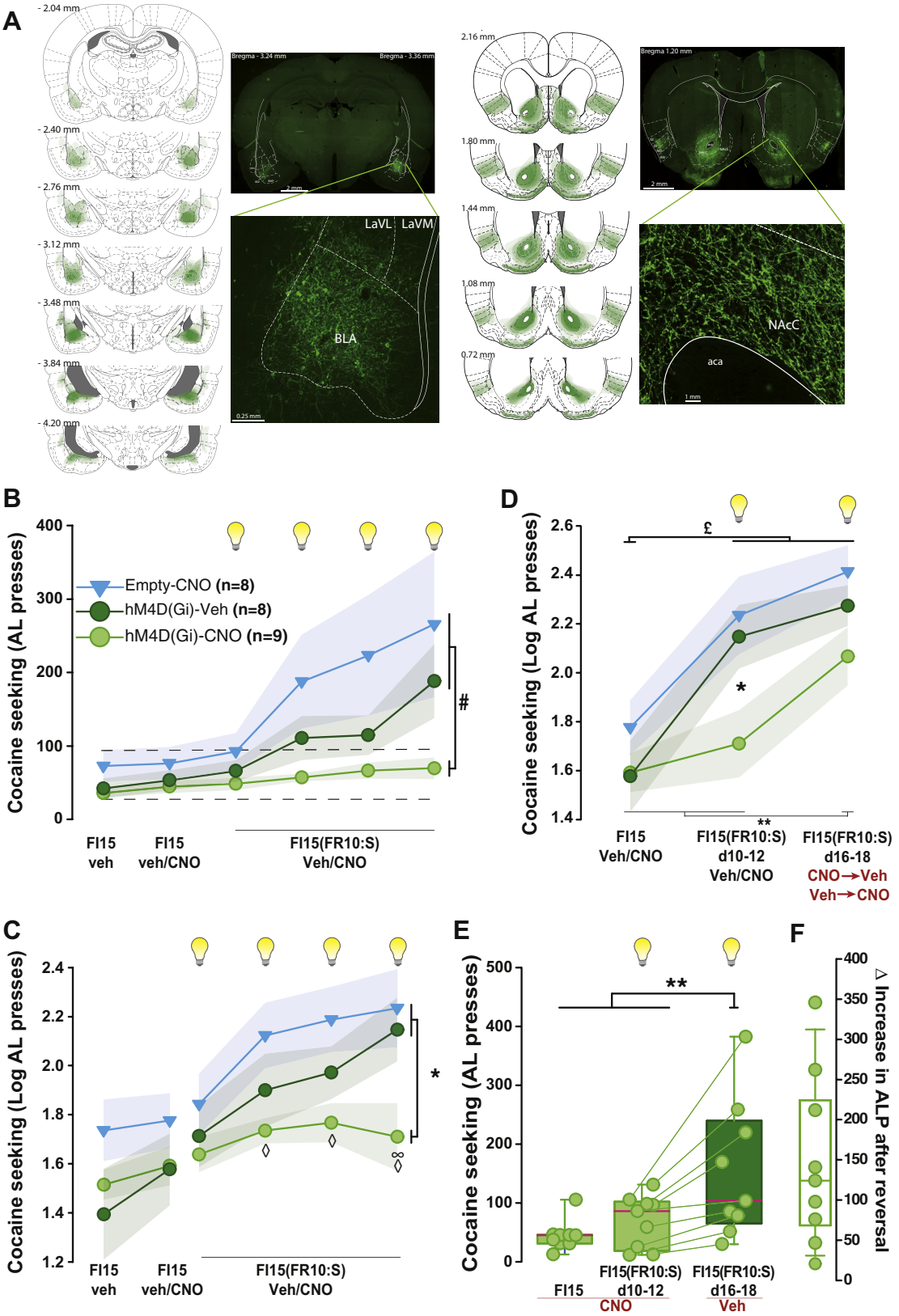
The expression of inhibitory hM4D(Gi) DREADDs or empty control virus, the reporters of which were localized in cell bodies and axon terminals of NAcC-projecting BLA neurons (Figure 2A), had no effect on the acquisition of cocaine self-administration under continuous reinforcement (FR1) or on cocaine seeking under FI schedules of reinforcement in the absence of presentation of cocaine-associated CRfs (Figure S1). Thus, the 3 experimental groups showed similar increase in their active lever presses over the 7 daily sessions under FR1 (main effect of session [$F_{6,132} = 2.25$, $p = .042$, $\eta_p^2 = .09$] and group × session interaction [$F_{12,132} < 1$, $\eta_p^2 = .06$]) and the subsequent 8 sessions under FI of increasing durations, from 1 to 15 minutes (main effect of session [$F_{7,154} = 28.94$, $p = 10^{-7}$, $\eta_p^2 = .57$] and group × session interaction [$F_{14,154} = 1.75$, $p = .051$, $\eta_p^2 = .14$]), to that previously described (40,41,51), even if the empty controls tended to respond at a slightly higher rate than the 2 hM4D(Gi) groups under FI.

The CNO-induced activation of inhibitory hM4D(Gi) DREADDs had no effect on seeking responses under a FI15 schedule of reinforcement (Figure 2B, C and Figure S2). Thus, as compared with pretreatment baseline performance measured over 3 days of training under FI15 (Figure 2B, C and Figure S2), the introduction of differential treatment (Veh vs. CNO) had no influence on the drug-seeking responses made by any of the groups (i.e., hM4D(Gi)-Veh, hM4D(Gi)-CNO, empty-CNO) over 3 days of training under FI15 (main effect of block [$F_{1,22} = 10.3$, $p = .004$, $\eta_p^2 = .32$], main effect of group [$F_{2,22} = 1.35$, $p = .28$, $\eta_p^2 = .11$], and block × group interaction [$F_{2,22} = 1.83$, $p = .18$, $\eta_p^2 = .14$]).

However, as predicted, chemogenetic inhibition of NAcC-projecting BLA neurons prevented the potentiation of instrumental drug-seeking responses seen in both the empty-CNO and hM4D(Gi)-Veh control groups, following the introduction of contingent presentations of the cocaine-paired CS (Figure 2B, C and Figure S2A, B) (main effect of block [$F_{4,88} = 23.13$, $p = 10^{-7}$, $\eta_p^2 = .51$] and group × block interaction [$F_{8,88} = 2.67$, $p = .011$, $\eta_p^2 = .19$]) (see Table S1 for additional analyses). While the 2 control groups similarly increased their instrumental seeking responses under the SOR (empty control vs. hM4D(Gi)-Veh: main effect of group [$F_{1,14} = 0.98$, $p = .338$, $\eta_p^2 = .06$], main effect of block [$F_{4,56} = 25.45$, $p < .001$, $\eta_p^2 = .64$], and group × block interaction [$F_{4,56} = 0.5$, $p = .735$, $\eta_p^2 = .03$]) from the second block onward, eventually to reach levels of responding 400% that of baseline by the last block (Figure S2C) (post hoc analysis vs. FI15 baseline: all $ps < .048$ [see Table S2 for details]), hM4D(Gi)-CNO rats failed to show this increase and instead maintained a low level of responding similar to that seen under FI15 baseline conditions throughout (main effect of block for the hM4D(Gi)-CNO group [$F_{4,32} = 1.59$, $p = .199$, $\eta_p^2 = .17$]).

Consequently, hM4D(GI)-CNO rats progressively diverged from the 2 control groups in which cocaine seeking steadily increased over the four 3-day session blocks of training under FI15(FR10:S) (planned comparison hM4D(Gi)-CNO vs. control groups for SOR vs. FI15 [$F_{1,22} = 5.49$, $p < .03$]), eventually to differ statistically from them on the last blocks of SOR (Figure 2 and Figure S2; see Table S3 for detailed post hoc results).

As soon as the treatment was reversed and hM4D(Gi)-CNO rats started to receive Veh instead of CNO (hM4D(Gi)-CNO→Veh), they quickly increased their cocaine seeking responses under the same FI15(FR10:S) conditions (main effect of block [$F_{2,44} = 59.25$, $p = 10^{-7}$, $\eta_p^2 = .72$] and group × block interaction [$F_{4,44} = 3.04$, $p = .027$, $\eta_p^2 = .22$]). Therefore, hM4D(Gi)-CNO→Veh rats eventually reached levels of responding that were similar to those of the empty-CNO→Veh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Δ Increase in ALP after reversal

and hM4D(Gi)-Veh→CNO control groups (post hoc analysis pre/post reversal performance under SOR: p = .3969). In contrast, the performance of the 2 control groups under SOR prior to reversal, already different from that under FI15 baseline (post hoc analysis FI15 vs. SOR prereversal: p = .00009 and p = .00003, respectively) was maintained throughout (post hoc analysis SOR prereversal vs. SOR postreversal: p = .087 and p = .2234, respectively) (Figure 2D). Hence, hM4D(Gi)-CNO→Veh rats eventually showed potentiation of cocaine seeking following the response-contingent presentations of the cocaine-paired CS as compared with their previous performance under FI15 (post hoc analysis: p = .0009) (40,51) and SOR with CNO (post hoc analysis: p = .00189), which otherwise did not differ from each other (post hoc analysis FI15 vs. SOR prereversal: p = .2314) (Figure 2D). The reversibility of the effect of chemogenetic inhibition of the NAcC-projecting BLA neurons on the acquisition of cue-controlled cocaine seeking was further supported by an analysis of individual performance upon reversal of treatment (Figure 2E). Thus, each of the 9 rats in that group increased their seeking behavior for cocaine on reversal of CNO treatment ($F_{2,16}$ = 9.33, p = .002, η_p^2 = .54), with a potentiation ranging from 20% to 345% (Figure 2F).

The introduction of CNO treatment in the hM4D(Gi)-Veh (hM4D(Gi)-Veh→CNO) group after a period of 18 days of training to seek cocaine had no effect on well-established cue-controlled cocaine seeking (Figure 2D) (post hoc analysis: p = .3969).

Taken together, these results show that the NAcC-projecting BLA neurons are necessary for the potentiation of cocaine seeking by the conditioned reinforcing properties of cocaine-paired cues. However, histological assessment of the pattern and spread of expression of the transgenes revealed that in addition to the dense expression in the BLA→NAcC circuit, robust expression was also systematically observed in the anterior insular cortex (AI). This demonstrates that NAcC-projecting BLA neurons send substantial collateral projections to the AI, confirming earlier observations, but the functional significance of this was not investigated (36).

We therefore investigated the possible involvement of the AI in pavlovian mechanisms and instrumental conditioning (52,53) by studying the influence of chemogenetic inhibition of excitatory neurons in the AI on the impact of conditioned reinforcement on cocaine seeking.

An independent cohort of rats had virus-mediated expression of hM4D(Gi) or reporter only under the CAMKII (Ca^{2+}/calmodulin-dependent protein kinase II) promoter bilaterally in the AI (Figures 1 and 3A) prior to being tested in the same procedure as that described above. The expression of inhibitory hM4D(Gi) DREADDs or empty control virus, the reporters of which were heavily expressed in cell bodies of the AI (Figure 3A), had no effect on the acquisition of cocaine self-administration under FR1 (Figure S3A) or cocaine seeking under FI schedules of reinforcement. The 3 experimental groups showed similar increases in their active lever presses over the 7 daily sessions under FR1 (main effect of session [$F_{6,84}$ = 8,32, $p < .01$, η_p^2 = .37] and group × session interaction [$F_{12,84} < 1$, η_p^2 = .07]) and the subsequent 8 sessions under FI of increasing durations, from 1 to 15 minutes (main effect of session [$F_{7,98}$ = 22.95, $p < .01$, η_p^2 = .62] and group × session interaction [$F_{14,98} < 1$, η_p^2 = .06]) (Figure S3B) to that of those of the first experiment.

As previously, the CNO-induced activation of inhibitory hM4D(Gi) DREADDs had no effect on cocaine seeking under an FI15 schedule of reinforcement (Figure 3B, C). Thus, cocaine-seeking responses made during the first drug-free intervals over 3 days of training under FI15 did not differ between groups (i.e., hM4D(Gi)-Veh, hM4D(Gi)-CNO, empty-CNO) or from baseline performance measured over 3 days

Figure 2. Chemogenetic inhibition of NAcC-projecting BLA neurons specifically prevents the potentiation of cocaine-seeking behavior by drug paired cues acting as conditioned reinforcers. **(A)** Cre-mediated projection-specific expression of hM4D(Gi) in the NAcC-projecting BLA neurons resulted in dense expression of the reporter both in the cell bodies and in the terminals of targeted neurons, as shown in representative photos of hM4D(Gi)-expressing neurons (mCherry tag revealed by immunofluorescence in green) in the BLA (left) and their axon terminals in the NAcC (right). Alongside is a schematic of coronal sections of the brain covering the anteroposterior extent of the BLA (left) or the NAcC (right), with density maps depicting in green the spread of mCherry expression in each structure across all individuals. The majority of rats displayed labeling restricted to the NAcC, with strong labeling in the anterior insula to which NAcC-projecting BLA neurons send collateral projections. Some rats had expression spreading to the dorsolateral part of the NAc shell and the piriform cortex. **(B, C)** The introduction of the cocaine-paired cue contingent on responding resulted in a substantial increase in cocaine seeking over time in both the empty-CNO and hM4D(Gi)-Veh groups during the first 15-minute drug-free period of daily sessions represented as either **(B)** nontransformed data or **(C)** log-transformed data. In marked contrast, potentiation of responding was not seen in hM4D(Gi)-CNO rats, whose seeking responses were much lower than those of the 2 control groups over the four 3-session blocks of training under FI15(FR10:S) (# indicates planned comparison hM4D(Gi) vs. the 2 control groups across 5 blocks [1 FI15 block vs. 4 second-order schedule of reinforcement blocks], $p < .03$; ◇ indicates different from empty-CNO control, $p < .05$; ∞ indicates different from hM4D(Gi)-Veh, $p < .05$) and actually never differed from that seen under FI15 [the dotted lines on panel **(B)** represent the SEM of the entire population during the first FI15 block]. The effect of chemogenetic inhibition of the NAcC-projecting BLA neurons was specific of the potentiation of cocaine-seeking responses by the conditioned reinforcing properties of the CS, as it had no effect on performance under FI15, prior to the introduction of response-contingent presentations of the CS. **(D, E)** The prevention of acquisition of cue-controlled cocaine seeking by chemogenetic inhibition of the NAcC-projecting BLA neurons was reversible. **(D)** Thus, upon reversal of treatment, e.g., when hM4D(Gi)-CNO rats, whose drug seeking under FI15(FR10:S) had been much lower than that of both control groups (*post hoc, $p < .05$) and similar to that seen under FI15, received Veh in place of CNO, they immediately showed sensitivity to the conditioned reinforcing properties of the CS as they displayed a potentiation of responding that, within a week, became higher than that under FI15 (**post hoc, $p < .01$) and similar to that of the empty and hM4D(Gi)-Veh control groups (£ indicates group × block interaction, $p < .05$). **(F)** Further analysis of the behavior displayed by each individual of the hM4D(Gi)-CNO→hM4D(Gi)-Veh group revealed that the increase in responding shown at the group level on reversal of treatment (as compared with both FI15 baseline and the last 3-day block of FI15(FR10:S)) reflected an increase, in each of the 9 individuals, of at least 20% and up to 345% in ALPs, calculated as [(Xr – Xt)/Xt] × 100, where Xr is individual performance at reversal and Xt is individual performance prior to reversal (0 represents no increase and 100 represents 100% increase). Empty-CNO, hM4D(Gi)-Veh, and hM4D(Gi)-CNO are represented in blue triangles, dark green circles, and light green circles, respectively. aca, anterior commissure; AL, active lever; ALP, active lever presses; BLA, basolateral amygdala; CNO, clozapine N-oxide; CS, conditioned stimulus; DV, dorsoventral; FI, fixed interval; FR, fixed ratio; LaVL, lateral amygdala ventrolateral; LaVM, lateral amygdala ventromedial; NAcC, nucleus accumbens core; Veh, vehicle.

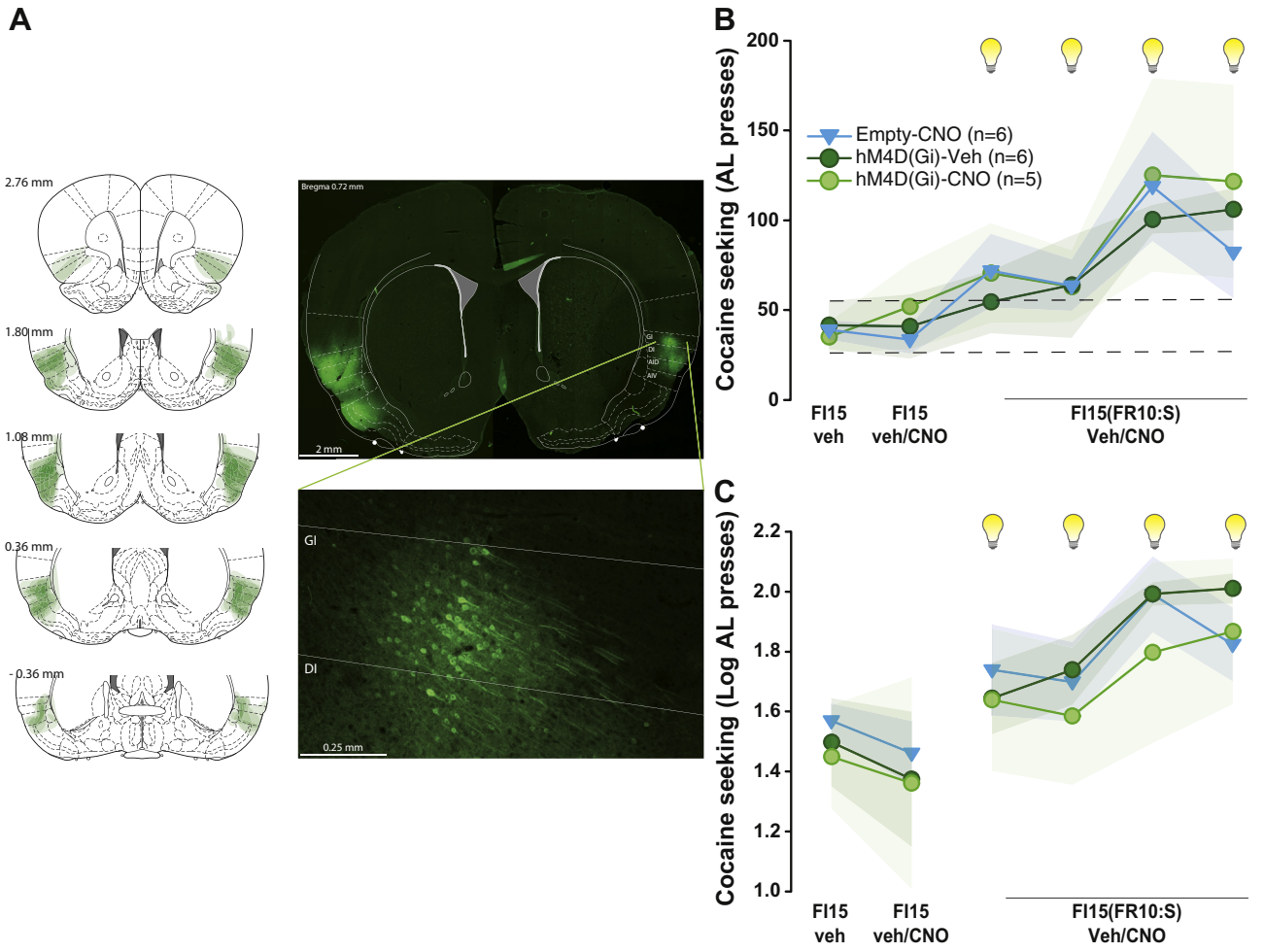

Figure 3. Chemogenetic inhibition of the AI does not influence the potentiation of drug seeking by cocaine-paired conditioned stimuli acting as conditioned reinforcers. **(A)** Viral-mediated expression of hM4d(Gi) in the AI resulted in dense expression of the reporter in the cell bodies of targeted neurons, as shown in representative photos of hM4D(Gi)-expressing neurons (mCherry tag revealed by immunofluorescence in green) in the AI (left). Alongside is a schematic of coronal sections of the brain covering the anteroposterior extent of the AI, with density maps depicting in green the spread of mCherry expression in each structure across all individuals. A dense expression of the reporters was almost exclusively restricted to the AI, with some spread observed in some rats in adjacent territories in the dorsal part of the 3 layers of the piriform cortex, the dorsal endopiriform nucleus, and the claustrum. **(B, C)** Chemogenetic inhibition of the AI had no effect on cocaine seeking under FI15 or on the potentiation of drug-seeking responses by the conditioned reinforcing properties of drug-paired conditioned stimuli. Indeed, **(B)** nontransformed or **(C)** log-transformed AL presses measured during the first 15-minute drug-free interval of 12 daily sessions represented in blocks of 3 sessions never differed between the hM4D(Gi)-CNO and the 2 control groups, namely empty-CNO and hM4D(Gi)-Veh. Thus, upon introduction of the FI15(FR10:S) second-order schedule of reinforcement, all groups showed a similar steady increase in responding as compared with the FI15 baseline, thereby demonstrating their sensitivity to the conditioned reinforcing properties of the cocaine-paired conditioned stimuli. The dotted lines on panel **(B)** represent the SEM of the entire population for the first FI15 block. Empty-CNO, hM4D(Gi)-Veh, and hM4D(Gi)-CNO are represented in blue triangles, dark green circles, and light green circles, respectively. AI, anterior insular cortex; AL, active lever; CNO, clozapine N-oxide; DI, dysgranular insular cortex; FI, fixed interval; FR, fixed ratio; GI, granular insular cortex; Veh, vehicle.

prior to the introduction of treatment (Figure 3B, C) (main effect of group [$F_{2,14} < 1$, $\eta_p^2 = .01$] and block × group interaction [$F_{2,14} < 1$, $\eta_p^2 = .12$]).

However, in marked contrast to the effect of chemogenetic inhibition of NAcC-projecting BLA neurons, inhibition of the AI had no effect on the potentiation of cocaine seeking that accompanies response-contingent presentation of the drug-paired CS under a FI15(FR10:S) SOR (Figure 3B, C). Thus, on the introduction of the CRf, hM4D(Gi)-CNO rats showed the same increase in drug-seeking responses as that shown by the hM4D(Gi)-Veh and empty-CNO control groups (main effect of block [$F_{4,56} = 11.82$, $p < .01$, $\eta_p^2 = .46$] and group × block interaction [$F_{8,56} < 1$, $\eta_p^2 = .06$]).

DISCUSSION

Taken together, the data presented here show that the direct BLA→NAcC circuit mediates the impact of conditioned reinforcement on cocaine seeking, measured as a prolonged period of responding prior to the eventual intravenous infusion

of cocaine, in this case after a 15-minute FI had timed out (25,42). Therefore, chemogenetic inhibition of the NAcC-projecting BLA neurons prevented the potentiation of responding that follows seeking response-contingent presentation of cocaine-associated CSs (26). Importantly, CNO had no effect itself (50) on responding because the empty-control CNO-treated animals tended, if anything, to increase lever presses, a minor effect opposite that seen in hM4D(Gi)-CNO rats. Moreover, these effects were specific to the BLA→NAcC, as chemogenetic inhibition of the AI, to which NAcC-projecting BLA neurons send substantial collateral projections, had no effect on either instrumental seeking responses or their potentiation by cocaine cues.

These results extend understanding of the amygdalo-striatal mechanisms involved in conditioned reinforcement (13,25,54,55), and particularly its impact on the seeking of stimulant drugs (5,25,55,56).

Drawing on early evidence that the BLA and its functional interactions with the NAcC mediate the impact of conditioned reinforcers on instrumental responding for natural reinforcers such as water or a sexual partner (32,57), studies using a variety of pharmacological and physical lesion-based manipulations of the amygdalo-striatal system, including functional disconnections, have provided evidence of a causal role of the BLA (31,33), the NAcC (27), and their functional interaction in mediating the conditioned reinforcing properties of drug-paired CSs and their impact on the reinstatement of extinguished drug-seeking instrumental responses (27,28,33,58,59). Bilateral BLA (33) or NAcC (but not NAc shell) (27) excitotoxic lesions prevented the acquisition of cocaine seeking under an SOR. Additionally, functional disconnection of the BLA and the NAcC showed that coordinated dopaminergic activity in the BLA and glutamatergic activity in the NAcC are involved in the acquisition of cue-controlled cocaine seeking (28).

Here, we extended these findings by showing that these functional interactions depend on a specific BLA→NAcC pathway, whereby glutamatergic inputs from the BLA influence downstream processes in the NAcC to mediate the effects of the conditioned reinforcing properties of cocaine-paired cues on instrumental drug-seeking behavior.

This observation is consistent with the previous demonstration that BLA neurons gate, in a glutamate-dependent manner, the activity of NAcC medium spiny neurons and consequent reward-seeking behavior (34), and that plasticity at the BLA→NAcC synapse is involved in the acquisition of responding reinforced by the contingent presentation of a cocaine-paired cue after withdrawal (59).

In addition, the present results reveal that unlike zif-268 knockdown–mediated long-lasting disruption of the reconsolidation of the CS-cocaine memory in the BLA (60), chemogenetic inhibition of the BLA→NAcC pathway did not permanently disrupt the mechanisms underlying the pavlovian-instrumental interactions involved in the potentiation of cocaine seeking by the conditioned reinforcing effects of cocaine-paired cues. The effect of chemogenetic inhibition of the BLA→NAcC pathway to prevent the impact of cocaine-associated conditioned reinforcement seeking responses was reversible. When hM4D(GI) rats previously receiving CNO were instead administered Veh, their seeking responses were potentiated by response-contingent cocaine CS presentation within 6 days of treatment reversal. This observation suggests that the two structures mediate complementary aspects of drug memory (61) and that the BLA→NAcC circuit is necessary for bridging the motivational value of the cocaine-paired cue stored in the BLA with the pavlovian-instrumental interactive processes supported by the NAcC (12,13).

This observation is consistent with the evidence provided here that chemogenetic inhibition of the BLA→NAcC pathway did not influence cocaine seeking per se, as instrumental responding for cocaine under FI15 conditions in the absence of conditioned reinforcement was completely unaffected. This confirms that the acquisition of drug-seeking behavior in anticipation of, and reinforced by, the eventual delivery of a drug infusion (42) does not depend on the BLA or its functional interactions with the NAcC. The observation that cocaine seeking under an FI15 schedule of reinforcement is impervious to the inhibition of the BLA→NAcC pathway at first sight seems inconsistent with the previous demonstration that response-contingent optogenetic activation of this pathway in mice supports instrumental responding under continuous reinforcement (36). However, these results, taken together with the present data, provide further evidence that instrumental seeking responses are mediated by neural circuits that are dissociable from those mediating directly reinforced taking responses (25), even when emitted within the same behavioral sequence (2).

Finally, the present results confirm that the BLA is necessary for the acquisition and early-onset performance of cue-controlled cocaine seeking but not its longer-term maintenance (40). Thus, following extensive training under an SOR, cue-controlled cocaine seeking was well established, and chemogenetic inhibition of BLA→NAcC pathway, which completely prevented the conditioned reinforcement impact of cocaine cues, had no effect. This observation is in agreement with our previous demonstration that the BLA is necessary for the acquisition of cue-controlled cocaine seeking and the recruitment of dorsolateral striatum dopamine-dependent control over behavior, but that it is the central amygdala that assumes a critical role in maintaining well-established dorsolateral striatum, dopamine-dependent cue-controlled cocaine seeking (40). These findings therefore suggest that the engram of the instrumental association potentiated by the conditioned reinforcing properties of drug-paired cues may be distributed across amygdalo-striatal systems.

The results of the present study reveal the first node of an intricate and shifting amygdalo-striatal circuit that mediates the marked influence of pavlovian drug cues that act as conditioned reinforcers to invigorate drug seeking over prolonged time periods in order to obtain intravenous cocaine (41,56).

ACKNOWLEDGMENTS AND DISCLOSURES

This work, carried at the Department of Psychology of the University of Cambridge, was supported by a Programme Grant from the Medical Research Council (Grant No. MR/N02530X/1 [to BJE and DB]), a research grant from the Leverhulme Trust (Grant No. RPG-2016-117 [to DB]), a José Castillejo Grant of the Ministry of Education of Spain (to AH-M), and the University of Tours and the University Hospital of Tours (to PB).

DB, MP, BJE, and AH-M designed the experiment. MP, PB, and AH-M carried out the experiments. MP, AH-M, and DB analyzed the data. AH-M, MP, BJE, and DB wrote the manuscript.

Cocaine hydrochloride and clozapine N-oxide were provided to DB by the National Institute on Drug Abuse Drug Supply Programme. We thank the reviewers for their constructive comments as well as Dr. Steven Simmonds and Dr. Yan Dong for their review of the preprint of this paper published on bioRxiv.

This article was published as a preprint on bioRxiv: https://doi.org/10.1101/2020.05.05.078329.

The authors report no biomedical financial interests or potential conflicts of interest.

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Received May 8, 2020; revised Jul 22, 2020; accepted Jul 27, 2020.

Supplementary material cited in this article is available online at https://doi.org/10.1016/j.biopsych.2020.07.022.

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