ARCHIVAL REPORT

Infralimbic D2 Receptors Are Necessary for Fear Extinction and Extinction-Related Tone Responses

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Background: Fear extinction is dependent on plasticity in the infralimbic prefrontal cortex, an area heavily innervated by midbrain dopaminergic inputs. Dopamine D2 receptors are concentrated in infralimbic output neurons that are involved in the suppression of conditioned fear after extinction. Here, we examined the specific role of infralimbic D2 receptors in mediating associative learning underlying fear extinction using the selective D2 antagonist raclopride.

Methods: Raclopride was administered systemically or infused into the infralimbic prefrontal cortex before fear extinction, and extinction retention was tested the following day. Rats were also prepared for single-unit recording in the infralimbic prefrontal cortex to assess the effect of raclopride on firing properties.

Results: We found that systemic injection of raclopride given before extinction impaired retrieval of extinction when rats were tested drug-free the next day but also induced catalepsy during extinction training. To determine whether impaired extinction was due to impaired motor function or disruption of extinction consolidation, we infused raclopride directly into the infralimbic prefrontal cortex. Raclopride infused immediately before extinction training did not produce motor deficits but impaired recall of extinction when tested drug-free. Furthermore, in animals that underwent extinction training, systemic raclopride reduced the tone responsiveness of infralimbic prefrontal cortex neurons in layers 5/6, with no changes in average firing rate.

Conclusions: We suggest that D2 receptors facilitate extinction by increasing the signal-to-noise of infralimbic prefrontal cortex neurons that consolidate extinction.

Key Words: Anxiety disorder, dopamine, fear conditioning, medial prefrontal cortex, raclopride, schizophrenia

The D2 family of dopamine receptors is intimately involved in affective, motor, and cognitive functions, many of which are mediated by the prefrontal cortex (1,2). D2 receptor signaling in prefrontal cortex has been linked to working memory function (3), reversal learning (4), and behavioral flexibility (5), and blocking these receptors plays a key therapeutic role in diminishing the positive symptoms of schizophrenia (6,7). Previous work has shown that D2 actions in prefrontal circuits are associated with motor control functions such as the suppression of prepotent responses (8). Similar suppression of behavioral responses is observed after extinction of conditioned fear, and converging evidence implicates the medial prefrontal cortex, particularly the infralimbic subregion (IL), in the consolidation of fear extinction (9–11). Whether D2 receptors in IL contribute to extinction consolidation, however, remains unknown.

D2 receptor density is particularly high in IL compared with other prefrontal regions (12), and IL is heavily innervated by dopaminergic projections from the ventral tegmental area (13). D2 receptor binding and transcripts are most prominent in layer V neurons (14), which are the output neurons of the cortex. These neurons are thought to inhibit fear after extinction training by impeding amygdala output (15,16). The localization of D2 receptors on IL output neurons and the function of these neurons in fear suppression suggest that D2 receptors have a significant function in fear extinction. Thus, D2 receptor activity may critically modulate IL output, and extinction-related tone responses generated in IL are hypothesized to suppress fear responses (10). Moreover, fear extinction induces dopamine release in IL (17), suggesting that dopamine is involved in the consolidation of extinction.

While one might expect D2 receptor antagonists to impair extinction, a previous study in mice showed that antagonizing D2 receptors with systemic administration of sulpiride facilitated extinction (18). Therefore, to clarify the role of D2 receptors in extinction, we used raclopride, which has greater specificity and is more potent at antagonizing D2 receptors than sulpiride (19). We administered raclopride both systemically and intra-IL to determine the role of D2 receptors in the acquisition and retrieval of extinction. We also evaluated the effect of raclopride on the firing properties of IL neurons, specifically on tone responses after extinction.

Methods and Materials

Subjects

Male Sprague-Dawley rats (270–320 g) were obtained, housed, and handled as described previously (20). Rats were restricted to 18 g of standard laboratory rat chow daily and were subsequently trained to bar press for food pellets on a variable interval schedule (VI 60 sec). Throughout behavioral experiments, rats were able to press for food to maintain a constant level of activity against which freezing could be reliably measured (20). All procedures were approved by the Institutional Animal Care and Use Committee at the University of Puerto Rico in compliance with National Institutes of Health guidelines.

Surgery

For infusion experiments, rats were implanted with a single 26-gauge stainless steel guide cannula (Plastics One, Roanoke, Virginia) aimed at IL (anterior-posterior: +2.9, midline: −1.0, dorsal-ventral: −4.1 mm relative to bregma, angled 11° toward the midline in the coronal plane). After behavioral testing, rats were perfused with .9% saline followed by 10% buffered formalin. Brains were removed and stored in a 30% sucrose/10% formalin solution. Coro-

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nal sections (40 μm) were cut, mounted on slides, and stained for Nissl bodies to visualize the injector tip location.

A separate group of rats was surgically implanted with recording electrodes that consisted of drivable bundles of 16 microwires (22 μm, Stablohm 650; California Fine Wire, Grover Beach, California) as previously described (21). Electrodes were aimed at IL, located 2.9 mm anterior, .6 mm lateral, and 5.0 mm ventral from bregma. At the conclusion of the experiment, lesions were made at the tip of the recording wires by passing an anodal current of 25 μA for 18 seconds. Rats were then perfused with 10% buffered formalin, and the brains were removed to mark the microlesions with a blue reaction of 6% ferrocyanide while fixing the tissue in 30% sucrose/10% buffered formalin. Locations of lesions were reconstructed onto coronal drawings adapted from Paxinos and Watson (22) from 40 μm Nissl-stained sections.

Fear Conditioning and Extinction

All fear conditioning and extinction procedures were carried out in four identical operant boxes (Colbourn Instruments, Allentown, Pennsylvania), located within sound-attenuating chambers. Between rats, shock grids were cleaned with soap and water and conditioning chamber walls were wiped clean. On day 1, rats received seven conditioning trials in which a tone (30 sec, 4 kHz, 75 dB) co-terminated with a mild footshock (.5 mA, .5 sec). The intertrial interval varied, averaging 3 min. On day 2, rats were injected with either saline ($n = 9$) or raclopride (.3 mg/kg, intraperitoneal [IP], $n = 11$) 10 min before extinction training (experiment 1); injected with either saline ($n = 4$) or raclopride (.1 mg/kg, IP, $n = 5$) 10 min before extinction training (experiment 2); or infused with saline ($n = 9$) or raclopride (5 μg in .5 μL, $n = 11$) into IL before extinction training (experiment 3), which consisted of 15 tone presentations in the absence of footshock. On day 3, rats were tested drug-free with 15 presentations of the tone alone. In experiment 4, we infused saline ($n = 5$) or raclopride ($n = 6$) into IL 10 min before test (day 3). Conditioned fear was assessed by measuring the percentage of time spent freezing during the tone.

Open Field Testing

To test the effects of raclopride on locomotor activity, rats were given injections of saline or raclopride (.1 or .3 mg/kg, IP) 10 min before testing in an open field ($n = 4$ per group). Grid lines drawn on the floor of the arena (91.5 × 91.5 × 61 cm) divided it into a peripheral region (within 15.25 cm of the walls) and central region (61 × 61 cm) of approximately equal area. The number of line crosses and time spent in the central region were scored by an observer blind with respect to experimental groups.

Infusions of Raclopride in IL

Infusions of raclopride (5 μg, Sigma, St. Louis, Missouri) dissolved in saline or saline alone were made 10 min before extinction training in a volume of .5 μL at a rate of .2 μL/min. Following infusions, injectors were left in place for 2 min to allow the drugs to diffuse. The tip of the injection cannula extended 1.0 mm beyond that of the guide cannula. This dose of raclopride was chosen as it impairs acquisition of fear conditioning when infused in the amygdala (23) and has been infused into the medial prefrontal cortex (24).

Behavioral Data Analysis

Digital video was recorded during the behavioral procedures and was analyzed with Freezescan software (Clever Systems, Reston, Virginia). Total seconds freezing during the tone presentations were scored for each rat, and this number was expressed as a percentage of the total tone presentation time. All data are included in the analysis, but data are shown in blocks of two trials (necessarily excluding trial 7 of conditioning and trial 15 of extinction). Group comparisons were made using analysis of variance or Student *t* tests (SPSS for Windows, SPSS, Inc., Chicago, Illinois). Significant main effects were followed by Tukey post hoc comparisons.

Multi-Channel Unit Recording

After surgery, rats were allowed 6 days to recover. Rats ($n = 3$) were fear conditioned with five tone-shock pairings, followed by extinction training consisting of 20 tone-alone presentations. The next day, rats received an additional five tone-alone presentations to assess extinction retention. Rats were then acclimated to recording procedures in the same chambers as in the behavioral experiments, and electrodes were driven in increments of 44 μm until single units were isolated with principle components analysis and template matching (Offline Sorter; Plexon, Dallas, Texas). Once cells in IL were well isolated, we assessed the effects of injections of saline or raclopride (.3 mg/kg, IP) on spontaneous activity while rats were in the operant chamber pressing for food. Five-minute sessions of spontaneous activity and 4-min sessions consisting of three tone presentations were recorded at four time points: 30 min before and 10 min after saline injection and 30 min before and 10 min after raclopride injection. Firing rates before and after injections were compared with a Wilcoxon matched-pairs test. After recording the four sessions at a given location, the electrode drive was advanced in 80 μm steps until new cells were found, and the experiment was repeated. Spike trains were analyzed with NeuroExplorer (NEX Technologies, Littleton, Massachusetts) to obtain firing rate and bursting. Bursts were defined as three or more successive spikes in which the first interspike interval was < 25 msec and subsequent intervals were < 50 msec (21). To measure tone-induced activity of neurons, the firing rate in the first 400 msec bin after tone onset was compared with the firing rate of 20 pretone bins of equal duration using a *Z*-score transformation as previously described (10).

Results

Systemic Blockade of Dopaminergic D2 Receptors Impairs Consolidation of Extinction Memory

We first examined whether dopamine, acting at D2 receptors, is necessary for extinction learning. Rats were fear conditioned on day 1 and were systemically injected with the D2 receptor antagonist raclopride (.3 mg/kg IP) or saline 10 min before extinction training on day 2. Rats injected with raclopride expressed significantly higher levels of freezing on average than rats injected with saline throughout the extinction session (Figure 1A). Analysis of variance revealed a main effect of group [$F(1,15) = 15.6$, $p < .001$] but no group by trial interaction [$F(14,252) = 1.2$, $p = .26$], indicating that raclopride-treated rats expressed more freezing overall during the session than saline-treated control rats. Thus, raclopride augmented freezing behavior during extinction training. In a drug-free test the following day, raclopride-treated rats expressed higher levels of freezing to the tone than saline-treated rats. Analysis of variance revealed a main effect of group [$F(1,18) = 6.7$, $p = .02$] and a group by trial interaction [$F(14,252) = 2.3$, $p = .005$], indicating that raclopride-treated rats expressed higher levels of freezing than saline-treated rats at the beginning of the extinction retention test but gradually expressed less freezing across the test. Thus, it would appear that raclopride interfered with both extinction acquisition and consolidation. However, it is well known that D2 receptor antagonists can induce catalepsy (25,26), which could interfere with

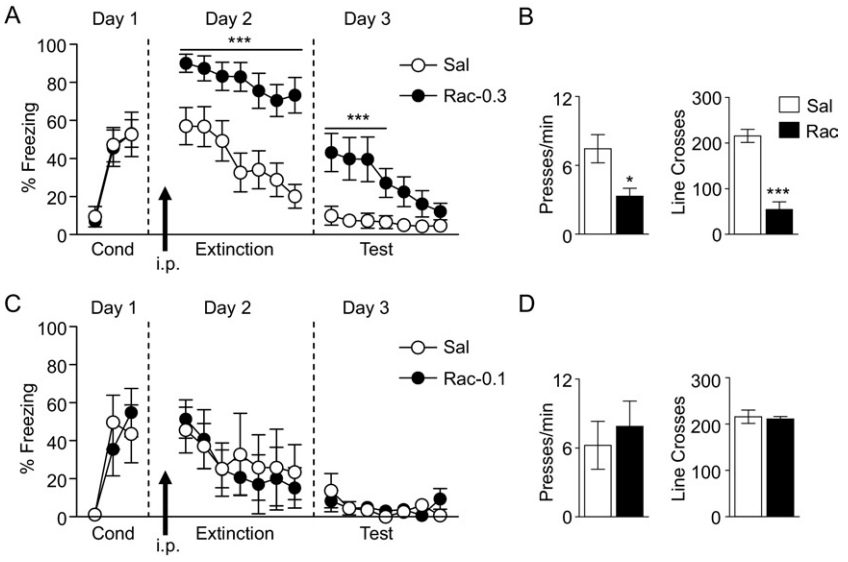

Figure 1. Systemic raclopride induces mild catalepsy and impairs long-term retention of fear extinction. **(A)** Systemic injections of .3 mg/kg raclopride (arrow) before extinction on day 2 led to a significant increase in percent freezing and impaired retention of extinction on day 3. **(B)** This dose of raclopride (.3 mg/kg) reduced pretone bar-press rates during extinction and reduced locomotor activity as measured by line crosses in an open field. **(C)** Systemic injections of .1 mg/kg raclopride (arrow) before extinction did not affect percent freezing or impair retention of extinction on day 3. **(D)** This dose of raclopride (.1 mg/kg) did not affect pretone bar-press rates during extinction or locomotor activity in an open field. Data shown in blocks of two trials for **(A)** and **(C).** $^{*}p < .05$, $^{***}p < .001$. Cond, conditioning; i.p., intraperitoneal; Rac, raclopride; Sal, saline.

extinction. Indeed, rats injected with .3 mg/kg (IP) raclopride showed significantly reduced pretone bar-press rates before the first extinction trial [Figure 1B; $t(18) = 2.3$, $p = .03$] and reduced locomotor activity in an open field compared with saline-injected control rats [Figure 1B; $t(6) = 7.3$, $p < .001$]. Thus, it is possible that the apparent blockade of extinction acquisition could be due instead to locomotor side effects of raclopride.

In an attempt to dissociate locomotor from extinction effects of raclopride, we repeated the experiment with a lower dose of raclopride (.1 mg/kg IP; Figure 1C). Raclopride-treated rats expressed similar levels of freezing as saline-treated rats throughout extinction training. Analysis of variance revealed no effect of group or a group by trial interaction (all F's < 1.0, $p > .75$). In a drug-free test the following day, both the raclopride- and saline-injected groups showed equally good retention of extinction. Analysis of variance revealed no effect of group or group by trial interaction on extinction retention (all F's < 1.5, $p > .15$). The lower dose of raclopride also had no effect on pretone bar-press rates before the first extinction trial [$t(7) = .58$, $p = .6$; Figure 1D] and did not affect locomotor activity in an open field compared with saline-injected control rats [$t(6) = .30$, $p = .78$; Figure 1D]. Thus, the increased freezing observed during extinction training with the .3 mg/kg dose correlated with the cataleptic effects of the drug. Because we were unable to dissociate locomotor effects from impaired retrieval of extinction, it remains possible that the apparent extinction deficit induced by systemic raclopride resulted from locomotor side effects.

Blockade of D2 Receptors in IL Impairs Consolidation of Extinction Memory

D2 receptor antagonists are thought to induce catalepsy by acting in the striatum (27–29). Therefore, to dissociate locomotor effects from effects on extinction consolidation, we infused raclopride directly into IL, a structure strongly implicated in consolidation of fear extinction. Rats were infused with raclopride (5 μg in .5 μL) or saline into IL 10 min before extinction training (see Figure 2A for infusion site for all rats). This dose of raclopride was chosen as it impairs acquisition of fear conditioning when infused in the amygdala (23). Unlike systemic administration, intra-IL raclopride did not affect expression of freezing or within-session extinction (day 2; Figure 2B). Analysis of variance revealed no effect of group or a group by trial interaction (all F's < 1.0, $p > .60$). In addition, raclopride did not affect pretone bar-press rates before the first extinc-

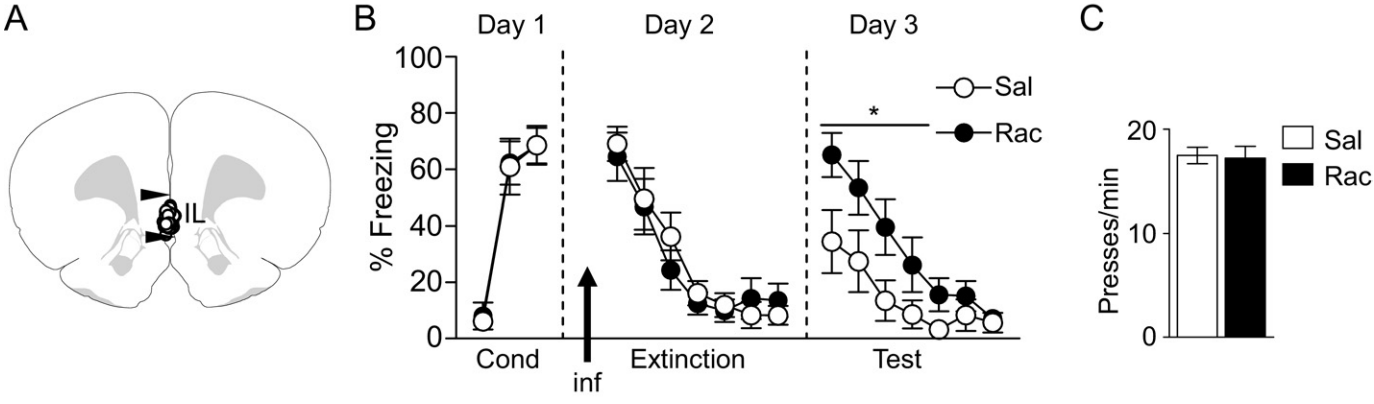

Figure 2. Infusion of raclopride into infralimbic subregion (IL) impairs long-term retention of fear extinction, without inducing catalepsy. **(A)** Coronal drawings (bregma, 3.20 mm; reprinted from [45] Copyright [1992]) show placements of injector tips for all rats in IL. **(B)** Before extinction, rats were infused (arrow) with either the D2 receptor antagonist raclopride or saline. Rats infused with raclopride into IL showed normal within-session extinction but were impaired in their recall of extinction on day 3. Data shown in blocks of two trials. **(C)** Raclopride infusions into IL did not affect pretone bar-press rates during extinction. $^{*}p < .05$. Cond, conditioning; IL, infralimbic subregion; inf, infusion; Rac, raclopride; Sal, saline.

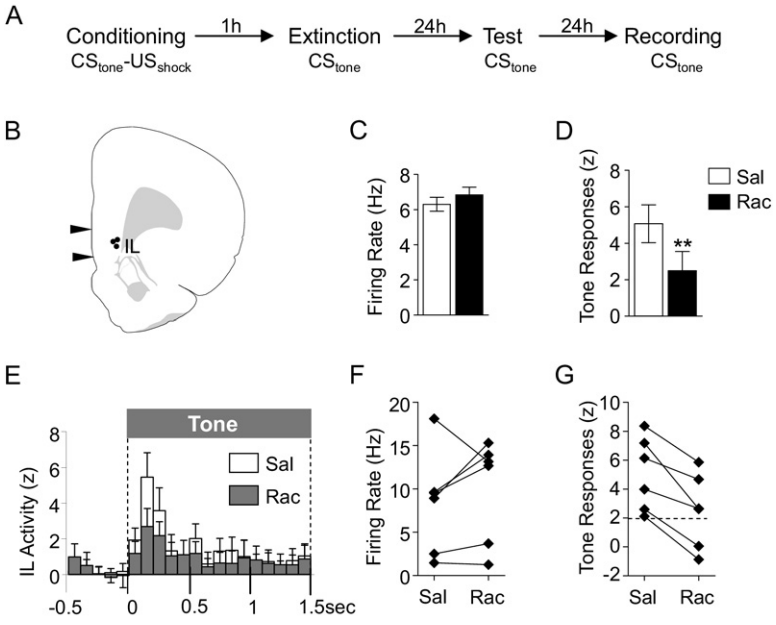

Figure 3. Raclopride attenuates extinction-related tone-evoked activity in infralimbic subregion units. **(A)** Diagram representing the experimental procedure. **(B)** Coronal drawing shows location of recording electrodes (bregma + 2.80 mm; reprinted from 45 Copyright [1992]). **(C)** Systemic raclopride had no effect on firing rate relative to saline. **(D)** In tone-responsive neurons, systemic raclopride attenuated the magnitude of tone-evoked responses in the first 400 msec after tone onset. **(E)** The reduction in tone responses are shown in the group perievent time histogram (bin width 100 msec). **(F)** Raclopride did not alter firing rate of these tone-responsive neurons, but **(G)** markedly reduced tone responsiveness in each of these individual neurons (dashed line indicates significant tone response, $p < .05$). $^{**}p < .01$. CS, conditioned stimulus; IL, infralimbic subregion; Rac, raclopride; Sal, saline; US, unconditioned stimulus.

tion trial relative to saline-infused control rats [Figure 2C; $t(18) = .06$, $p = .95$]. The following day, however, raclopride-infused rats showed impaired retrieval of extinction relative to saline-infused rats (Figure 2B). Analysis of variance revealed a main effect of group [$F(1,18) = 4.8$, $p = .04$] and a group by trial interaction [$F(14,252) = 1.9$, $p = .03$], indicating that raclopride-infused rats expressed higher levels of freezing than saline-infused rats at the beginning of the extinction retention test and gradually extinguished across the test. In a separate experiment, raclopride infusions before test did not impair the recall of extinction (Figure S1 in Supplement 1). Thus, dopaminergic D2 receptor blockade in IL impairs consolidation of fear extinction but not the expression of extinction previously learned.

Blockade of D2 Receptors Reduces Extinction-Related Tone Responses in IL

Given the preferential expression of D2 receptors in the output layer of cortex (14), we expected that D2 receptors would have distinct effects on the firing properties of IL neurons engaged in fear extinction. To determine the physiological effects of D2 receptor blockade, we recorded from IL neurons within layers 5/6 in behaving rats that had received conditioning and extinction training (procedure shown in Figure 3A and placement of electrodes shown in Figure 3B). All rats showed significant within-session extinction, as demonstrated by an overall effect of trial [$F(2,38) = 4.0$, $p < .001$], and good retention of extinction, as they showed similar freezing levels to a tone CS at test compared with the last trial of extinction [$t(2) = .8$, $p = .52$]. Following the test, single units were isolated and spontaneous activity was recorded for 5 min followed by three tone CS presentations. This was repeated 10 min after a saline injection, 30 min before raclopride injection, and 10 min after raclopride injection, resulting in a within-cell statistical design. A total of 20 single units were analyzed.

Raclopride did not affect averaged firing rate (Wilcoxon matched-pairs test: $Z = 1.53$, $p = .13$; Figure 3C) or high-frequency bursting [$t(19) = 1.46$, $p = .16$] compared with saline. Tone responses in IL neurons reflect extinction learning, so we examined the effect of raclopride administration on the magnitude of extinction-related tone responses. Tone-evoked activity was recorded before and after injection of saline or raclopride (.3 mg/kg, IP). Following saline administration, a subset of IL neurons (6/20, or 30%) exhibited significant conditioned tone responses within the first 400 msec after tone onset relative to the preceding 8 sec before tone onset (all Z's > 1.96, $p < .05$). Raclopride administration significantly attenuated tone responses in these IL neurons from an average $Z = 5.07$ to $Z = 2.50$ [$t(5) = 5.38$, $p = .003$; Figure 3D] and reduced the number of neurons exhibiting significant tone responses (4/6, or 67%). The perievent time histogram for this subset of neurons shows that tone responses were robustly expressed at the onset of the tone, but activity rapidly returned to baseline levels in the first 400 msec of the tone period (Figure 3E). This decrease in tone-evoked activity was independent of changes in firing rate or high-frequency bursting, as raclopride did not affect firing rate (Wilcoxon matched-pairs test: $Z = 1.57$, $p = .12$; Figure 3F) or high-frequency bursting [$t(5) = .10$, $p = .92$] in these tone-responsive neurons. Furthermore, each individual tone-responsive unit showed a marked decline in tone-evoked activity after raclopride treatment (Figure 3G), and no previously nonresponsive units became tone responsive after raclopride treatment. The decline in tone response with raclopride could not be attributed to extinction across repeated testing, because no such decline was observed in saline-treated rats (Figure S2 in Supplement 1). Taken together, these results suggest that blockade of D2 receptors impairs fear extinction by disrupting IL tone responses that arise during extinction (30,31).

Discussion

The present study demonstrates that D2 receptor signaling is necessary for extinction of conditioned fear. Blockade of D2 receptors in IL during fear extinction impairs later retrieval of extinction, implicating D2 receptor signaling in this structure. Although raclopride did not affect firing rate or bursting in IL, it attenuated extinction-related tone responses. Thus, D2 receptor signaling in IL promotes fear extinction, presumably by enabling tone responses during extinction that are necessary for extinction consolidation (10,30,31).

Raclopride-induced impairment of fear extinction generally agrees with previous findings. Haloperidol, a predominantly D2 receptor antagonist with notable affinity for the D1 receptor, was

recently shown to impair extinction when given systemically or into the nucleus accumbens (32). In addition, lesions of the dopaminergic terminals in medial prefrontal cortex impair contextual fear extinction (33). Our results implicate dopaminergic actions at the D2 receptor and parallel the finding that D4 receptor activation in IL is also required for fear extinction (34). Ponnusamy *et al.* (18), however, observed accelerated extinction in mice using a different systemically administered D2 receptor antagonist, sulpiride. The disparity with our results may be explained by the greater D2 receptor selectivity and binding strength of raclopride over sulpiride (19). Sulpiride is known to bind non-D2 receptor sites, an effect not observed with other neuroleptics (35). It should be noted that sulpiride did not enhance extinction of a cocaine-induced conditioned place preference (36). Our results cannot be explained by state dependent effects, as raclopride is quickly metabolized, and infusing the drug before extinction retrieval did not impair retrieval of extinction.

We identified IL as a site of action of raclopride in fear extinction and observed a significant reduction in tone responses of IL neurons after systemic raclopride injections. Tone responses in IL are correlated with extinction retention (10), and electrical stimulation designed to mimic tone responses in this region during extinction enhances extinction retention (10,37). Output from IL neurons inhibits fear after extinction training by impeding amygdala output (15,16). Because D2 receptors are located on IL output neurons and conditioned responses in these neurons are observed during extinction (30,31), we argue that activation of D2 receptors augments tone responsiveness during extinction learning.

In addition to direct modulation of tone responses, D2 receptor activation also stimulates mitogen-activated protein kinases (MAPKs) (38,39). The MAPK signaling cascade is critical for protein synthesis, and reducing the activity of MAPK (9,40) or blocking protein synthesis (41,42) in IL impairs extinction retention. Thus, activation of D2 receptors on IL output neurons would facilitate consolidation of fear extinction.

Our results indicate that optimal levels of dopamine acting at D2 receptors are required for fear extinction to occur. The D2 antagonists are commonly prescribed in psychiatry and are useful for the treatment of schizophrenia, a condition that is often comorbid with anxiety disorders. Our results suggest that the use of these drugs could lead to resistance to extinction, which may impede exposure therapy for anxiety disorders in patients currently using D2 receptor antagonists. Recently, it was shown that patients with schizophrenia exhibit extinction recall deficits (43), an effect that could be attributed, in part, to the use of medications that block D2 receptors (44). Whether chronic use of these medications reduces their potential to impair extinction learning, however, remains to be determined.

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Supplementary material cited in this article is available online.

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