ARTICLE

Chemogenetic inhibition of lateral habenula projections to the dorsal raphe nucleus reduces passive coping and perseverative reward seeking in rats

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The lateral habenula (LHb) processes information about aversive experiences that contributes to the symptoms of stress disorders. Previously, we found that chemogenetic inhibition of rat LHb neurons reduced immobility in the forced swim test, but the downstream target of these neurons was not known. Using an intersectional viral vector strategy, we selectively transduced three different output pathways from the LHb by injecting AAV8-DIO-hM4Di into the LHb and CAV2-CRE (a retrograde viral vector) into one of the three target areas as follows: dorsal raphe nucleus (DRN), ventral tegmental area (VTA), or rostromedial tegmentum (RMTg). Using the forced swim test, we found that chemogenetic inhibition of DRN-projecting LHb neurons reduced passive coping (immobility), whereas inhibition of the other pathways did not. Chemogenetic activation of DRN-projecting neurons using hM3Dq in another cohort did not further exacerbate immobility. We next examined the impact of inhibiting DRN-projecting LHb neurons on reward sensitivity, perseverative behavior, and anxiety-like behavior using saccharin preference testing, reward-omission testing, and open-field testing, respectively. There was no effect of inhibiting any of these pathways on reward sensitivity, locomotion, or anxiety-like behavior, but inhibiting DRN-projecting LHb neurons reduced perseverative licking during reward-omission testing, whereas activating these neurons increased perseverative licking. These results support the idea that inhibiting LHb projections to the DRN provides animals with resilience during highly stressful or frustrating conditions but not under low-stress circumstances, and that inhibiting these neurons may promote persistence in active coping strategies.

INTRODUCTION

Stress disorders including depression are very common and yet treatment developments have lagged behind our rapidly expanding knowledge about the contribution of various brain regions to the component symptoms of these disorders. One brain region in particular, the lateral habenula (LHb), has received a significant amount of attention in recent years. The LHb is a highly conserved structure, existing in lamprey, fish, reptiles, and mammals [1]. It receives convergent input from the basal ganglia and the limbic system, and projects to dopaminergic [2], serotonergic, and GABAergic midbrain nuclei [3], making it poised to integrate cognitive and emotional stimuli, and influence behavioral output. The LHb is activated by unexpected negative events and aversive stimuli [4–6], and numerous studies indicate that effective anti-depressants reduce activity in the LHb and direct inhibition may promote resilience in depressed individuals [7–9]. There is also evidence that LHb is the primary site of action for the newest Food and Drug Administration-approved antidepressant, S(+) Ketamine [10]. However, the precise contribution of LHb to motivated behavior is complex and is an active area of investigation. The LHb has been shown to impact diverse affective processes and behaviors such as attentional regulation [11], positive and negative reward-based decision-making [12–15], flexible and passive coping [16, 17], cognitive flexibility [18, 19], and generalized anxiety [20].

Although the medial habenula is organized into circumscribed subregions of neurons with distinct molecular phenotypes, the organization of LHb has been more difficult to define. Most LHb neurons are glutamatergic [21], but there are numerous neuropeptides and receptors that could contribute to functional specializations of subsets of LHb neurons [22, 23]. Furthermore, there is no clear subregional organization of gene expression in LHb [24] and neurons expressing transcripts of common interest, such as neuropeptides, monoamine receptors, and potassium channels, tend to be scattered across the LHb. Several reports indicate that LHb neurons that project to particular target areas such as the dorsal raphe nucleus (DRN), ventral tegmental area (VTA), or rostromedial tegmentum (RMTg) do so without branching to more than one of these targets [23, 25]. It is not known whether these sets of neurons subserve different functional roles nor whether these pathways diverge in terms of their molecular phenotypes, but given the functional diversity of their targets, we hypothesized that LHb neurons projecting to these different target areas would have different impacts on stress-associated behaviors.

Given that the neurons projecting to DRN, VTA, or RMTg cannot be selectively targeted with microinjections to subregions and there are no known molecular phenotypes as of yet, we decided to use an intersectional viral vector strategy by injecting Cre-dependent adeno-associated virus (AAV) vectors into the LHb in combination with a retrograde canine adenovirus-2 (CAV2) Cre

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vector into each of these downstream targets. We used chemogenetic inhibition via hM$_4$Di, a technique that has been well validated in the LHb [26–28], to reveal the distinct contributions of each individual LHb output pathway to stress-associated behaviors. As might be expected, we found that manipulating LHb neurons impacted behaviors observed only during stressful or frustrating conditions but had no observable impact when the animals were under a low-stress challenge. The LHb-DRN circuit has long been hypothesized to be involved in psychiatric illness [29] and accordingly we found that selectively inhibiting LHb neurons projecting to DRN increased passive coping associated with immobility during inescapable swim stress and decreased inflexible, perseverative saccharin seeking when the sweet reward was omitted. Conversely, selective activation of the same pathway increased perseverative saccharin seeking. These data suggest LHb neurons that project to the DRN may be an important target for modulating specific component symptoms of depression.

METHODS

Experimental design and statistical analyses

Our overarching experimental strategy was to use an intersectional viral vector approach to express DREADDs (hM$_4$Di or hM$_3$Dq) in LHb neurons selectively projecting to the VTA, RMTg, or DRN. This was achieved through injection of Cre-dependent adeno-associated viral vectors (AAV8-hSyn-DIO-hM$_4$Di-mCherry or AAV8-hSyn-DIO-hM3Dq-mCherry) into the LHb and a retrogradely transported CAV2-expressing Cre (CAV2-Cre) into one of the three output regions (Fig. 1a). Separate groups of animals were used for each pathway and for each DREADD. In three separate experiments, the effect of inhibiting the LHb's outputs using hM$_4$Di was examined during a forced swim test (FST; experiment 1), during saccharin preference testing and reward omission (experiment 2), and during open-field exploration (experiment 3). The effect of activating DRN-projecting LHb neurons using hM$_3$Dq was also tested in all three experimental conditions. All experimental procedures were approved by the University of Washington Institute institutional animal care and use committee, and were conducted in accordance with National Institutes of Health (NIH) guidelines.

We used linear mixed effect modeling (LME; MATLAB) to analyze all behavior. This study required large numbers of animals, meaning that animals receiving DREADDs in different brain regions were run as separate waves. This produced a high level of control within the brain region, but somewhat less control between waves. As such, we first ran LMEs with cohort and treatment as the main effects, and a cohort × treatment interaction, to determine whether there were differences between behavioral cohorts.

$$lme = fitlme\left(\begin{array}{l} data, \text{'Time} \sim \text{Region} * \text{Treatment'}, \\ \text{'DummyVarCoding', 'effects'} \end{array}\right);$$

If such differences existed, separate LMEs were run for each wave, with treatment (Clozapine-N-oxide (CNO) vs. vehicle) as the only fixed effect.

$$lme = fitlme\left(\begin{array}{l} region\_data, \text{'Time} \sim \text{Treatment'}, \\ \text{'DummyVarCoding', 'effects'} \end{array}\right);$$

Analysis of variances were run on the LMEs to determine the significance and effect sizes were calculated.

$$Result = ANOVA(lme); \quad d = computeCohen\_d(Group\,A, Group\,B);$$

Viral vectors

The Cre-dependent DREADD vectors used in these experiments consisted of AAV (serotype 8) driven by the human synapsin promotor and encoded hM$_4$Di-mCherry or hM$_3$Dq-mCherry in double inverted orientation were obtained from Addgene (AAV8-hSyn-DIO-hM$_4$Di-mCh and AAV8-hSyn-DIO-hM$_3$Dq-mCh; titers of $\sim 4 \times 10^{12}$ vg/µl). CAV2-CRE was packaged in our laboratory using DKZeo cells [30], purified by CsCl$_2$ step gradient followed by a second CsCl$_2$ purification, and functionally titered using TE26 cells (titer of $\sim 4 \times 10^{10}$ vg/µl [31]).

Drugs

CNO was procured from the NIMH Chemical Synthesis and Drug Supply Program (C-929), and was dissolved in 2% dimethyl sulfoxide (DMSO) (D8418-50ML, Sigma-Aldrich) and physiological saline solution (18-807-50, Henry Schein Animal Health). CNO solution was prepared at 3 mg/ml for 1 ml/kg injections (0.25–0.5 ml). Vehicles were made identically to drug solutions, except CNO was omitted. Cocaine was procured from the NIDA Drug Supply Program and prepared at 10 mg/ml in 0.9% sterile saline for 1 ml/kg injections (0.25–0.5 ml). Meloxicam (6451602845, Henry Schein Animal Health) was prepared at 1 mg/ml in 0.9% sterile saline for 1 ml/kg injections (0.25–0.5 ml).

Experiment 1 (forced swim test)

Male Sprague–Dawley rats ($n = 73$, Charles River) weighing $\sim$250–300 g were pair housed in a temperature- and humidity-controlled vivarium on a 12:12 h light–dark cycle and maintained on ad libitum food and water access. For intersectional surgeries, rats were anesthetized with 1–3% isoflurane. Using a custom robotic stereotaxic instrument [32], 58 animals received hM$_4$Di injected into the LHb. Blunt 28 g needles were inserted bilaterally at a 10° angle terminating at A/P −3.2, M/L ± 0.7, and D/V −5.25 and 1 µl of AAV8-hSyn-DIO-hM$_4$Di-mCherry was injected at a rate of 0.2 µl/min. Twenty-two animals received bilateral 1 µl injections of CAV2-CRE into the DRN at a 15° angle terminating at A/P −7.8, M/L ± 0.23, and D/V −6.85. Eighteen animals received bilateral 1 µl injections of CAV2-CRE into the VTA at a 10° angle terminating at A/P −5.8, M/L ± 0.6, and D/V −8.6. Eighteen animals received bilateral 1 µl injections of CAV2-CRE into the RMTg at a 10° angle terminating at A/P −7.6, M/L ± 0.62, and D/V −8.5. Finally, 15 animals received of AAV8-hSyn-DIO-hM$_3$Dq-mCherry in the LHb and CAV2-CRE into the DRN at the same locations and speed. After surgeries, rats were given meloxicam (1 mg/kg, subcutaneously (s.c.)) for pain management and monitored for at least 3 days. Accuracy of injection coordinates was confirmed by visualization of mCherry in the LHb; these injection volumes and coordinates were optimized to produce selective transduction of LHb neurons with minimal expression adjacent regions. Furthermore, the first five surgeries for each output region included 2.5% by volume CAV2-zsGreen, a direct fluorescence marker of retrograde infection, to ensure the output region coordinates were accurate.

Twenty-one days after the viral infusions, rats were subjected to a FST [33], a procedure commonly used to test the efficacy of antidepressants [34]. Briefly, the animal is placed in an inescapable cylinder containing water (50 cm tall, 20 cm diameter, 30 cm water depth, 23–25 °C) for 15 min and then removed and dried. The animal is then treated with either vehicle (2% DMSO in saline) or CNO (3 mg/kg intraperitoneally (i.p.); dissolved in 2% DMSO in saline) three times at 1, 5, and 23.5 h before the 5 min post-test swim session, which is performed 24 h after the first swim. These injection timings were chosen, as they are identical to those used to test traditional anti-depressants in the FST [35]. For strong behavioral control, CNO and vehicle animals were selected from pair-housed males that received viral injections on the same day. These pairs then recovered together and were run through the FST together in side-by-side swim chambers. These paired (CNO and vehicle) swim sessions were recorded from the side at 30 frames per second and rats were tracked using Ethovision XT11 software (Noldus, NL). The tracking data were analyzed using custom MATLAB scripts (Mathworks, Natick MA) to
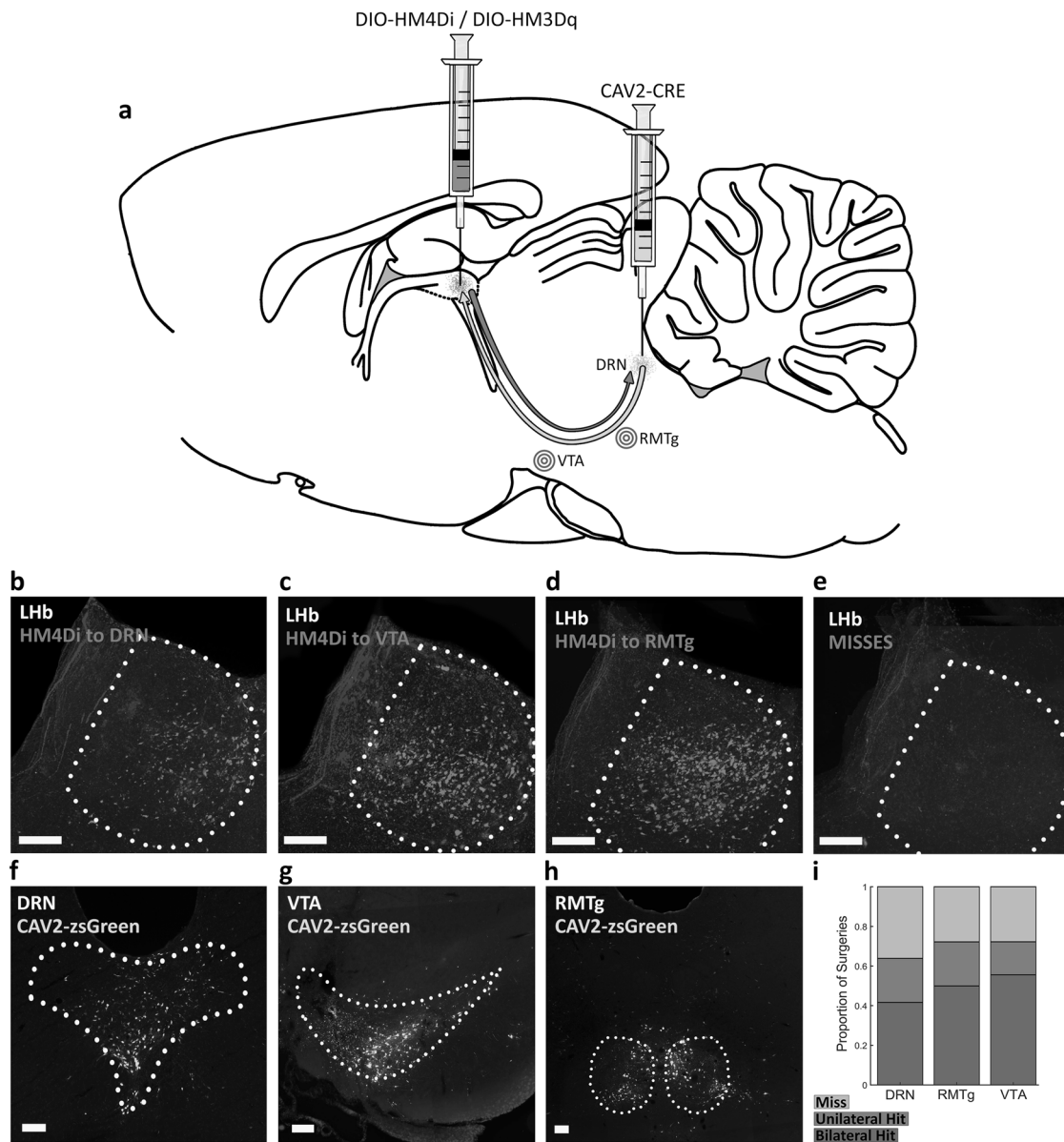
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Fig. 1 Schematic diagram of intersectional DREADD expression. a DIO-hM$_4$Di or DIO-hM$_3$Dq was injected bilaterally into the lateral habenula and CAV2-CRE was injected bilaterally into one of the three targeted LHb outputs (VTA, RMTg, or DRN). CAV2-CRE is taken up into terminals and transported retrogradely to LHb cell bodies where it drives the expression of DREADDs. **b–d** Maximum intensity projections of DREADD expression in each pathway. One representative slice from each successful surgery in the first experiment (FST) was automatically aligned and overlaid to provide a visual representation of hM$_4$Di expression and the anatomical layout of neurons in each pathway. **e** Maximum intensity projection of LHb slices from "miss" animals. **f–h** A representative injection of CAV2-CRE mixed with 2.5% CAV2-zsGreen for visualization of terminal targets in the midbrain. **i** Proportion of surgeries deemed bilateral hit, unilateral hit, or miss. Scale bars = 200 µm.

determine time spent immobile, defined as movement of <2 cm/s. Although the cutoff for movement is technically arbitrary, using an automated approach ensured an unbiased analysis that was applied equally to all subjects.

Experiment 2 (saccharin preference testing)
Male Sprague–Dawley rats ($n = 53$, Charles River) weighing ~250–300 g were pair housed identically to experiment 1. Intersectional DREADD expression was performed identically to experiment 1. Animals ($n = 39$) received hM$_4$Di injected into the LHb as described above. Animals ($n = 14$) received bilateral 1 µl injections of CAV2-CRE into the DRN, 13 animals received bilateral 1 µl injections of CAV2-CRE into the VTA, and 12 animals received bilateral 1 µl injections of CAV2-CRE into the RMTg. A further 14 animals received hM$_3$Dq vector in the LHb and CAV2-CRE into the

DRN at the same locations and speed. After surgeries, rats were given meloxicam (0.2 mg/kg, s.c.) for pain management and monitored for at least 3 days. At 21 days following viral infusions, rats were tested for saccharin preference using a two-bottle lickometer chamber (Lafayette Instruments, IN) with a water bottle and a bottle of 0.1% saccharin for 1 h, with a maximum of 35 mL of saccharin solution and water available. Bottle location was counterbalanced within groups. Animals underwent 4 days of preference training. On the fifth day, animals received either vehicle or CNO (3 mg/kg i.p.) 30 min prior to saccharin preference testing; this was referred to as the test session. On the sixth day, animals received whichever injection they did not receive on day 5, either vehicle or CNO (3 mg/kg i.p.). The injection order was randomized and balanced within brain regions. Following the probe sessions, animals underwent 1 more day of normal

saccharin preference testing on day 7, followed by a reward-omission session on day 8. During the reward-omission session, animals were randomly assigned to receive either vehicle or CNO (3 mg/kg i.p.) 30 min prior to the test in which both bottles were filled with water. Licks were counted automatically using an impedance meter connected to each bottle with computerized data collection.

Experiment 3 (open field)
Male Sprague–Dawley rats from experiment 2 also underwent an open-field exploration procedure 3 days after the end of saccharin preference testing. Thirty minutes prior to the test, animals received either vehicle or CNO (3 mg/kg i.p.). Animals were placed into the center of a 75 cm diameter circular open field with 65 cm tall walls. Animals were recorded from the top down against a black floor at 30 fps for 10 min and were tracked using Ethovision XT11 software (Noldus, NL). Distance traveled, rearing, center time, and center crossings were calculated for each subject.

Validation of hM_4Di function in the lateral habenula
Male, Long–Evans rats (390–420 g) ($n = 8$) were injected with AAV8-hSyn-hM_4Di-mCherry in the LHb as described above. A separate set of animals ($n = 9$) were injected with AAV8-hSyn-DIO-hM_4Di-mCherry in the LHb and CAV2-CRE into the VTA, as described above (Supplementary Fig. S2). Half of the animals in each group were pretreated with either vehicle or CNO (3 mg/kg, ip). Twenty minutes following pre-treatment all rats were injected with cocaine (10 mg/kg, i.p.). Ninety minutes following cocaine injections, rats were deeply anesthetized with an i.p. injection of sodium pentobarbital and phenytoin sodium, and were perfused transcardially with 100 ml of 0.1 M phosphate-buffered saline (PBS), followed by 250 ml of cold 4% paraformaldehyde (pH 7.4). Brains were dissected, post fixed in 4% paraformaldehyde overnight at 4 ℃, and transferred to PBS (also at 4 ℃). Sections (40 μm) across the rostro-caudal axis of the LHb were collected on a Leica VT1000S vibrating blade microtome and stored in 30% ethylene glycol cryoprotectant until processing.

For cFos immunohistochemistry, free floating sections were rinsed in 1× PBS (3×, 10 min), permeabilized with 0.5% Triton PBS, and blocked with 5% normal goat serum in 0.25% Triton PBS at room temperature. Sections were incubated with primary anti-rabbit antibody diluted 1:400 (Abcam; ab87655) in 2.5% normal goat serum and 0.25% Triton PBS for 48 h at 4 ℃. Sections were then rinsed with PBS (3 ×, 10 min), incubated with 1: 400 dilution of Alexa Fluor Goat anti-rabbit secondary antibody (Invitrogen) for 60 min at room temperature, washed with PBS (3 × 5 min), mounted on gelatin-coated slides, air-dried, and cover-slipped with Pro-Long Gold mounting medium (ThermoFisher Scientific). Cells immunolabeled for cFos were visualized under a Nikon Eclipse E600 microscope and were manually counted in the central LHb at the bregma level −3.6 mm.

RESULTS
Histological verification and localization of DREADD expression
One major pitfall of viral-mediated gene transfer is the ability to infect cells outside of the intended target region, potentially confounding the interpretation of results. However, our experience with intersectional expression in LHb has been more in line with all or nothing expression. As both viruses need to intersect in order to induce DREADD expression in a cell, only neurons that project from LHb to the target regions can express the transgene. LHb is surrounded by the medial habenula and thalamus, regions with few, if any, projections to the DRN, RMTg, and VTA. Thus, subjects were included as "hits" if either the left or right LHb contained HM_4Di or HM_3Dq expression with at least 90% of cells confined to the LHb. For experiment 1, of the 72 animals injected

with HM_4Di or HM3Dq, 13 animals were "hits" (4 unilateral and 9 bilateral) for the LHb to DRN pathway, whereas 9 were "misses"; 13 animals were "hits" (3 unilateral and 10 bilateral) for the LHb to VTA pathway, whereas 5 were "misses"; 13 animals were "hits" (4 unilateral and 9 bilateral) for the LHb to RMTg pathway, whereas 5 were "misses". Regarding hM_3Dq experiments, ten animals were "hits" (four unilateral and six bilateral) for the LHb to DRN pathway, whereas four were "misses". Two videos containing data from four animals were corrupted and could not be analyzed. A microscopic view of a single section from each subject included as a hit in this experiment was aligned to the others and was used to produce a maximum intensity projection of the DREADD expression in each pathway (Fig. 1b–h). DREADD expression using this intersectional technique transduced neurons situated within several hundred micrometers around the injection site. Still, we found that DREADD expression was distributed across the medial–lateral extents of the LHb, regardless of the pathway (Fig. 1b–d). DREADD expression was also apparent in the terminals in VTA, RMTg, and DRN (Supplementary Fig. S1). Targeting of CAV2-CRE was visualized with CAV-ZsGreen and was well-confined to the intended brain target regions (Fig. 1f, g, h), and collateralization between these pathways is very low, i.e., 0–3% [23, 36]. The number of animals that did not show successful intersectional expression of DREADD (i.e., "misses") was relatively high, but these surgeries produced matched control rats for the effect of CNO vs. vehicle on animals without DREADD expression (Fig. 1e), a control that has become necessary in the wake of recent discoveries that CNO can be back-metabolized to clozapine and have potential off-target effects [37].

Inhibition of DRN-projecting LHb neurons reduces immobility in the forced swim test
Inhibiting DRN-projecting LHb neurons produces a similar effect to classic anti-depressants in the FST. Animals with hM_4Di expression in LHb neurons projecting to the DRN and who were administered CNO had significantly decreased immobility compared with vehicle administered animals ($d = 1.32$; $F(10,1) = -6.29$, $p = 0.031$; Fig. 2b). Although immobility in vehicle-treated animals rises steadily throughout the 5 min test swim, immobility in the CNO-treated animals remains low throughout the swim (Fig. 2f). Visualizing activity tracks from these animals exemplifies the increase in swimming and attempts to climb the walls of the swim chamber (Fig. 2j). None of the VTA ($d = 0.07$; Fig. 2c), RMTg ($d = 0.55$; Fig. 2d), or Miss ($d = 0.72$; Fig. 2e) groups showed significant differences between CNO and vehicle animals. Animals in the Miss group came from failed intersectional surgeries from all brain regions and represent a strong control group for the effects of CNO in animals that do not express DREADDs.

Inhibition of DRN-projecting LHb neurons reduce saccharin licking during a reward-omission test
Animals were trained to lick saccharin in a two-bottle saccharin and water lickometer chamber. All groups of animals learned to increase saccharin licking across four training sessions, with no region by session interaction ($F(121,3) = 7.04$, $p = 0.0002$; Fig. 3b, f, j, n). All animals then received CNO and vehicle on alternating test sessions to determine whether inhibition of LHb output pathways had any effect on saccharin licking, a hedonic behavior. Inhibition of the LHb outputs to the DRN ($d = 0.04$; Fig. 3c), VTA ($d = 0.05$; Fig. 3g), RMTg ($d = 0.18$; Fig. 3k), and Miss ($d = 0.48$; Fig. 3o) did not have a significant effect on saccharin licking. On the final session, saccharin was omitted, two water bottles were presented, and licks on the previously saccharin-paired bottle were measured. Compared with vehicle-treated animals, those with inhibition of DRN-projecting LHb neurons licked less at the previously saccharin-paired bottle ($d = 2.45$; $F(5,1) = -16.00$, $p = 0.007$; Fig. 3d) and licked more at the previously water-paired bottle

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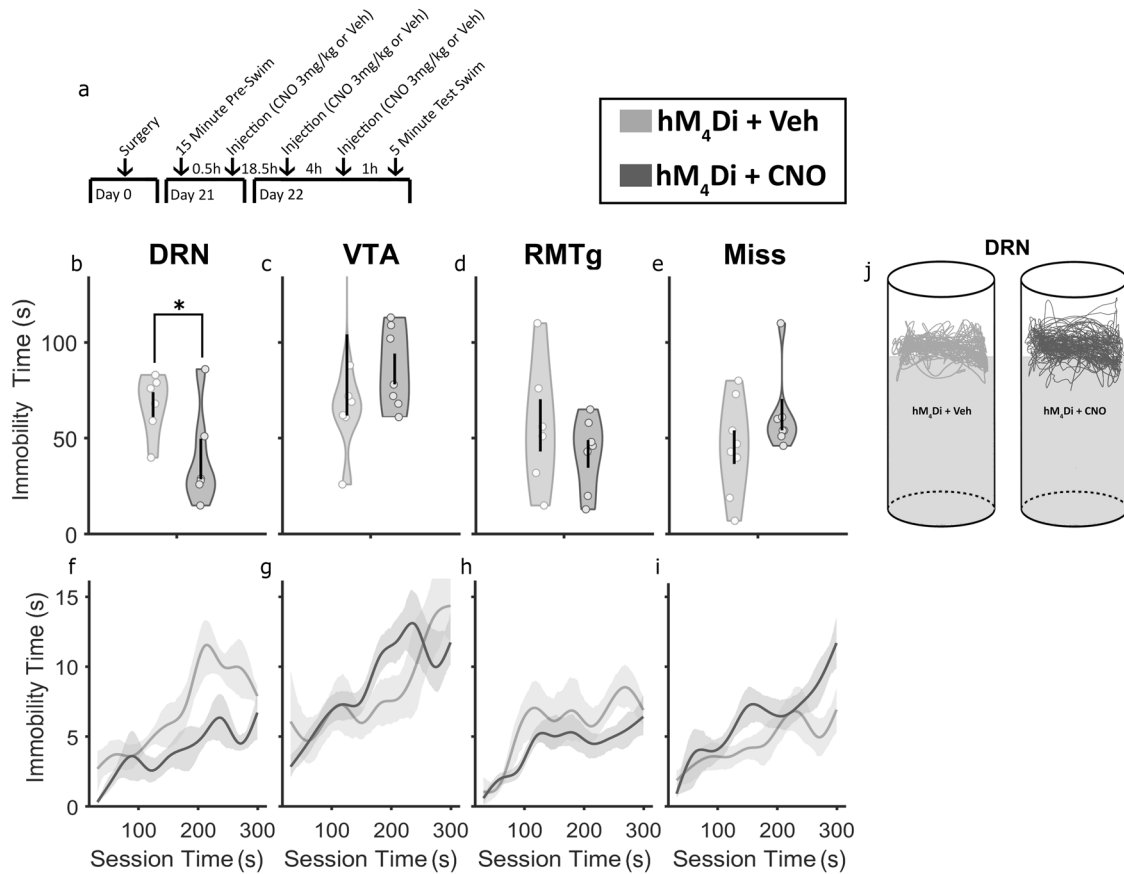**Fig. 2 Inhibition of DRN-projecting LHb neurons reduces immobility in the forced swim test. a** Experimental timeline and legend. **b** Animals with hM$_4$Di expression in DRN-projecting LHb neurons, which were administered CNO ($n = 6$), had significantly reduced immobility in the FST compared with vehicle-treated ($n = 6$) animals ($d = 1.32$; F(10,1) = −6.29, $p = 0.030$). **c–e** CNO had no effect in animals with hM$_4$Di expression in LHb neurons projecting to the VTA (CNO $n = 7$, Veh $n = 7$; outlier not shown) or RMTg (CNO $n = 6$, Veh $n = 7$), or in animals with no DREADD expression (CNO $n = 8$, Veh $n = 7$). **f** Immobility remained low throughout the FST for animals with hM$_4$Di expression in DRN-projecting LHb neurons, which were administered CNO. **g–i** Immobility trajectories were not different for CNO- and vehicle-treated animals from all other groups. **j** Example swim tracks from two animals with hM$_4$Di expression in DRN-projecting LHb neurons. Animals were housed together and run side-by-side. *$p < 0.05$; **$p < 0.01$.

($d = 2.02$; F(5,1) = 10.90, $p = 0.016$; Fig. 3e). During the reward-omission session, inhibition of the LHb outputs to the VTA ($d = 0.81$; Fig. 3h), RMTg ($d = 0.52$; Fig. 3l), and Miss ($d = 0.09$; Fig. 3p) did not have a significant effect on previously saccharin-paired bottle. Inhibition of the LHb outputs to the VTA ($d = 0.23$; Fig. 3i), RMTg ($d = 0.08$; Fig. 3m), and Miss ($d = 0.72$; Fig. 3q) also did not have a significant effect on water licking. There were no differences in saccharin acquisition between animals prior to being randomly assigned to the CNO or vehicle groups for reward omission (Supplementary Fig. S3).

Inhibition of LHb neurons projecting to DRN, VTA, and RMTg had no effect on locomotor activity and open-field behavior
After saccharin preference testing, animals were tested in an open-field arena. Open-field testing provided both a control for CNO's effect on locomotion as well as a test of anxiety-like behavior. There were no differences in distance traveled between CNO and vehicle groups for any of the three pathways or the Miss animals (DRN, $d = 0.02$; VTA, $d = 0.84$; RMTg, $d = 0.10$; Miss, $d = 0.47$; Fig. 4a–d). This is important for interpretation of forced swim behavior, as it shows the reduction in immobility caused by inhibition of the LHb to DRN pathway was not solely due to an increase in general motor activity. There was no evidence of "anti-anxiety" effects of LHb inhibition in any of the pathways, as evidenced by no significant CNO-induced change in center time (DRN, $d = 0.17$; VTA, $d = 0.36$; RMTg, $d = 0.00$; Miss, $d = 1.31$;

Fig. 4e–h), center crossings (DRN, $d = 0.35$; VTA, $d = 0.01$; RMTg, $d = 0.34$; Miss, $d = 0.03$; Fig. 4i-l), of rearing (DRN, $d = 0.95$; VTA, $d = 1.26$; RMTg, $d = 0.29$; Miss, $d = 0.80$; Fig. 4m–p) for any pathway.

Excitation of DRN-projecting LHb neurons had the opposite effect of inhibition on previously saccharin-paired bottle licking during reward-omission testing
All three experiments were repeated with hM$_3$Dq expression in the LHb to DRN pathway. Excitation of DRN-projecting LHb neurons had no effect on immobility in the FST ($d = 0.50$; Fig. 5a, b) or on saccharin licking during the saccharin preference test ($d = 0.13$; Fig. 5d). However, excitation of DRN-projecting LHb neurons increased licking at the previously saccharin-paired bottle during the reward-omission test ($d = 2.55$; F(8,1) = 19.49, $p = 0.002$; Fig. 5e), the opposite effect of chemogenetic inhibition of DRN-projecting LHb neurons. Excitation of DRN-projecting LHb neurons had no effect on licking at the previously water-paired bottle ($d = 0.64$; Fig. 5f) or on distance ($d = 0.95$; Fig. 5g), center time ($d = 0.71$; Fig. 5h), center crossings ($d = 0.90$; Fig. 5i), or rears ($d = 0.59$; Fig. 5j) in the open-field test. The hM$_3$Dq*vehicle animals from this experiment had markedly less licking at the previously saccharin-paired bottle then the hM$_4$Di*vehicle animals ($257.5 \pm 87.06 < 658.25 \pm 102.16$), but the hM$_3$Dq*CNO animals still licked more at the previously saccharin-paired bottle than the hM$_4$Di*vehicle animals ($754.67 \pm 74.80 > 658.25 \pm 102.16$).

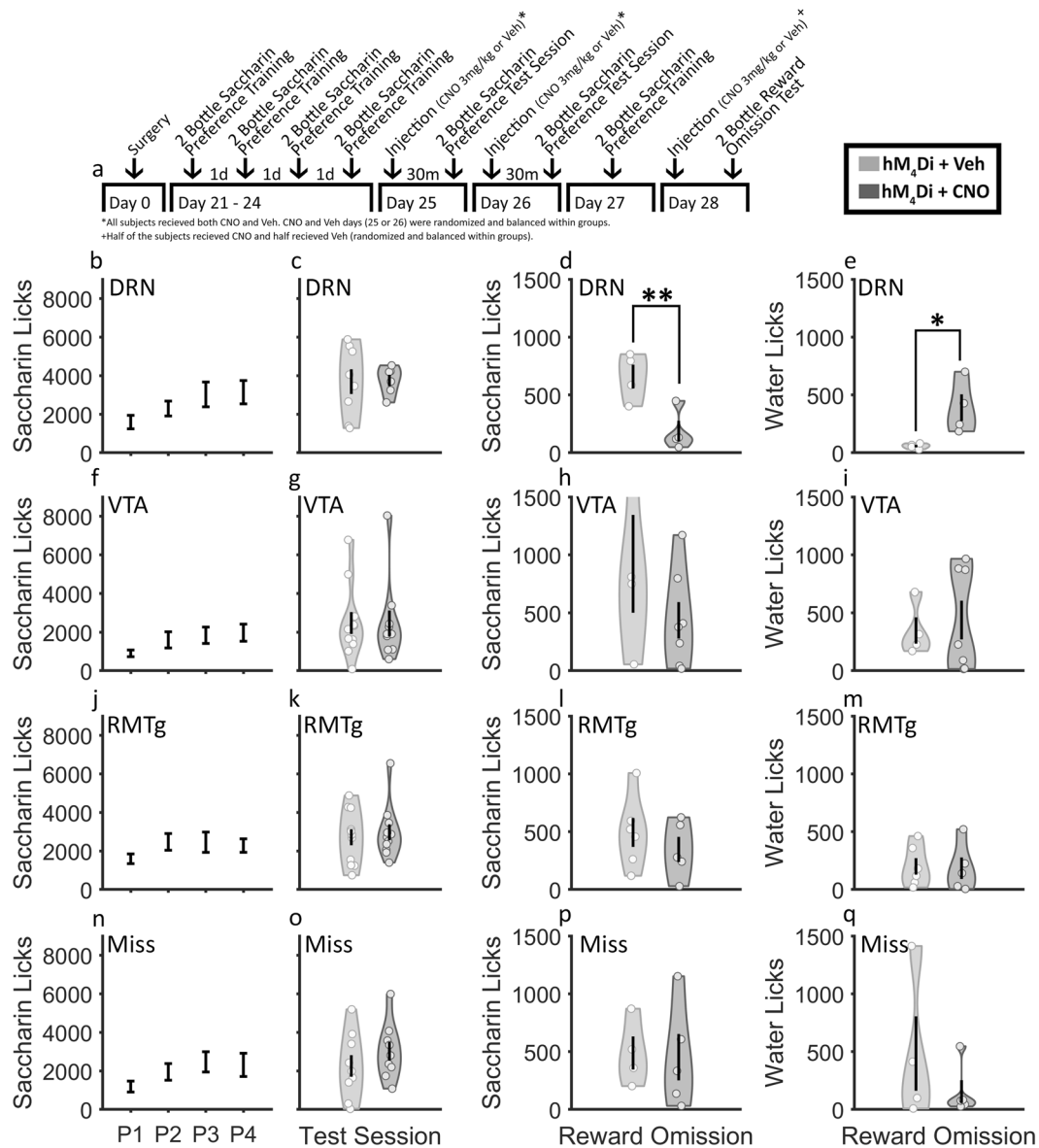Chemogenetic inhibition of lateral habenula projections to the dorsal…
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Fig. 3 Inhibition of DRN-projecting LHb neurons decreases perseverative saccharin seeking. a Experimental timeline and legend. **b, f, j, n** All animals learned to increase saccharin licking across four training sessions in a two-bottle choice lickometer chamber. **d, h, l** Inhibition of the LHb output pathways (DRN, $n = 8$ paired; VTA, $n = 11$ paired; RMTg, $n = 11$ paired) had no effect on saccharin licking, **p** and CNO also had no effect on saccharin licking in animals lacking DREADD receptors ($n = 9$, paired). **d** During a reward-omission test, inhibition of the LHb to DRN pathway reduced licking at a previously saccharin-paired bottle (CNO $n = 4$, Veh $n = 4$; $d = 2.45$; $F(5,1) = -16.00$, $p = 0.007$). **e** During a reward-omission test, inhibition of the LHb to DRN pathway also increased licking at a previously water-paired bottle (CNO $n = 4$, Veh $n = 4$; $d = 2.02$; $F(5,1) = -10.90$, $p = 0.016$). **h, i, l, m** Inhibition of LHb outputs to VTA (CNO $n = 7$, Veh $n = 4$; outlier not shown) and RMTg (CNO $n = 6$, Veh $n = 5$) had no effect on the reward-omission test. **p, q** CNO also had no effect on reward-omission testing in animals lacking DREADD expression (CNO $n = 5$, Veh $n = 4$). *$p < 0.05$; **$p < 0.01$.

Activation of hM4Di with CNO reduces cocaine-induced cFos immunoreactivity in the lateral habenula

To test for hM4Di-mediated inhibition of LHb neurons, we stimulated cFos expression with a single injection of cocaine (10 mg/kg i.p.) in rats that were pretreated with CNO (3 mg/kg i.p.) or vehicle. There was significantly less cocaine-induced (10 mg/kg) cFos expression (# of cells) in LHb of rats expressing hM4Di and treated with CNO compared with those treated with vehicle ($d = 3.02$; $t(6) = -24.32$; $p = 0.002$; Supplementary Fig. S2c). Similarly, we tested a set of animals with intersectional hM4Di expression in LHb neurons projecting to VTA; again, CNO reduced cFos expression compared with those treated with vehicle ($d = 2.66$; $t(5) = -17.049$; $p = 0.009$; Supplementary Fig. S2f). Immunohistochemical staining

for hM4Di failed in these slices, so we were unable to analyze double labeling for DREADD and cFOS, but these results agree with the well-established literature that hM4Di stimulation by CNO can meaningfully reduce activation of LHb neurons [26–28].

DISCUSSION

The LHb is a phylogenetically conserved structure that acts as a gateway between the limbic system and the monoaminergic midbrain nuclei. This makes the LHb well positioned to integrate complex emotional and motivational signals, and relay this information to the midbrain nuclei responsible for setting the monoaminergic tone of the whole brain. Although the role of the
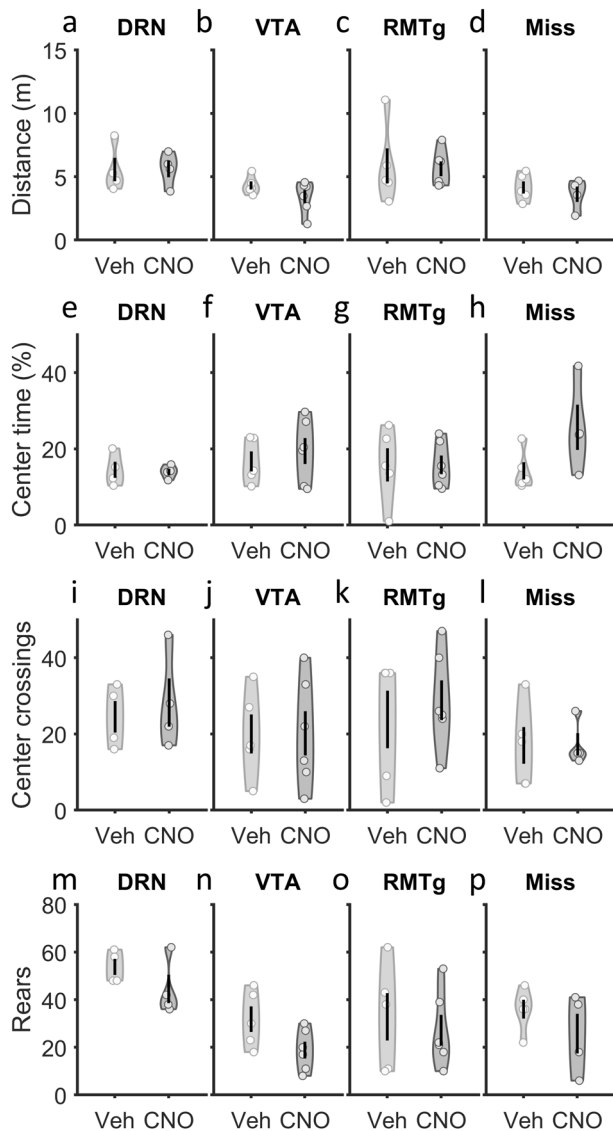
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Fig. 4 Inhibition of LHb neurons projecting to DRN, VTA, or RMTg had no effect on locomotor activity or open-field behavior. Inhibition of LHb neurons projecting to DRN (CNO n = 4, Veh n = 4), VTA (CNO n = 6 Veh n = 5), or RMTg (CNO n = 6, Veh n = 5) had no effect on (**a, e, i, m**) distance traveled, (**b, f, j, n**) center time, (**c, g, k, o**) center crossings, or (**d, h, l, p**) rearing in the open field. CNO also had no effect on animals without DREADD expression (CNO n = 5, Veh n = 4).

LHb in processing aversive stimuli has been well studied as a whole, the relative contributions of its output circuitry to different brain regions are less understood. To probe those circuits, we employed an intersectional viral approach that allowed us to express the inhibitory DREADD hM4Di exclusively in neurons that project to DRN, VTA, or RMTg. We then tested a variety of behaviors that induce stress, passive coping, anxiety, reward seeking, and frustration, as each of these can be components of neuropsychiatric disorders such as depression, anxiety, and substance abuse. Our goal was to transiently inhibit or activate the LHb-mediated circuits projecting to these individual midbrain nuclei, thereby altering behaviors associated with those pathologies.

Behavioral testing strategies
We first used the modified Porsolt FST [35]. Each stress model has strengths and limitations, and the FST has been used for more

than 30 years and has proved to have excellent predictive validity for antidepressant interventions. For many years, researchers interpreted the rodent's floating response as depressive-like behavior, but this interpretation has fallen out of favor. Swimming and immobility are now seen as the expression of different coping strategies. Although active and passive coping can both be useful survival strategies, drugs that promote active coping tend to improve symptoms of depression in humans, and animals who display a propensity toward active or passive coping have underlying neurobiological differences [38]. We next used the saccharin preference test followed by a reward-omission session. Reduced preference for sweet solutions is a measure of anhedonia that can be tested under low-stress conditions, whereas reward omission assesses coping flexibility in a frustrating condition. Both anhedonia and decreased coping flexibility are associated with clinical depression and anxiety conditions [39], and a decreased resilience following stressful life events [40, 41]. Finally, we used the open field to test for nonspecific locomotor effects of CNO as well as provide a simple readout of anxiety-like behavior. Overall, we found that inhibition of DRN-projecting LHb neurons may promote active coping and coping flexibility, two important goals in treating depression. This key connection from LHb to DRN has been discussed by previous studies, but had not been directly tested. Inescapable stress induces cFos in DRN-projecting LHb neurons and inhibition of the entire LHb reduces extracellular 5-HT in the basolateral amygdala [42]. Non-intersectional expression of hM4Di DREADD receptors and inhibition of LHb neurons also decreased immobility and increased swimming behavior in the FST, a pattern of behavior that also suggested the involvement of the serotonin system [17].

Inhibition of DRN-projecting LHb neurons reduces passive coping in the FST
Lesions or inhibition of the entire LHb reduces passive coping [7–9] and we previously found that DREADD-mediated inhibition of LHb neurons had similar antidepressant-like properties [17]. Here we show that hM4Di-induced inhibition of a subset of LHb neurons, which project to the DRN was sufficient to elicit this response. Inhibition of DRN-projecting LHb neurons reduced immobility in the FST, and by attenuating activity in the LHb's glutamatergic projections to the DRN, we can increase resilience to an inescapable stressor by reducing passive coping. CNO concentration in cerebrospinal fluid peaks about 15 min after i.p. injection and is mostly cleared by 60 min, but recent evidence has shown that appreciable levels of back-metabolized clozapine are present in the cerebrospinal fluid at 60 min post injection [43]. We used a standard three-dose protocol to comport with the typical rat FST procedure [33], to allow ready comparisons with previous studies. Our results indicate that the LHb to DRN projection is critical, but does not determine the exact cognitive mechanism involved; this would require a different battery of behavioral tests to assess whether the animals experience less "helplessness" or an impairment in some aspect of learning. However, we do not believe that this result reflects an impairment in extinction learning. In the reward-omission test, activating hM4Di in LHb projections to DRN decreased licking at the saccharin bottle (faster extinction, if anything) and increased licking at the water bottle, showing a shift in strategy that we interpret as increased coping flexibility.

The LHb's connections to the serotonin system are complex and reciprocal, with LHb neurons likely synapsing on serotonin neurons, GABAergic neurons, and even glutamatergic neurons [44–46]. There is some conflicting evidence as to what the physiological consequences to serotonin neurons are by inhibiting LHb inputs. Electrophysiological evidence suggests that LHb neurons synapse onto DRN GABAergic interneurons, and that inhibiting these neurons would reduce drive on GABA interneurons, thereby disinhibiting serotonin neurons and increasing
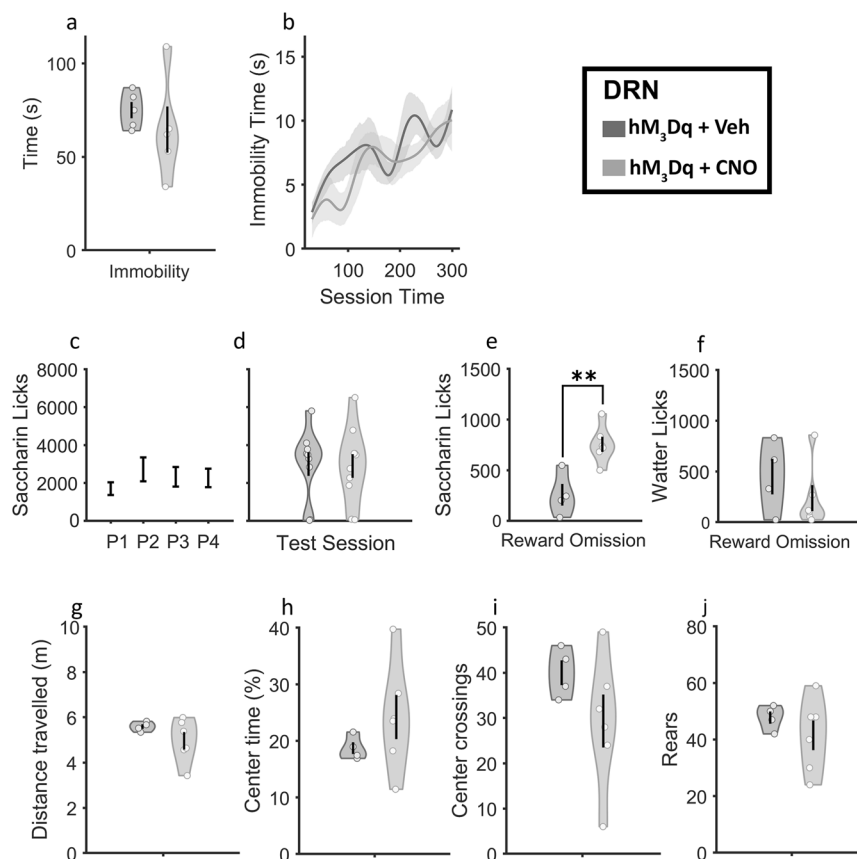
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Fig. 5 Excitation of DRN-projecting LHb neurons increases perseverative reward seeking. a, b Excitation of DRN-projecting LHb neurons (CNO $n = 5$, Veh $n = 5$) had no effect on immobility in the FST. **c** hM₃Dq-expressing animals ($n = 10$, paired) learned to increase saccharin licking across four pre-exposures and CNO had no effect on saccharin licking during the test session. **e** Excitation of DRN-projecting LHb neurons did however increase licking at the previously saccharin-paired bottle during the reward-omission test (CNO $n = 6$, Veh $n = 4$; $d =$ 2.55; $F_{(5,1)} = 19.49$, $p = 0.002$), which is the opposite result of inhibiting DRN-projecting LHb neurons. Excitation of DRN-projecting LHb neurons (CNO $n = 6$, Veh $n = 4$) had no effect on **f** licking at the previously water-paired bottle, **g** distance traveled, **h** center time, **i** center crossings, or **j** rearing in the open field. *$p < 0.05$; **$p < 0.01$.

serotonergic tone [47, 48]. GABA injection into the DRN also increases immobility time in the FST and interestingly reduces cFos expression in LHb neurons [49]. This observed decrease in LHb activity may be due to a negative feedback loop between the DRN and the LHb. Alternatively, there is also evidence that optogenetic silencing of the LHb during inescapable stress blocks increases in extracellular serotonin (5-HT) in the basolateral amygdala [42]. Unfortunately, LHb inhibition in that study was not specific to DRN-projecting neurons and it was difficult to separate the direct effect of inhibiting the DRN-projecting LHb neurons with the effect of inhibiting RMTg-projecting LHb neurons, which are GABAergic and also project to the DRN.

Inhibition of DRN-projecting LHb neurons does not increase general locomotor activity, exploration, or anxiety-like behaviors in the open field, strengthening our interpretation that reduced immobility in the FST represents reduced passive coping rather than a nonspecific enhancement of motor activity. Although it known that the FST induces cFos in the LHb [50], we did not specifically demonstrate that cFos is induced in the LHb to DRN pathway during FST. As we gave CNO after the first swim, we think that decreasing activity in this pathway in the interval between the first and second swim sessions alters plasticity that otherwise develops, but we cannot say with certainty that this pathway was or was not silenced during the second swim session. However, some drugs such as Ketamine and Mibefradil block LHb neuron bursting but do not affect baseline firing, yet are effective in reducing immobility in the FST [10]. Exciting DRN-projecting LHb

neurons with hM₃Dq did not increase immobility in the FST, whereas general LHb activation can reduce locomotion in an open field but not on the Rotarod [51], so a nonspecific effect on motor activity is unlikely to explain these results. Rather, there may be a ceiling effect for stress-induced excitation of the LHb that is not exacerbated by hM₃Dq.

Several other recent reports have manipulated LHb projections to the midbrain in tests designed to probe passive coping or effort exertion. Proulx et al. [52] found that acute optogenetic inhibition of RMTg-projecting LHb neurons transiently decreased immobility during a single FST session, whereas Cerniauskas et al. [36] found that chemogenetic inhibition of LHb neurons projecting to the VTA in mice exposed to 8 weeks of chronic mild stress (CMS) reduced immobility in the tail suspension test. Although on the surface these results do not paint a cohesive story, the fundamental differences between these approaches makes direct comparisons difficult. Proulx et al. [52] utilized a forced swim procedure consisting of alternating 2 min light on/off epochs for RMTg-projecting LHb neurons during a single 20 min swim. Optogenetic inhibition causes almost complete silencing of neurons, often followed by rebound excitation during the "off" periods, which may confound data interpretation. This study also did not employ a true intersectional approach, as it expressed inhibitory opsin into LHb non-selectively but administered laser light only into the RMTg. LHb projections to DRN pass through and around the RMTg, meaning they may be inhibited by this manipulation as well. We utilized a truly intersectional technique

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and found that only inhibition of projections to the DRN reduced passive coping. Cerniauskas et al. [36] found that after 8 weeks of CMS, LHb projections to the VTA were sensitized, whereas DRN projections were not. They then show that hM₄Di-based inhibition of LHb projections to the VTA reduced passive coping in CMS animals, but they did not test inhibition of LHb projections to the DRN. It is possible that CMS changes habenula activity in such a way that makes the projection to the VTA more important for passive coping, whereas our results suggest that during acute stress, the projections to DRN are promoting passive coping. Finally, the current study was only powered sufficiently to find large effect sizes, so modest effects in other pathways may have been missed.

Inhibition and excitation of DRN-projecting LHb neurons have opposite effects on perseverative reward seeking

Inhibiting LHb neurons projecting to the DRN, VTA, and RMTg had no effect on saccharin licking during a saccharin preference test, during which the animal reliably received as much saccharin as it wished, i.e., any frustration associated with the lack of reward is quite low. This is not surprising given that LHb is generally thought to process primarily aversive stimuli, although unexpected reward delivery can actually inhibit LHb [13]. However, reward omission is known to activate the LHb, suggesting that frustration can be sufficiently aversive to activate LHb, and this may in turn modulate decision-making [4, 15]. When we inhibited DRN-projecting LHb neurons during reward-omission testing, rats spent less time licking at the previously saccharin-paired bottle. Continued licking at the previously saccharin-paired bottle may represent perseverative reward seeking, whereas promptly reducing effort to obtain an unavailable reward is adaptive. Inhibiting the LHb's projections to the DRN reduced perseverative licking at a bottle previously containing saccharin. This reward-omission condition revealed that frustration activates this pathway and interferes with flexibility, as hM₄Di-mediated inhibition reduced, but hM₃Dq-mediated activation increased, perseverative licking at the bottle previously associated with saccharin. Inability to cope with stressful events flexibly is a correlate of the development of depression in humans [40, 41]. There was a nonsignificant trend for inhibition of VTA-projecting LHb neurons to reduce this perseverative licking at the bottle previously associated with saccharin. These results differ from previous reports using pathway nonspecific manipulations of the LHb, but it is particularly difficult to compare pathway-specific manipulations of the LHb with the global LHb manipulations, as the regions the LHb projects to form complex and reciprocal networks both with each other and with the LHb. In either case, the LHb appears to be important during circumstances requiring flexible, adaptive coping when the likelihood of outcomes is changing [53, 54]. By alternatively inhibiting or activating DRN-projecting LHb neurons, our data indicate that this particular pathway plays an important role in flexible coping. Future studies might utilize additional experimental designs that investigate probabilistic learning and responding.

Targeting single circuits with DREADDs may lead to treatments for specific domains of psychiatric illness

Although "sledge hammer" approaches to LHb manipulation (e.g., lesions) cause dramatic effects in aversion and reward processing, DREADD-induced manipulation of individual pathways caused only subtle changes in particular behaviors. In addition to providing a great degree of anatomical precision, chemogenetic approaches allow for transient, bidirectional manipulation of discrete circuits and makes balanced order and within-subjects experimental designs possible. Current treatments for psychiatric illness are plagued by side-effects that are often the root of non-compliance [55]. Our ability to isolate a single circuit with a limited effect on behavior could be crucial to finding treatments with fewer off-target effects. Still, even with our limited subset of

behavioral outputs, we found two distinct behaviors that were affected by inhibiting LHb's projections to DRN. Further studies will be needed to tease these behaviors apart and to determine the effect that these manipulations have on the serotonin system. These changes are also highly acute and need to be explored in a chronic setting, as chronic stress in humans is more likely to lead to psychiatric illness than acute stressors.

Data accessibility

The data and code necessary to reproduce the figures and statistics are available at https://github.com/DrCoffey/Manuscripts/tree/master/LHb%20Pathways%20(Neuropsychopharmacology%202020) (unpublished).

AUTHOR CONTRIBUTIONS
KC performed the experiment, analyzed the data, and wrote the manuscript. RM performed the experiment and analyzed the data. EV performed the experiment. SN performed the experiment and analyzed the data. JN designed the experiment and wrote the manuscript.

ADDITIONAL INFORMATION
Supplementary Information accompanies this paper at (https://doi.org/10.1038/s41386-020-0616-0).

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