No evidence that post-training D2R dopaminergic drug administration affects differential fear responding in rats

Natalie Schroyens[a,b], Laura Vercammen[a,b,c], Burcu Özcan[a], Victoria Aurora Ossorio Salazar[b,c], Jonas Zaman[d], Dimitri De Bundel[e], Tom Beckers[a,b]*, Laura Luyten[a,b]*

[a] KU Leuven, Centre for the Psychology of Learning and Experimental Psychopathology, Faculty of Psychology and Educational Sciences, Tiensestraat 102 box 3712, 3000 Leuven, Belgium;

[b] KU Leuven, Leuven Brain Institute, O&N V Herestraat 49 box 1020, 3000 Leuven, Belgium;

[c] KU Leuven, Laboratory of Biological Psychology, Faculty of Psychology and Educational Sciences, Tiensestraat 102 box 3714, 3000 Leuven, Belgium;

[d] KU Leuven, Health Psychology, Tiensestraat 102 box 3726, 3000 Leuven, Belgium

[e] Research Group Experimental Pharmacology, Department of Pharmaceutical Sciences, Center for Neurosciences, Vrije Universiteit Brussel, 1090 Brussel, Belgium.

*Both authors contributed equally to this work

Corresponding authors

Tom Beckers at KU Leuven, Tiensestraat 102 bus 3712, 3000 Leuven, Belgium

Laura Luyten at KU Leuven, Tiensestraat 102 bus 3712, 3000 Leuven, Belgium

Email addresses

laura.luyten@kuleuven.be (L. Luyten)

tom.beckers@kuleuven.be (T. Beckers)

Word, figure and table counts

Abstract: 180 words

Article: 5353 words

Figure: 4

Tables: 2

Supplement: 1

Conflict of interest

The authors have no conflict of interest to disclose.

Abstract

The neurotransmitter dopamine plays an important role in the processing of emotional memories, and prior research suggests that dopaminergic manipulations immediately after fear learning can affect the retention and generalization of acquired fear. The current study focuses specifically on the role of dopamine D2 receptors (D2Rs) in differential fear responding in male Wistar rats. In a series of five experiments, D2R dopaminergic drugs were injected systemically after differential cued fear conditioning. One day later, the differential expression of acquired fear was assessed. All five experiments involved the administration of the D2R agonist quinpirole at different doses or saline (*n* = 12, 16 or 44 rats/treatment condition). Additionally, one of the studies administered the D2R antagonist raclopride (*n* = 12 rats/treatment condition). We found no indications for an effect of quinpirole or raclopride on differential fear responding during a later drug-free test. In addition, there were no effects of treatment on baseline freezing levels (i.e., before tone onset) or freezing during the conditioned tones (CS+) at test. We did observe acute behavioral effects of both drugs on a locomotor activity test.

Keywords

differential cued fear conditioning, generalization, rats, dopamine, quinpirole, raclopride

Author contributions

Conceptualization: N.S., T.B. and L.L.; Data curation: N.S.; Formal analysis: N.S.; Funding acquisition: N.S., T.B. and L.L.; Investigation: N.S., L.V., B.Ö. and V.A.O.S.; Methodology: N.S., J.Z., D.D., T.B. and L.L.; Project administration: T.B. and L.L.; Resources: T.B.; Supervision: T.B. and L.L.; Validation: N.S.; Visualization: N.S. and L.L.; Writing – original draft: N.S.; Writing - review & editing: N.S., L.V., B.Ö., V.A.O.S., J.Z., D.D., T.B. and L.L.

Acknowledgements

We acknowledge the financial support of the European Research Council (ERC Consolidator Grant to T. Beckers, grant number 648176), the Research Foundation – Flanders, Belgium (FWO Doctoral Fellowship to N. Schroyens, grant number 1114018N) and KU Leuven Research Grant C16/19/002 (to T. Beckers and L. Luyten). A previous version of this paper was published on bioRxiv (https://www.biorxiv.org/content/10.1101/2022.02.02.478781).

65 **1. Introduction**

66 The neurotransmitter dopamine has been shown to play a role in the processing of emotional
67 memories (Iemolo et al., 2015). For instance, genetic and pharmacological studies suggest that
68 activation of dopaminergic D1 and D2 receptors immediately after Pavlovian conditioning or inhibitory
69 avoidance training is required for subsequent expression of acquired fear (Castellano et al., 1991;
70 Fadok et al., 2009; Greba et al., 2001; LaLumiere et al., 2004; Manago et al., 2008). In addition,
71 dopamine has been found to modulate long-term potentiation (Bissière et al., 2003; Frey et al., 1991;
72 Lemon and Manahan-Vaughan, 2006; Rosenkranz and Grace, 2002; Rossato et al., 2009), a form of
73 synaptic plasticity that is assumed to be essential for memory formation (Lisman and Grace, 2005).
74 The focus of the present paper is on the effect of dopaminergic signaling after fear conditioning with
75 two cues, differentially predicting safety (CS-) or threat (CS+), on subsequent differential fear
76 responding to those cues.

77 Although most studies regarding the influence of dopamine on fear memory processing have
78 focused on its effects on memory retention specifically, more recent studies have also investigated
79 dopamine's role in generalization of fear towards safe or novel stimuli after contextual, cued, or
80 differential cued fear conditioning (De Bundel et al., 2016; Jo et al., 2018; Jones et al., 2015; Sarinana
81 et al., 2014; Zweifel et al., 2011). Investigating if and how dopaminergic manipulations during or after
82 learning affect the retention and spreading of fear towards safe situations is highly relevant to
83 understand the neurobiology of anxiety, given that fear generalization is often found to be elevated
84 in patients with anxiety disorders (Hermans et al., 2013; Lee et al., 2016).

85 Prior research indicated that knock-out mice lacking hippocampal dopamine D1 receptors
86 (D1Rs) exhibit equal levels of freezing during exposure to a novel context as to a context that was
87 previously paired with shock (Sarinana et al., 2014). In addition, using differential cued fear
88 conditioning procedures in mice, it has been shown that differential responding between CS+ and CS-
89 can be increased by optogenetic stimulation of dopaminergic neurons during conditioning (Jo et al.,
90 2018) or by systemic injection of a D2R agonist immediately after conditioning (De Bundel et al., 2016).

91 The latter study, by De Bundel and colleagues, found the dopaminergic effect to be bidirectional, with
92 post-learning injection or intra-amygdala infusion of a D2R antagonist impairing differential
93 responding. A PET imaging study in humans (Frick et al., 2021) found evidence consistent with these
94 findings in mice. Likewise, recent rat studies (often with more elaborate behavioral procedures that
95 also included reward cues, or using compound or contextual stimuli, rather than simple discrimination
96 learning with a tone CS+ and CS-, (Colucci et al., 2019; Moaddab and McDannald, 2021; Ng et al., 2018;
97 Yau and McNally, 2022)) have provided accumulating evidence for the involvement of dopamine
98 signaling in the discrimination between danger and safety. The pharmacological and recording
99 techniques that were used in these rat studies did, however, not allow for conclusions regarding the
100 specific involvement of D2 receptors.

101 The abovementioned neurobiological studies have proposed a range of different mechanisms
102 that may underlie dopamine's effect on the spreading of fear towards safe situations. For example, it
103 has been suggested that the role of dopamine in the encoding of novel *perceptual* information may
104 lie at the basis of its effects on fear generalization (Sarinana et al., 2014). Alternatively, it has been
105 proposed that the generalized fear observed in knockout mice with impaired dopaminergic signaling
106 can be attributed to inadequate learning of CS-US *associations* due to aberrant salience detection
107 (Zweifel et al., 2011) and the erratic assignment of negative valence to both the CS+ and CS- during
108 learning (Jo et al., 2018). Yet another possible way in which dopamine may affect differential fear
109 responding between stimuli that predict danger versus safety is via enhancement of the *stabilization*
110 or 'consolidation' of acquired fear memory (De Bundel et al., 2016). Related to this idea, it has been
111 suggested that ensuring adequate stabilization of the fear memory trace may be required for the
112 establishment of a sufficiently precise memory to prevent generalization to non-threatening
113 situations (Robertson, 2018). Overall, dopamine's effects on fear memory generalization have thus
114 been attributed to several aspects of learning (i.e., encoding of perceptual information, association
115 formation, differential learning), at different points in time (i.e., during versus shortly after learning),

116 and via various hypothetical mechanisms (i.e., by coding salience or valence, or by enhancing memory
117 stabilization).

118 Our ultimate goal was to study, in rats rather than mice, to what extent dopamine D2 signaling
119 after differential fear learning plays a role in subsequent differential fear responding and, if so, which
120 aspect(s) of learning (perceptual, associative, and/or differential) would be involved. To this aim, we
121 first embarked on a conceptual replication of published findings in mice by De Bundel and colleagues
122 (De Bundel et al., 2016), in which it was shown that systemic injection of a D2R agonist or antagonist
123 immediately after differential cued fear conditioning can attenuate or enhance subsequent fear
124 generalization (i.e., enhanced fear responding to a safe cue in a new context), respectively. One
125 advantage of administering dopaminergic drugs *after* learning is that it allows to distinguish between
126 the importance of dopaminergic signaling during versus after learning. It may thus provide useful
127 insight into the role of dopamine during the memory stabilization phase specifically.

128 While most prior evidence in non-human animals regarding the role of dopamine D2Rs in CS+
129 versus CS- discrimination has been obtained in mice, the translation of those findings to rats remains
130 unclear. Despite the many similarities between both species, rats and mice are characterized by
131 important differences in functional brain anatomy and physiology, including neurochemistry
132 (Ellenbroek and Youn, 2016). Rats are an important model organism for behavioral neuroscience, in
133 particular in the study of fear learning and interventions to influence fear memory specificity.

134 We describe a series of five experiments (Exp. 1-5) that investigated the behavioral effects of
135 post-training dopaminergic drug injection on subsequent differential fear responding in rats. These
136 experiments were preceded by a series of protocol optimization studies (reported as Exp. A-C) that
137 provided insight into the effects of shock intensity and testing order on differential fear responding.
138 In a last experiment (Exp. 6), the acute effects on locomotor activity of the drugs used throughout our
139 experiments was evaluated (i.e., 0.3 mg/kg raclopride and 0.05, 1, and 5 mg/kg quinpirole). All details
140 regarding Experiments A-B-C and Experiment 6 can be found in the ***Supplement***.

141

2. Materials and Methods

2.1. Preregistration, data and code availability

All study protocols and statistical analyses were preregistered on the Open Science Framework (OSF) at https://osf.io/kzcyt. Relevant datasets, analysis scripts, and results of all preregistered statistical analyses can be found there as well.

2.2. Subjects

Male Wistar rats (Janvier Labs, Le Genest-Saint-Isle, France) were housed in groups of 3 or 4 rats per cage on a 12 h/12 h day-night cycle (lights on at 8 am). Cage enrichment, in the form of a tunnel hanging from the top grid, was provided and with food and water available ad libitum. Experiments were carried out between 9 am and 5 pm. All experiments were approved by the KU Leuven animal ethics committee (in accordance with the Belgian Royal Decree of 29/05/2013 and European Directive 2010/63/EU).

2.3. Drug administration

Depending on the experiment (see ***Table 1***), raclopride HCl (RACLO; 0.3 mg/kg; Santa Cruz Biotechnology), (-)-quinpirole HCl (QUIN; 0.05 mg/kg, 1 mg/kg or 5 mg/kg; Tocris), or saline was administered immediately after conditioning. Both drugs were dissolved in saline (0.9%, w/v) and injected intraperitoneally at a volume of 1 ml/kg. Drug doses were based on the study in mice by De Bundel and colleagues (De Bundel et al., 2016). Similar doses have previously been used in rats (de Lima et al., 2011; Mueller et al., 2010).

2.4. Differential cued fear conditioning

Four Med Associates test chambers were used to train and test the animals. Details can be found in the **Supplement**. Fear conditioning in context A (CTX-A) consisted of presentations of a

168 dangerous tone (5 x CS+, always co-terminated with a foot shock (US)) and a safe tone (5 x CS-, never
169 paired with the shock) in a semi-random order (see ***Table 1*** and ***Fig. 1***). Next, rats received an
170 intraperitoneal injection of saline, quinpirole (0.05, 1 or 5 mg/kg) or raclopride (0.3 mg/kg) at a volume
171 of 1 ml/kg. Twenty-four hours after training, rats underwent a drug-free test in a novel context (CTX-
172 B), during which the CS+ and CS- were repeatedly presented (4 times each) in a semi-random order.
173 Depending on the experiment, additional drug-free test sessions were performed, in either CTX-A or
174 CTX-B.
175
176 **2.5. Statistical analyses**
177 Percentage freezing during tones was scored by an experienced observer, blinded to
178 treatment condition, tone frequency and CS type (CS- or CS+). Average % freezing during CS+ and CS-
179 was calculated for each rat and for each test session. According to a preregistered exclusion criterion,
180 rats showing less than 10% freezing during CS+ presentations on average at Test 1 were excluded from
181 the analysis, as these low freezing scores may indicate a lack of acquisition of the CS-US association.
182 Analyses were performed with and without those rats. R was used for frequentist and Bayesian
183 analyses. For Bayesian analyses, we used a default Cauchy prior on the standardized effect size with a
184 scale of 0.707 in the BayesFactor package. Bayes factors indicate how likely the obtained data are
185 under H_A, relative to H_0 (i.e., BF_{10}).
186 Primary analyses for Experiments 1-5 (secondary analyses are described in the ***Supplement***)
187 included mixed ANOVAs with between-subjects factor Treatment (saline or drug) and within-subjects
188 factor CS type (CS- or CS+) to investigate whether treatment affected differential fear responding
189 during tests. Mixed ANOVAs were performed for each drug (versus saline) separately. The Bayes factor
190 (BF) quantifying the evidence for including the Treatment x CS type interaction was calculated by
191 dividing the BF for the model with interaction and main effects by the BF for the model with only main
192 effects, (see doi 10.31234/osf.io/spreb; referred to as 'BFinclusion across matched models'). One-

193 sided paired t-tests assessed whether freezing during CS+ was higher compared to CS- for each
194 treatment condition.

195 Primary statistical analyses for Experiment 5 were the same as for Exp. 1-4, but in contrast
196 with the previous experiments, this study used a sequential design, for which the total sample size
197 depended on the value of the Bayes factor for the CS type x Treatment interaction observed during
198 interim assessments (see ***Table 2***). The first assessment took place after testing a total of 40 rats (batch
199 1; n = 20 rats/treatment condition) and the second assessment after testing a total of 64 rats (batch
200 2; 24 additional rats; n = 12 rats/treatment condition). The maximum sample size (N = 88) was
201 determined based on frequentist power calculations using the results obtained in Experiment 4 (CS
202 Type x Treatment interaction; power = 92%, alpha = .05, partial eta squared = .13).

3. Results

All details regarding the behavioral protocol optimization studies (Experiments A-B-C) that preceded Experiments 1-5 can be found in the ***Supplement***.

3.1. Experiment 1: No evidence for an effect of post-training injection of raclopride (0.3 mg/kg) or quinpirole (1 mg/kg) on subsequent differential fear responding

Based on the results of De Bundel and colleagues in mice (De Bundel et al., 2016), we hypothesized that 0.3 mg/kg raclopride or 1 mg/kg quinpirole would increase or decrease differential fear responding at test, respectively.

Primary analyses provided no evidence for Treatment (dopaminergic drug versus saline) x CS type (CS- versus CS+) interactions at Test 1 (RACLO: $F(1, 22) = .04$, $p = .847$, $\eta 2p < .01$, BF = .38; QUIN1: $F(1, 22) = .20$, $p = .662$, $\eta 2p = .01$, BF = .38). One-sided paired t-tests suggested higher freezing during CS+ than CS- presentations at test in saline control rats ($t(11) = -2.42$, $p = .017$, $d = -.64$, BF = 4.35), and raclopride rats ($t(11) = -2.6$, $p = .012$, $d = -.67$, BF = 5.66), but not in quinpirole rats ($t(11) = -1.38$, $p = .098$, $d = -.33$, BF = 1.09).

Although freezing during CS+ presentations at Test 1 was on average lower in quinpirole rats than in saline control rats (see ***Fig. 2a***), two-sided t-tests (preregistered secondary analysis, see OSF) suggested no evidence for differences in freezing during CS+ presentations between treatment conditions (i.e., QUIN1 versus SAL or RACLO versus SAL). In addition, there was no evidence for differences in baseline freezing (i.e., before onset of the first tone, see OSF) or the proportion of generalizers between treatment conditions.

During Test 2, which took place in the training context on Day 3 (***Fig. 2b***), there was again no evidence for Treatment x CS type interactions (RACLO: $F(1, 22) = .67$, $p = .421$, $\eta 2p = .03$, BF = .45; QUIN1: $F(1, 22) = 1.25$, $p = .276$, $\eta 2p = .05$, BF = .63) and one-sided paired t-tests suggested higher freezing during CS+ than CS- presentations at test in saline control rats ($t(11) = -2.09$, $p = .03$, $d = -.60$,

229 BF = 2.75), raclopride rats (t(11) = -2.31, p = .021, d = -.29, BF = 3.75), and in quinpirole rats (t(11) = -
230 3.75, p = .002, d = -1.14, BF = 30.08). Secondary analyses suggested no evidence for differences in
231 freezing during CS+ presentations between treatment conditions (i.e., QUIN1 versus SAL or RACLO
232 versus SAL). In addition, there was no evidence for differences in baseline freezing (i.e., before onset
233 of the first tone), average freezing during CS+ presentations, or the proportion of generalizers
234 between treatment conditions.
235
236 ***3.2. Experiments 2 & 3: No evidence for an effect of post-training injection of quinpirole (0.05 or 5***
237 ***mg/kg) on subsequent differential fear responding***
238 Locomotor activity studies in rats suggest that quinpirole differentially affects presynaptic
239 versus postsynaptic D2 receptors depending on the applied dose (Eilam and Szechtman, 1989;
240 Hartesveldt et al., 1992; Horvitz et al., 2001; Kropf and Kuschinsky, 1991). Experiments 2 and 3 aimed
241 to assess how different doses of quinpirole influence differential fear responding. We hypothesized
242 that a low dose of quinpirole (0.05 mg/kg) would decrease differential responding (due to the
243 assumed activation of presynaptic autoreceptors), whereas a higher dose of 5 mg/kg would increase
244 differential responding (due to the assumed activation of postsynaptic D2Rs). Experiments 2 and 3
245 adopted the exact same behavioral procedures and drug doses (apart from additional tests 2 and 3),
246 and are therefore presented together.
247 In Experiment 2 (***Fig. 3a-b***), three rats (1 SAL and 2 QUIN5 rats) were excluded due to the
248 preregistered exclusion criterion of showing less than 10% freezing on average during CS+
249 presentations at Test 1, so all preregistered analyses were performed with and without those three
250 rats. Statistical results are shown here for the sample in which the exclusion criterion was applied, and
251 results for both samples (i.e., with and without exclusions) are provided only when both analyses
252 reached different overall conclusions. All results can be found at https://osf.io/kzcyt.
253 Primary analyses suggested no evidence for Treatment (dopaminergic drug versus saline) x CS
254 type (CS- versus CS+) interactions (QUIN (0.05 mg/kg): F(1, 21) = 2.01, p = .171, η2p = .09, BF = .89;

QUIN (5 mg/kg): $F(1, 19) = .53$, $p = .477$, $\eta 2p = .03$, BF = .48) at Test 1. Contrary to Experiment 1, in which the same behavioral procedures were used for training and testing, there was no evidence for more freezing during CS+ compared to CS- presentations at test in saline control rats ($t(10) = -.39$, $p = .353$, $d = -.12$, BF = .40). A similar pattern of responding was observed in rats treated with the high dose of quinpirole ($t(9) = -1.34$, $p = .106$, $d = -.51$, BF = 1.1). However, rats that received the low quinpirole dose did show statistically significant differential responding between tones ($t(11) = -2.67$, $p = .011$, $d = -.59$, BF = 6.25). Contrary to our hypotheses, neither of the quinpirole doses thus induced a statistically significant effect on differential fear responding. If anything, the low quinpirole dose (0.05 mg/kg) seemed to *enhance*, rather than impair, differential responding, although this mere trend did not reach statistical significance.

Average freezing during CS+ presentations at Test 1 was numerically higher in the 'low quinpirole' condition than in saline control rats (see ***Fig. 3a***), but this between-group difference did not receive statistical support when excluding the saline rat showing on average less than 10% freezing during CS+ presentations ($t(21) = 1.79$, $p = .088$, $d = .75$, BF = 1.16). When including all rats, the effect was statistically significant, although the obtained evidence was indifferent according to the Bayesian analysis ($t(22) = 2.1$, $p = .047$, $d = .86$, BF = 1.72). Furthermore, no between-group differences were found in baseline freezing (i.e., before onset of the first tone) or the proportion of generalizers.

During Test 2, which took place in the training context on Day 27 (***Fig. 3b***), there was again no evidence for Treatment x CS type interactions (QUIN (0.05 mg/kg): $F(1, 21) = .06$, $p = .801$, $\eta 2p < .01$, BF = .37; QUIN (5 mg/kg): $F(1, 19) < .01$, $p = .945$, $\eta 2p < .01$, BF = .38). Furthermore, both QUIN0.05 and saline rats now showed significantly higher freezing during CS+ than CS- ($t(10) = -2.96$, $p = .007$, $d = -.77$, BF = 8.98; $t(11) = -3.85$, $p = .001$, $d = -1.20$, BF = 34.85, respectively), whereas QUIN5 rats did not show statistically significant differential responding ($t(9) = -1.61$, $p = .071$, $d = -.63$, BF = 1.51). In addition, secondary analyses suggested no evidence for differences in baseline freezing (i.e., before onset of the first tone), average freezing during CS+ presentations, or the proportion of generalizers between treatment conditions.

281 In Experiment 3 (***Fig. 3c-e***), eight rats (2 SAL, 2 QUIN0.05, and 4 QUIN5 rats) were excluded
282 due to the preregistered exclusion criterion of showing less than 10% freezing on average during CS+
283 presentations at Test 1, so all preregistered analyses were performed with (N = 36) and without (N =
284 28) those eight rats.

285 Primary analyses suggested no evidence for Treatment (dopaminergic drug versus saline) x CS
286 type (CS- versus CS+) interactions (QUIN (0.05 mg/kg): $F(1, 18) = .55$, $p = .468$, $\eta 2p = .03$, BF = .47;
287 QUIN (5 mg/kg): $F(1, 16) = .88$, $p = .362$, $\eta 2p = .05$, BF = .54) at Test 1 (***Fig. 3c***). One-sided paired t-tests
288 suggested higher freezing during CS+ than CS- presentations at test in all treatment conditions (saline
289 control rats: ($t(9) = -3.64$, $p = .003$, $d = -.42$, BF = 20.35; QUIN (0.05 mg/kg): $t(9) = -2.82$, $p = .01$, $d = -$
290 $.76$, BF = 7.03; QUIN (5 mg/kg): $t(7) = -3.52$, $p = .005$, $d = -.98$, BF = 13.27). However, differential
291 responding between tones was smaller (and non-significant) when including all QUIN5 rats ($t(22) =$
292 $.08$, $p = .933$, $d = .03$, BF = .37), probably due to the fact that two rats showed no freezing during any
293 of the tones, and thus, no differential responding.

294 During Test 2 (***Fig. 3d***), which took place in the testing context (CTX-B) on Day 28, there was
295 again no evidence for Treatment x CS type interactions (QUIN0.05: $F(1, 18) = .57$, $p = .458$, $\eta 2p = .03$,
296 BF = .47; QUIN5: $F(1, 16) = .03$, $p = .861$, $\eta 2p < .01$, BF = .39). Saline control rats ($t(9) = -1.25$, $p = .121$,
297 $d = -.24$, BF = .99) and QUIN5 rats ($t(7) = -1.37$, $p = .107$, $d = -.77$, BF = 1.18) did no longer show
298 statistically significant differential responding between CS+ and CS-, whereas QUIN0.05 rats did show
299 significant differential responding between tones still ($t(9) = -2.76$, $p = .011$, $d = -.78$, BF = 6.47).
300 However, when including all rats, all groups showed statistically significant differential responding
301 (SAL: $t(11) = -1.86$, $p = .045$, $d = -.33$, BF = 2.01; QUIN0.05: $t(11) = -2.48$, $p = .015$, $d = -.61$, BF = 4.72;
302 QUIN5: $t(11) = -2.11$, $p = .029$, $d = -.65$, BF = 2.83).

303 During Test 3 (***Fig. 3e***), which took place in the training context (CTX-A) on Day 29, there was
304 no evidence for Treatment x CS type interactions (QUIN0.05: $F(1, 18) < .01$, $p = .982$, $\eta 2p < .01$, BF =
305 .41; QUIN5: $F(1, 16) = .74$, $p = .402$, $\eta 2p = .04$, BF = .58) and there was no statistical evidence for
306 differential responding between tones in any of the treatment conditions (SAL: $t(9) = -1.45$, $p = .09$, d

= -.5, BF = 1.25; QUIN0.05: t(9) = -1.48, p = .086, d = -.53, BF = 1.29; QUIN5: t(7) = .08, p = .531, d = .04, BF = .32).

Secondary analyses suggested no evidence for between-group differences in average freezing during CS+ presentations, baseline freezing, (i.e., before onset of the first tone), or the proportion of generalizers during any of the test sessions.

3.3. Experiment 4: No evidence for an effect of post-training injection of quinpirole (0.05 or 1 mg/kg) on differential fear responding when using higher shock intensity during training

Experiment 4 aimed to investigate whether quinpirole injection can enhance differential fear responding at a drug-free test when using a higher shock intensity during training, which we expected would result in limited differential responding in saline control rats at test (based on the results of Experiment C, see ***Supplement***). Although we initially hypothesized that the low dose of quinpirole (i.e., 0.05 mg/kg) would decrease differential responding, the results of Exp. 2 suggested that, if anything, this dose may *increase* differential responding. Therefore, the low dose was also used in this study.

Saline control rats showed differential responding between tones at test (t(15) = -1.8, p = .046, d = -.35, BF = 1.78). Numerically even higher levels of differential responding were observed in quinpirole rats, especially in rats that received the dose of 1 mg/kg (QUIN0.05: t(15) = -2.34, p = .017, d = -.44, BF = 4.01; QUIN1: t(15) = -5.04, p < .001, d = -.86, BF = 410.27) (***Fig. 4a***). There was, however, no reliable evidence for Treatment (dopaminergic drug versus saline) x CS type (CS- versus CS+) interactions (QUIN (0.05 mg/kg): F(1, 30) = .25, p = .622, η2p = .01, BF = .36; QUIN (1 mg/kg): F(1, 30) = 3.28, p = .08, η2p = .1, BF = 1.08).

Secondary analyses suggested no evidence for between-group differences in average freezing during CS+ presentations, baseline freezing, (i.e., before onset of the first tone), or the proportion of generalizers.

3.4. Experiment 5: No evidence for an effect of post-training injection of quinpirole (1 mg/kg) on differential fear responding when using higher shock intensity during training and large sample size

In Experiment 4 we observed numerically higher levels of differential responding in rats that received 1 mg/kg quinpirole. Therefore, we aimed to investigate this effect further by taking into account the size of the effect observed in Exp. 4 and using a sample size that is required to detect such an effect with a power of more than 90%. Experiment 5 followed the procedures from Experiment 4, but only included two of the groups from Experiment 4 (i.e., quinpirole 1 mg/kg versus saline). The experiment had a sequential design with a predetermined maximum sample size, and was performed in three separate batches. Given that we did not observe the predefined Bayes factor for the CS type x Treatment interaction after testing batch 1 or 2, the maximum sample size of 88 rats was tested.

One saline rat was excluded from analyses because it showed no freezing at all during testing (i.e., 0% freezing; exclusion due to preregistered criterion). In addition, the excluded rat did not show any freezing during training either, and a lack of responding at the time the shocks should have been given, so we decided not to perform analyses with this rat included. Note that we hereby deviate from the preregistration because we planned to perform the analyses with *all* subjects included, apart from applying the preregistered exclusion criterion. A total of 87 rats was thus included in the analyses described below.

There was no evidence for a CS type x Treatment interaction ($F(1, 85) = .17$, $p = .68$, $\eta 2p < .01$, $BF = .23$), and the obtained Bayes factor suggested substantial evidence for the absence of an interaction (Wetzels et al., 2011) (***Fig. 4b***). One-sided paired t-tests suggested statistically significant differential responding between CS+ and CS- in saline control rats ($t(42) = -4.93$, $p < .001$, $d = -.62$, $BF = 3032.43$) and quinpirole rats ($t(43) = -4.31$, $p < .001$, $d = -0.56$, $BF = 502.73$).

Secondary analyses suggested no difference in the proportion of generalizers between saline and QUIN1 rats ($X^2(1) < .01$, $p = 1$, $BF = .27$). In addition, the generalization index was not significantly lower in QUIN1 rats than in saline rats ($t(85) = .76$, $p = .777$, $d = .16$, $BF = .14$).

358 As mentioned before, we carried out an additional study (Experiment 6) to assess the acute
359 effects of raclopride and quinpirole on locomotor activity. All details can be found in the ***Supplement***.
360 In brief, we observed locomotor effects that were largely in line with prior research. Importantly, as
361 described in the literature (Horvitz et al., 2001), we found that 1 mg/kg quinpirole first suppressed
362 locomotor activity, followed by a statistically significant increase in locomotor activity compared to
363 saline (***Supplement, Fig. S4***). This indicates, that in a different task than the differential fear
364 conditioning procedure, the drug did produce the expected acute behavioral effect at the applied dose
365 in male Wistar rats.
366

367 **4. Discussion**

368 In a series of five experiments, the dopamine D2 receptor (D2R) agonist quinpirole and D2R
369 antagonist raclopride were injected systemically after differential cued fear conditioning in rats. One
370 day after treatment, differential expression of acquired fear was assessed by comparing average %
371 freezing during CS- versus CS+ presentations in a new context. Our studies aimed to evaluate in rats
372 the role of dopaminergic signaling in fear memory processing as established in mice, emulating the
373 protocol of a previously published study by De Bundel and colleagues (2016). They showed that post-
374 training systemic injection in mice of quinpirole or raclopride affects differential responding to a
375 learned danger cue (CS+) versus safety cue (CS-). Based on those published results, we expected
376 quinpirole (1 or 5 mg/kg) to increase differential fear responding, and raclopride (0.3 mg/kg) to impair
377 differential fear responding. In addition, we initially hypothesized that a low dose of quinpirole (0.05
378 mg/kg) would decrease differential responding due to the assumed activation of presynaptic
379 autoreceptors. We found no evidence in rats for an effect of systemically administered quinpirole or
380 raclopride on differential fear responding at test. In addition, there were no effects of treatment on
381 freezing levels during CS+ presentations or during the baseline period at test (i.e., before tone onset)
382 in any of the 5 experiments. We did, however, replicate previously described acute behavioral effects
383 of quinpirole (0.05, 1 and 5 mg/kg) and raclopride (0.3 mg/kg) during a locomotor activity test.

384 In the light of the inconsistent results between our studies (in rats) and the published studies
385 by De Bundel and colleagues (in mice) (De Bundel et al., 2016), a number of differences between
386 these two studies should be acknowledged. First and foremost is of course the difference in species,
387 given the reported species-dependent variations in ventral tegmental area dopamine signaling
388 (Courtney et al., 2012). The fact that DA neurons in mice and rats show different sensitivity to D2-
389 mediated inhibition could have important implications for presynaptic or postsynaptic effects of D2R
390 modulation. Similarly, differences in DA agonist-induced locomotor effects, prepulse inhibition, or
391 yawning between rats and mice have been described (Halberda et al., 1997; Li et al., 2010; Ralph and
392 Caine, 2005). Another relevant question is whether mice and rats express fear in the same manner

393 (e.g., comparable levels of freezing versus escape behavior) during presentations of tones with
394 different frequencies. If existent, between-species differences in prototypical fear behavior may
395 obviously complicate the comparison of studies in rats versus mice when differential freezing
396 responses are used as a measure of generalization.

397 Furthermore, and as expected, pilot studies in our lab indicated the need for different
398 behavioral parameters than those used in the mouse studies (De Bundel et al., 2016) in order to allow
399 for the study of differential fear responding in rats (e.g., tones of 5 and 10 kHz rather than 2.5 and 7.5
400 kHz, shocks of .5 s rather than 2 s, and intermixed rather than blocked testing). In addition, we opted
401 to leave out the habituation session prior to conditioning, and used a semi-random rather than a fixed
402 order of CS+ and CS- presentations during learning. Finally, our saline control animals showed, on
403 average, a different behavioral pattern than those in the De Bundel study. The control rats in most of
404 our dopamine studies (except for Experiment 2) showed statistically significant higher freezing during
405 CS+ presentations than during CS-, whereas the control mice in the prior quinpirole study showed a
406 lack of differential responding (see Fig. 1C of (De Bundel et al., 2016)). The observed variability in
407 freezing behavior of the control animals between some of our experiments could be perceived as a
408 limitation, but is probably a logical consequence of our attempt to tread a fine line, i.e., to create a
409 learning experience that still contained a degree of ambiguity (with the control animals of Experiment
410 2 showing statistically insignificant discrimination, and thus flipping to the other side than in
411 Experiments A, 1 and 3, where the effect was significant). As mentioned before, we did not want to
412 overtrain the animals in their discrimination between the CS+ and CS- (which could, for example, have
413 been achieved with multiple training days (Day et al., 2016)), nor did we want them to have
414 inadequate learning opportunities to discriminate between the CS+ and CS- (in such scenario, full
415 generalization between the CS+ and CS- might just reflect a perceptual discrimination deficit). Overall,
416 the observed behavioral variability between experiments is most likely not a major issue, given that
417 each of our experiments had its own appropriate saline control group.

418 As far as the effects of raclopride concern, our conclusions rely on a single study with a sample
419 size of 12 rats per treatment condition, yielding only anecdotal evidence for the absence of a CS type
420 x Treatment interaction effect. In contrast, we performed five studies with quinpirole, in which we
421 used three different doses of the drug (0.05, 1, or 5 mg/kg), two different shock intensities (.4 or 1
422 mA), and including a final study with a large sample size yielding a power of over 90% to detect an
423 effect if any. Moreover, Experiment 3 (N = 36) was a replication of Experiment 2 (N = 36) (except for
424 additional tests), and Experiment 5 (N = 88) was a replication of Experiment 4 (N = 48) (except for the
425 omission of the 0.05-mg/kg quinpirole group in Exp. 5). The final experiment provided substantial
426 evidence (Bayesian analyses) for the absence of an effect of quinpirole (1 mg/kg) on differential fear
427 responding. In other words, we have gathered evidence *against* an effect of quinpirole on differential
428 fear responding under the applied conditions in rats.
429 It should be noted that our studies are limited to the use of systemic drug injection and that
430 we did not directly target a particular brain region via, for example, drug infusion or optogenetic
431 manipulation. Given the apparent lack of generalizability to rats of the results of De Bundel et al.
432 (2016) of *systemic* application of dopaminergic drugs, a possible follow-up study to investigate the
433 involvement of post-learning D2R signaling in differential fear responding could include the use of
434 *intracerebral* drug administration. In this case, a replication in rats of previous experiments in which
435 the D2R antagonist raclopride was infused into the central or basolateral amygdala (see Fig. 2I and 3I
436 in (De Bundel et al., 2016)) would be optimal to inform about the replicability of the effect specifically
437 and the role of post-learning D2R signaling in differential fear responding in rats more generally.

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Tables

Exp.	N	Body weight	Acclim. period	Treatment*	Shock Intensity	Test 1 (Day 2, CTX-B) Order of tone presentations**	Test 2	Test 3
1	36	250 g	4 days	quinpirole 1 mg/kg raclopride .3 mg/kg	.4 mA	12112122	Day 3; CTX-A 21211212	/
2	36	321 g	4 days	quinpirole .05 mg/kg quinpirole 5 mg/kg	.4 mA	12112122	Day 27; CTX-A 21211212	/
3	36	317 g	4 days	quinpirole .05 mg/kg quinpirole 5 mg/kg	.4 mA	12112122	Day 28; CTX-B 21211212	Day 29, CTX-A 12211221
4	48	302 g	11 days	quinpirole .05 mg/kg quinpirole 1 mg/kg	1 mA	12211212	/	/
5	87	314 g	11 days	quinpirole 1 mg/kg	1 mA	12211212	/	/

562 ***Table 1. Study characteristics of Experiments 1-5.*** All experiments were preregistered on OSF at https://osf.io/kzcyt/registrations. Sample size (N) and average
563 body weight one day before training are shown. Acclim. period = interval between the rats' arrival in our animal facility and the start of handling (two
564 consecutive days of handling preceded the fear conditioning protocol). Rats underwent differential cued fear conditioning in context A (CTX-A), after which
565 they were injected with saline or a dopaminergic drug (quinpirole or raclopride). During subsequent drug-free sessions, they were tested in CTX-A or context
566 B (CTX-B).*All experiments contained a saline control group. **The order of the tones at test was counterbalanced (1 = CS+, 2 = CS- or 1 = CS-, 2 = CS+).

Batch	N	N_{total} (all batches combined)	Obtained power (alpha = .05)
1	40	40	.60
2	24	64	.80
3	24	88	.92

Table 2. Overview of the sequential design used for Experiment 5. The reported power was calculated based on the effect size for the CS Type x Treatment interaction observed in Experiment 4. Interim assessments – performed after collecting data for batch 1 and 2 – included the computation of the Bayes factor for the CS type x Treatment interaction (testing stopped if the obtained BF was larger than 10 or smaller than 1/10 at interim assessment).

Figures

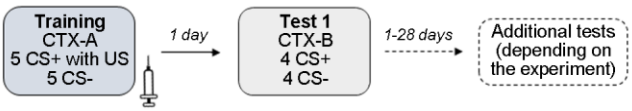
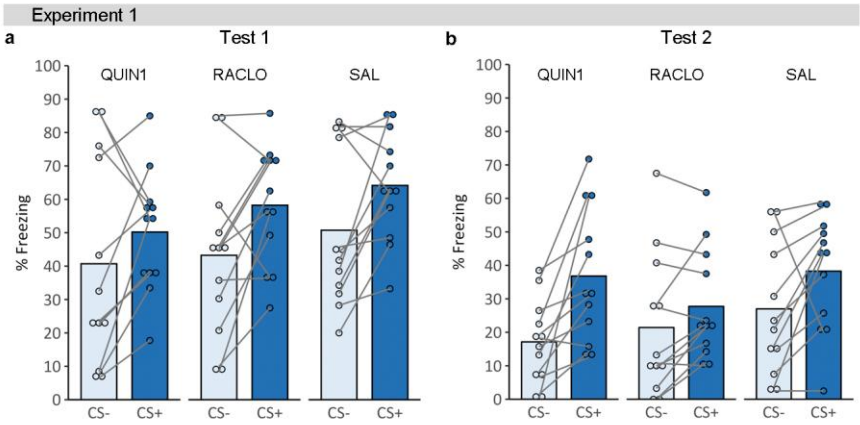


Fig. 1. Experimental procedure. Immediately after differential cued fear conditioning in context A (CTX-A), rats were systemically injected with saline, raclopride, or quinpirole (see ***Table 1*** for details). Twenty-four hours later, behavioral differentiation between CS+ and CS- was assessed in a novel context B (CTX-B, Test 1) using percentage of time spent freezing during tones as an outcome measure. Depending on the experiment, additional behavioral tests took place at least one day after Test 1.



587

588 ***Fig. 2. Experiment 1.*** Average % freezing per treatment condition during Test 1 (Day 2; CTX-B; panel

589 a) and Test 2 (Day 3; CTX-A; panel b). QUIN1 = quinpirole (1 mg/kg), RACLO = raclopride (0.3 mg/kg),

590 SAL = saline.

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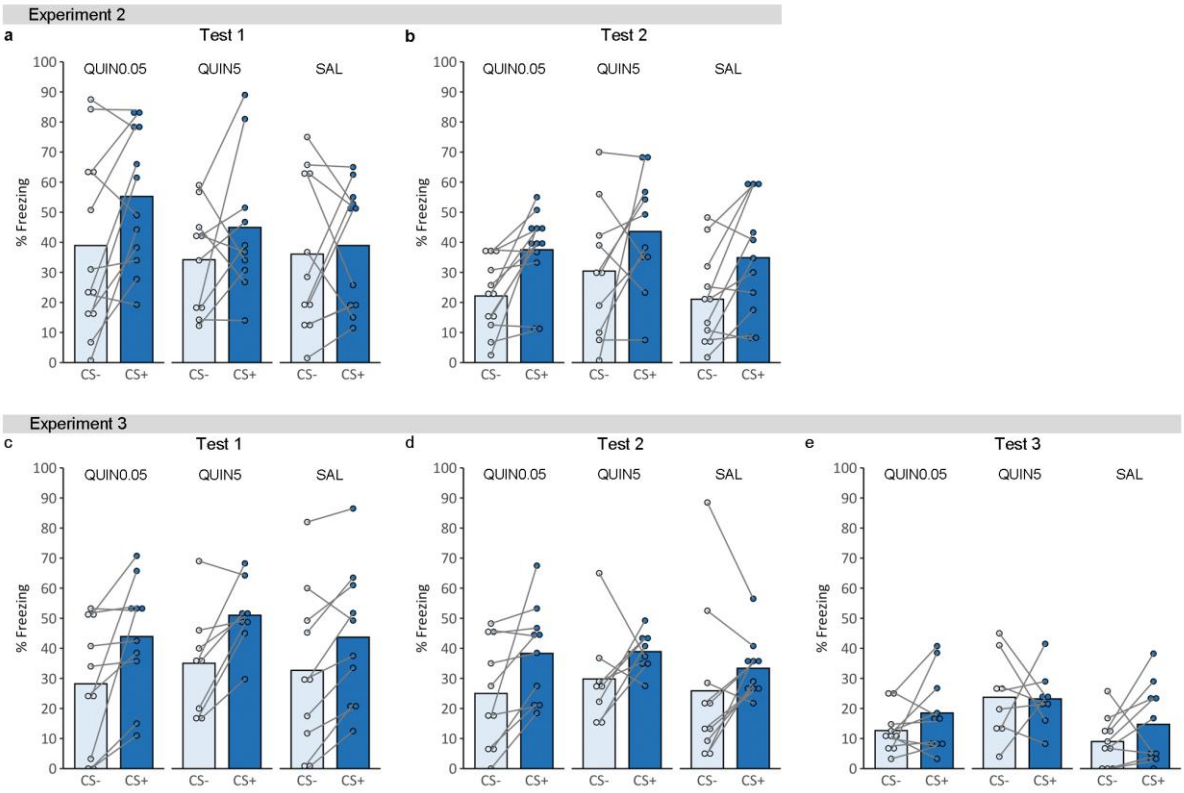

Fig. 3. Experiments 2 & 3. Average % freezing per treatment condition during Test 1 (Day 2; CTX-B; panel a) and Test 2 (Day 27; CTX-A; panel b) of Experiment 2, and during Test 1 (Day 2; CTX-B; panel c), Test 2 (Day 28; CTX-B; panel d), and Test 3 (Day 29; CTX-A; panel e) of Experiment 3. QUIN0.05 = quinpirole (0.05 mg/kg), QUIN5 = quinpirole (5 mg/kg), SAL = saline.

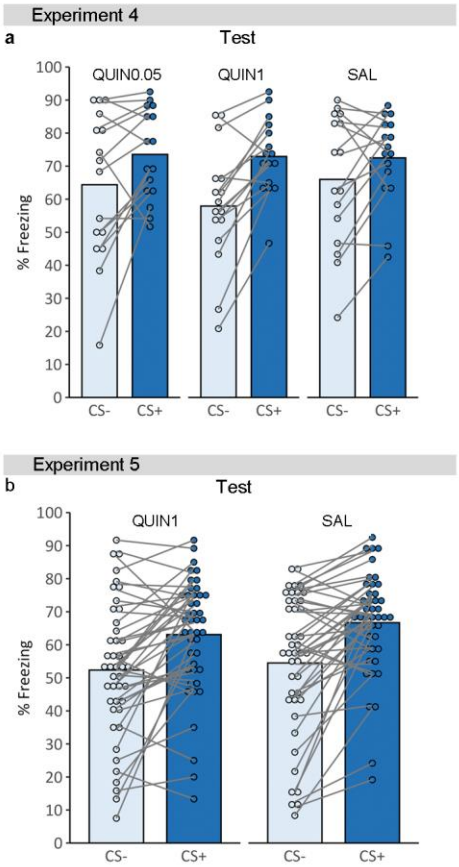

Fig. 4. Experiments 4 & 5. Average % freezing per treatment condition during testing (Day 2; CTX-B) in Experiment 4 (panel a) and Experiment 5 (panel b). QUIN0.05 = quinpirole (0.05 mg/kg), QUIN1 = quinpirole (1 mg/kg), SAL = saline.

No evidence that post-training D2R dopaminergic drug administration affects differential fear responding in rats

Natalie Schroyens, Laura Vercammen, Burcu Özcan, Victoria Aurora Ossorio Salazar, Jonas Zaman, Dimitri De Bundel, Tom Beckers, Laura Luyten

Supplementary Methods & Results

This Supplement contains details about the fear conditioning equipment and procedure. It also contains information related to three behavioral protocol optimization studies (i.e., Experiments A-C) which were carried out before the five psychopharmacological studies in the main text (i.e., Experiments 1-5), as well as details about secondary statistical analyses for those experiments. Finally, it contains information related to a locomotor activity study that assessed acute effects of quinpirole and raclopride (i.e., Experiment 6).

Supplementary Methods

Experiments A-B-C & Experiments 1-5

Apparatus

Four chambers (Contextual NIR Video Fear Conditioning System for Rats, Med Associates Inc., St. Albans, VT, USA, 30 cm (L) x 25 cm (W) x 21 cm (H)) illuminated by infrared and white light (45 lux), and with built-in ventilation fans (±67 dB) were used. Fear conditioning took place in context A (CTX-A), consisting of a grid floor (19 rods of 4.8 mm diameter, 16 mm center to center) and a triangular-shaped black insert. Before and/or after each behavioral session, the context was cleaned and scented with diluted cleaning product (5.55 % in water). The first test session took place in context B, consisting of a white plastic floor, a white plastic curved back wall, illuminated with infrared light only, and cleaned and scented with a different cleaning product.

Differential cued fear conditioning

Rats were placed in context A (CTX-A) and, after a baseline period of 120 s, two tones (5 and 10 kHz, counterbalanced) were presented five times each in semi-random order (80 dB, 30 s, ITI = 60 – 160 s, order of tone presentations = CS+, CS-, CS-, CS+, CS-, CS+, CS-, CS-, CS+, CS+). In Experiments B, C, 4, and 5, the rise time of the tones was changed from .1 s to 1s to prevent interference with freezing behavior due to startle responding at tone onset. One tone (i.e., CS+) always co-terminated

with a foot shock (.5 s, .4, .8 or 1 mA, see ***Tables 1 & S1*** for shock intensities), while the other tone (i.e., CS-) was never paired with shock. Sixty seconds after the last tone, rats were returned to their home cage. Depending on the experiment, rats received an intraperitoneal injection of saline, quinpirole (0.05, 1 or 5 mg/kg) or raclopride (0.3 mg/kg) at a volume of 1 ml/kg (see ***Table 1***).

Test of differential fear responding

Twenty-four hours after training, rats underwent a drug-free test in a novel context (CTX-B). After a baseline period of 180 s, the CS+ and CS- were repeatedly presented in a semi-random and counterbalanced order (4 presentations of each tone, 80 dB, 30 s, ITI = 80 – 140 s, see ***Tables 1 & S1*** for the order of tone presentations). Thirty seconds after the last tone, rats were returned to their home cage.

Depending on the experiment, additional drug-free test sessions were performed (see ***Tables 1 & S1***). Two (Exp. 1) or 26 days (Exp. 2) after training, rats were re-exposed to the training context (CTX-A, Test 2), and, after a baseline period of 180 s, the CS+ and CS- were repeatedly presented in a semi-random and counterbalanced order (4 presentations of each tone, 80 dB, 30 s, ITI = 80 – 140 s). Thirty seconds after the last tone, rats were returned to their home cage. In Experiment 3, a second test took place in CTX-B at day 28, and a third test in CTX-A on Day 29. The order of the tones and ITIs differed between test sessions.

Supplementary Methods

Experiments A-B-C: Behavioral protocol optimization

Preregistration

All study protocols and statistical analyses were preregistered on the Open Science Framework (OSF) at https://osf.io/bm76c/registrations.

Data and code availability

All relevant datasets, analysis scripts, and results of all preregistered statistical analyses can be found on the OSF at https://osf.io/bm76c.

Behavioral protocol optimization studies (Experiments A-C)

We performed experiments A, B and C to obtain appropriate behavioral protocols to investigate the effects of post-training dopaminergic drugs on fear memory generalization (see ***Table S1*** for an overview of study characteristics).

Experiment B investigated the effect of presenting tones in blocked versus intermixed order at test on differential fear responding, and used a higher shock intensity during training compared to Exp. A (.8 mA instead of .4 mA). Experiment C investigated the effect of shock intensity (.4 versus 1 mA) on differential fear responding. Smaller pilot studies were conducted to determine the optimal frequency and intensity of the tones; they are not reported here.

All rats received an intraperitoneal injection of saline (1 mg/kg) immediately after differential fear conditioning. The subjects, apparatus and behavioral procedures used in these studies are identical to those described below, except for the details that are listed below.

In Experiment A, a baseline period (i.e., before onset of the first tone) of 1 min. was used. This baseline period was prolonged to 3 min. in all other studies in order to measure baseline responding. Experiment B compared two different test sessions, i.e., with intermixed versus blocked tone presentations. The intermixed test session was as described below in the section *'Test of differential fear responding'***.** In the blocked testing group, either the CS+ or the CS- was presented three times (baseline period of 180 s, 80 dB, 30 s, ITI = 80 – 140 s) during a first test session one day after training. Thirty seconds after the last tone, rats were returned to their home cage. The next day, the other tone was tested under the same conditions. The order of tone presentations (i.e., CS- or CS+ as the first tone during test) was counterbalanced.

The behavioral protocol of Exp. A was used in Experiments 1-3 (with the addition of extra behavioral testing) and the behavioral protocol of Exp. C was used in Experiments 4-5 because we aimed to investigate dopaminergic drug effects when using a protocol that elicits low differential fear responding in control rats.

Statistical analyses

Detailed information regarding all statistical analyses can be found in the main text. Here, we only describe specifics for behavioral protocol optimization Experiments A-C.

For Experiments B-C, rats showing less than 10% freezing during CS+ presentations on average at Test 1 were excluded from the analysis according to a preregistered exclusion criterion. For Experiments A-C, one-sided paired t-tests assessed whether freezing during CS+ was higher compared to CS- for each condition (i.e., classification into conditions based on testing intermixed versus blocked in Exp. B and shock intensity in Exp. C). In order to investigate whether shock intensity during training affected differential fear responding at test in Exp. C, we performed a mixed ANOVA with between-subjects factor Shock intensity (.4 or 1 mA) and within-subjects factor Trial type (CS- or CS+).

Exp. ID	N	Body weight	Acclim. period	Groups	Shock Intensity	Test 1 (Day 2, CTX-B) Order of tone presentations*	Test 2
A	16	315 g	4 days	NA	.4 mA	12112122	NA
B	24	339 g	3 days	Intermixed vs. blocked test	.8 mA	12211212 (intermixed) vs. 111 or 222 (blocked)	CTX-B; Day 3 (only blocked group) 222 or 111
C	24	298 g	11 days	.4 vs. 1 mA	.4 or 1 mA	12211212	NA

Table S1. Behavioral protocol optimization studies. All experiments were preregistered on OSF at https://osf.io/bm76c/registrations. Sample size (N) and average body weight one day before training are shown. Acclim. period = interval between the rats' arrival in our animal facility and the start of handling (2 subsequent days of handling preceded the fear conditioning protocol). Rats were trained in context A (CTX-A) and tested in context B (CTX-B). *The order of the tones at test was counterbalanced (1 = CS+, 2 = CS- or 1 = CS-, 2 = CS+). During intermixed tests, 4 CS+ and 4 CS- presentations occurred in intermixed and semi-random order within one session, whereas blocked testing occurred in two separate test sessions with either 3 CS- or 3 CS+ presentations.

Supplementary Methods

Experiments 1-5: Effects of dopaminergic drugs on differential fear responding

Statistical analyses

In addition to the primary statistical analyses described in the main text, we also conducted secondary analyses. These secondary analyses included two-sided t-tests to assess whether treatment affected average freezing (1) during CS+ presentations and (2) during baseline at test.

For Experiments 1-4, Chi-squared tests were performed to investigate whether treatment influenced the proportion of generalizers. We preregistered to calculate a generalization index (GENINDEX = average % freezing during CS- divided by average % freezing during CS+ at test) for each rat. Rats were subdivided in 'discriminators' (GENINDEX smaller or equal to 0.75) and 'generalizers' (GENINDEX larger than 0.75, but smaller than 1.30). Rats showing considerably higher freezing during CS- compared to CS+ (GENINDEX larger or equal to 1.30) were not included in the Chi-Squared tests, as they could not be unambiguously classified as generalizers or discriminators.

Secondary analyses for Experiment 5 included calculation of the generalization index (GENINDEX = average % freezing during CS- divided by average % freezing during CS+) for each rat, and rats were subdivided into 'discriminators' (GENINDEX smaller or equal to 0.90) and 'generalizers' (GENINDEX larger than 0.90, but smaller than 1.10). Rats showing considerably higher freezing during CS- compared to CS+ (GENINDEX larger or equal to 1.10) were not included in the Chi-Squared tests, as they could not be unambiguously classified as generalizers or discriminators. The rationale for changing the criteria for classification into generalizers and discriminators can be found in the preregistration form for Experiment 5 at https://osf.io/gnqpw. As in our previous studies, Chi-Squared tests (quinpirole versus saline) were performed to assess whether quinpirole treatment affected the proportion of generalizers. In addition, we conducted one-sided t-tests to investigate whether the GENINDEX was lower in quinpirole-treated rats than in saline control rats.

Supplementary Methods

Experiment 6: Acute effects of raclopride and quinpirole on locomotor activity

Preregistration

All study protocols and statistical analyses were preregistered on the Open Science Framework (OSF) at https://osf.io/9d72h/registrations.

Data and code availability

All relevant datasets, analysis scripts, and results of all preregistered statistical analyses can be found on the OSF at https://osf.io/9d72h.

Rationale for the evaluation of the acute effects of raclopride and quinpirole on locomotor activity

Previous research in rats has shown acute effects of systemic administration of raclopride and quinpirole on locomotor activity (Archer et al., 2003; Eilam and Szechtman, 1989; Frantz and Van Hartesveldt, 1995; Hartesveldt et al., 1992; Horvitz et al., 2001; Kropf and Kuschinsky, 1991). As a positive control of drug efficacy, non-naïve rats received intraperitoneal saline or drug injection and acute effects on locomotor activity were assessed (Experiment 6). Based on the previously mentioned literature, we hypothesized that raclopride would generally suppress locomotor activity during a 1-h test session. Regarding quinpirole, it has been suggested that a dose of 1 mg/kg would first activate presynaptic autoreceptors, resulting in a transient decrease in dopamine synthesis and locomotor activity, followed by activation of postsynaptic dopamine D2 receptors, inducing hyperactivity (Kropf and Kuschinsky, 1991). On the other hand, a low dose of quinpirole (0.05 mg/kg) would solely activate presynaptic autoreceptors. Thus, quinpirole was expected to generally suppress locomotor activity at a dose of 0.05 mg/kg, whereas higher doses (1 or 5 mg/kg) would first suppress and later increase locomotor activity.

Subjects

At the age of 15-16 weeks, 35 rats that previously underwent a fear conditioning procedure (identical to the one adopted in Experiment 2) were used to test the acute drug effects during a locomotor activity test.

Drug administration

Rats received an intraperitoneal injection of saline, 0.3 mg/kg raclopride, 0.05 mg/kg quinpirole, 1 mg/kg quinpirole, or 5 mg/kg quinpirole (1 ml/kg; 7 rats/treatment condition).

Apparatus

Five minutes after the injection, rats were exposed to a novel testing box (38 x 38 x 60 (h) cm) for 1 h to assess effects on locomotor activity (distance travelled and % movement). The rats' behavior was recorded by a video camera that was mounted above the experimental set-up. Testing occurred in the dark. Distance travelled (cm) and % movement during the 1h-session were measured in 5-min time bins using Ethovision software XT (version 11.5, Noldus, Netherlands). The software's default criteria, i.e., start velocity of 2 cm/s and stop velocity of 1.75 cm/s, were used to calculate % movement.

Statistical analyses

Mixed Treatment x Time (twelve 5-min time bins) ANOVAs were used to assess treatment effects over time for raclopride (RACLO, SAL) and quinpirole (SAL, QUIN0.05, QUIN1, QUIN5) separately. Significant ANOVAs were followed up by Tukey post hoc tests to assess effects of treatment during each 5-min time bin, except for the first and the last time bin given that we had registered planned comparisons (one-sided t-tests) at those time points based on our *a priori* hypotheses (see https://osf.io/5bsh7). Specifically, we performed one-sided t-tests to assess treatment effects during the first and last 5 min of the test session. For the first 5 min (i.e., min 0-5) we expected for distance travelled and % movement a general suppression of locomotor activity by all treatment conditions, compared to saline (i.e., SAL > RACLO, QUIN0.05, QUIN1, QUIN5) and for the last 5 min (i.e., min 55-60) we expected an enhancement of locomotor activity in rats that received 1 and 5 mg/kg quinpirole, compared to saline (i.e., SAL < QUIN1, QUIN5).

Supplementary Results

Experiments A-B-C: Behavioral protocol optimization

Experiment A: Inducing intermediate levels of differential fear responding

The aim of Experiment A was to obtain a behavioral protocol that could be used to investigate the effects of post-training dopamine D2 receptor (ant)agonism on differential fear responding. Rats were trained using a differential cued fear conditioning protocol, injected intraperitoneally with saline immediately after training, and tested one day later in a new context B (CTX-B) to assess the amount of fear generalization between CS+ and CS-. About half of the rats showed higher freezing during the CS+ than the CS- at test, whereas the other half showed generalized fear responding (i.e., no or small difference in freezing between CS+ and CS-, see ***Fig. S1***). Average freezing was significantly higher during CS+ presentations, compared to CS- presentations (one-sided paired t-test; $t(15) = -2.08$, $p = .028$, $d = -.33$, $BF = 2.67$). We decided to use this protocol in Experiments 1, 2, and 3 as there were no apparent ceiling or floor effects, thus theoretically allowing the bidirectional modulation of generalization.

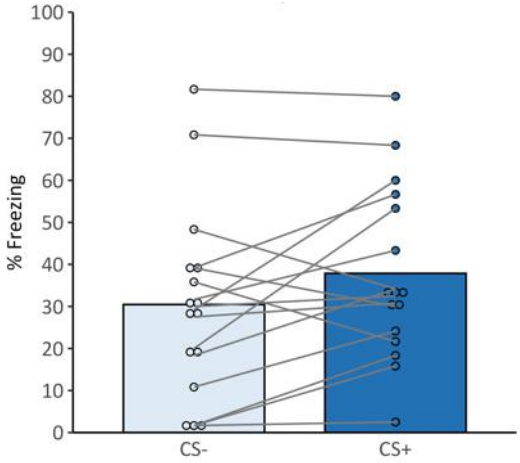

Figure S1. Average % freezing during testing (Day 2; CTX-B) of Experiment A.

Experiments B and C: Inducing lower levels of differential fear responding

The aim of Experiments B and C was to obtain a behavioral protocol that would induce lower levels of differential fear responding at test than in Experiment A, i.e., no reliable difference in freezing levels during CS+ versus CS-. This behavioral pattern would be more in line with what was observed in mice in the study by De Bundel et al. (2016) who reported no differential responding in saline control animals (and an enhancement of differential responding by post-training quinpirole injection; see Fig. 1C by De Bundel et al, 2016). To this aim, we increased shock intensity during training (i.e., from .4 mA used in Exp. A (as well as Exp. 1-3) to .8 mA in Exp. B and 1 mA in Exp. C (as well as Exp. 4-5)). In addition, Experiment B aimed to compare freezing levels between two different types of test sessions.

Intermixed tone presentations (i.e., CS- and CS+ presented in intermixed and semi-random order, in a single session) were compared to blocked tone presentations (i.e., CS- and CS+ presented in separate test session on different days, similar to De Bundel et al., 2016).

The results of Experiment B (***Fig. S2***) show that, despite the use of a higher shock intensity, rats still showed significantly higher freezing during CS+ than CS- (one-sided t-tests; intermixed test session: t(11) = -2.62, d = -.48, p = .012, BF = 5.82; blocked test: t(11) = -2.67, d = -.59, p = .011, BF = 6.24). A visual analysis suggested that the overall and differential fear responding during tones observed during the blocked and intermixed test are similar. Exploratory analyses suggested that there was a significant effect of testing order (i.e., whether CS+ or CS- was presented as the first tone during testing) on the amount of fear generalization when using the blocked tests, as suggested by a significant Testing order (CS- first versus CS+ first) x CS type (CS- versus CS+) interaction (F(1, 68) = 6.50, p = .013, η2p = .087, BF = 3.94). Visual analysis of supplemental graphs (see https://osf.io/kg8vb/, section 5.3) illustrates that, on average, rats that received CS+ presentations during the first test show high rates of differential responding, whereas rats that received CS- presentation during the first test do not show clear differential responding. There was no evidence for such an effect of testing order on generalization when using the intermixed test (F(1,92) = .42, p = .521, η2p = .004, BF = .36).

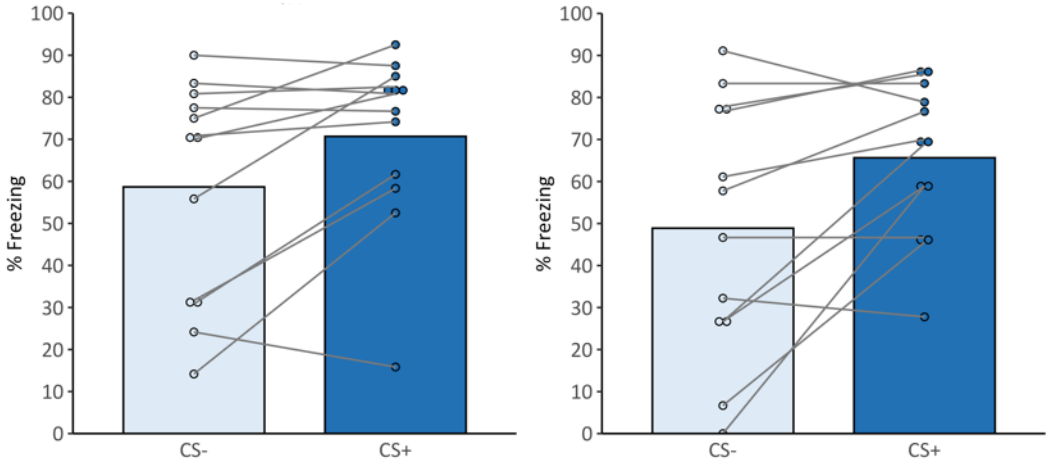

Figure S2. Average % freezing during intermixed testing (Day 2; CTX-B; left panel) and blocked testing (Day 2 and 3; CTX-B; right panel) of Experiment B.

In Experiment C (***Fig. S3***), two different shock intensities were used during training, i.e., .4 mA and 1 mA. There was a statistically significant difference in freezing between CS+ and CS- presentations at test if shocks of .4 mA were used during training, in line with what we observed in Experiment A (one-sided t-test; t(11) = -7.11, d = -1.1, p < .001, BF = 2380.46), whereas there was no clear evidence for a difference in freezing between CS+ and CS- when 1-mA shocks were used (one-sided t-test; t(11) = -1.23, d = -.34, p = .122, BF = 0.92). In addition, there was a significant Shock

intensity x CS type interaction ($F(1,22) = 20.37$, $\eta 2p = .48$, $p < .001$, BF = 394.35). To conclude, increasing the shock intensity during training from .4 to 1 mA induced a significant decrease in differential fear responding at test. The behavioral protocol from Experiment C (with 1-mA shocks) was therefore used in Experiments 4 and 5.

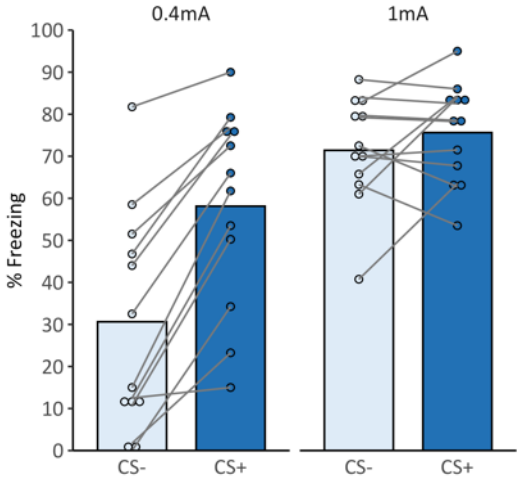

Figure S3. Average % freezing scores during testing (Day 2; CTX-B) of Experiment C illustrate that rats receiving 1-mA shocks during training show smaller differential fear responding between CS- and CS+ than rats that received .4-mA shocks.

Supplementary Results

Experiment 6: Acute effects of raclopride and quinpirole on locomotor activity

Raclopride (0.3 mg/kg) and quinpirole (0.05 mg/kg) temporarily suppress locomotor activity

In line with previously published studies and our preregistered hypotheses (see Methods section), raclopride (0.3 mg/kg) and the low dose of quinpirole (0.05 mg/kg) induced suppression of locomotor activity (compared to saline, ***Fig. S4***). For both drugs, mixed ANOVAs showed statistically significant main effects of Treatment and Time, and a significant interaction (see ***Table S2***). Tukey post-hoc tests showed that the raclopride- and quinpirole-induced differences in distance travelled and % movement compared to saline rats were temporary and became statistically insignificant during the second half of the test session. These findings corroborate previous research and illustrate that the drugs produced the expected acute behavioral effects at the applied doses in Wistar rats.

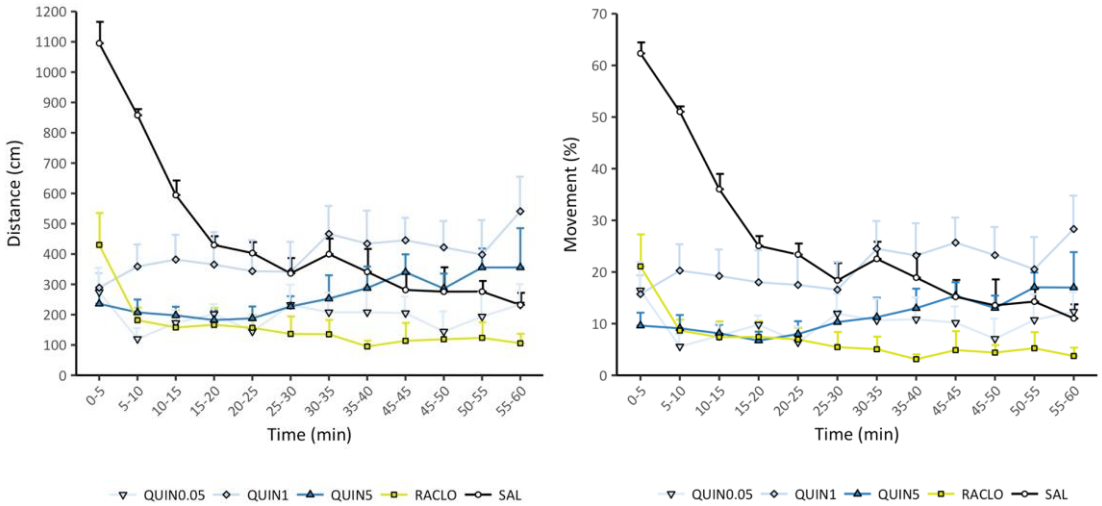

Figure S4. Acute effects of raclopride (RACLO; 0.3 mg/kg) and quinpirole (QUIN, different doses in mg/kg) on locomotor activity (means and SEMs) in Experiment 6. Distance travelled (cm) (left panel) and % movement (right panel) were measured to assess locomotor activity.

An intermediate dose of quinpirole (1 mg/kg) first suppresses and later increases locomotor activity

In line with previously published studies and our preregistered hypotheses (see Methods section), the intermediate quinpirole dose (1 mg/kg) first suppressed locomotor activity, followed by a statistically significant increase in locomotor activity compared to saline (***Fig. S4***). One-sided t-tests suggested that during the first 5 min of the test (i.e., 5-10 min after injection), quinpirole rats showed a statistically significant decrease in distance travelled and % movement compared to saline rats, whereas they showed a statistically significant increase compared to saline rats during the last 5 min of the test (see ***Table S3***). This is in line with previous research and illustrates that quinpirole (1 mg/kg) produced the expected acute behavioral effects.

A high dose of quinpirole (5 mg/kg) temporarily suppresses locomotor activity

We assumed that the biphasic behavioral pattern that was observed after injection of an intermediate 1 mg/kg-dose of quinpirole (i.e., brief suppression followed by an increase in locomotor activity after injection) would be even more pronounced when using a higher dose (5 mg/kg). One-sided t-tests suggested that the highest dose of quinpirole induced a statistically significant suppression of locomotor activity (i.e., distance travelled and % movement) during the first 5 min. of the test session (***Fig. S4***, ***Table S3***). However, contrary to our expectations, its stimulating effects at the end of the test session (i.e., about 1 hour after injection) were only moderate and insignificant when compared to saline.

Treatment	Main effect of Treatment	Main effect of Time	Treatment x Time interaction
RACLO, SAL	$F(1,12) = 52.68$, $\eta_p^2 = .81$, $p < .001$, BF = 187.45	$F(4.68, 56.19) = 27.67$, $\eta_p^2 = .70$, $p < .001$, BF = 3.85*10^18	$F(4.68, 56.19) = 8.48$, $\eta_p^2 = .41$, $p < .001$, BF = 2.71*10^8
QUIN0.05, QUIN1, QUIN5, SAL	$F(3, 24) = 6.30$, $\eta_p^2 = .44$, $p = .003$, BF = 14.83	$F(5.29, 127.04) = 4.71$, $\eta_p^2 = .16$, $p < .001$, BF = 3.91	$F(15.88, 127.04) = 8.64$, $\eta_p^2 = .52$, $p < .001$, BF = 3.55*10^23

Table S2. Results of preregistered mixed ANOVAs show statistically significant main effects of Treatment and Time, and significant Treatment x Time interactions for raclopride and quinpirole during the locomotor activity test (Experiment 6).

Treatment	First 5 min. of test session (min. 0-5)	Last 5 min. of test session (min. 55-60)
RACLO	$t(12) = 5.26$, $d = -2.81$, $p < 0.001$, BF = 103.19	NA
QUIN0.05	$t(12) = 7.66$, $d = -4.10$, $p < 0.001$, BF = 2.049.88	NA
QUIN1	$t(12) = 9.45$, $d = -5.05$, $p < 0.001$, BF = 13802.42	$t(12) = 2.55$, $d = 1.36$, $p = 0.013$, BF = 2.76
QUIN5	$t(12) = 10.29$, $d = - 5.50$, $p < 0.001$, BF = 31009.8	$t(12) = 0.91$, $d= 0.49$, $p = 0.190$, BF = 0.58

Table S3. Results of preregistered one-sided t-tests during the first and last five minutes of the locomotor activity test (Experiment 6).

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