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

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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. Importantly, the evidence for the absence of an effect of quinpirole (1 mg/kg) on CS+ versus CS- discrimination was substantial according to Bayesian analyses and was observed in a highly powered experiment (N = 88). We did observe acute behavioral effects of both quinpirole and raclopride during 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.

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64 **1. Introduction**

65 The neurotransmitter dopamine has been shown to play a role in the processing of emotional
66 memories[1]. For instance, genetic and pharmacological studies suggest that activation of dopaminergic
67 D1 and D2 receptors immediately after Pavlovian conditioning or inhibitory avoidance training is
68 required for subsequent expression of acquired fear[2–6]. In addition, dopamine has been found to
69 modulate long-term potentiation[7–11], a form of synaptic plasticity that is assumed to be essential for
70 memory formation[12]. The focus of the present paper is on the effect of dopaminergic signaling after
71 fear conditioning with two cues, differentially predicting danger (CS+) or the absence of danger (CS-),
72 on subsequent differential fear responding to those cues.

73 Although most studies regarding the influence of dopamine on fear memory processing have
74 focused on its effects on memory retention specifically, more recent studies have also investigated
75 dopamine's role in generalization of fear towards safe or novel stimuli after contextual, cued, or
76 differential cued fear conditioning[13–17]. Investigating if and how dopaminergic manipulations during
77 or after learning affect the retention and spreading of fear towards safe situations is highly relevant
78 to understand the neurobiology of anxiety, given that fear generalization is often found to be elevated
79 in patients with anxiety disorders[18,19].

80 Prior research indicated that knock-out mice lacking hippocampal dopamine D1 receptors
81 (D1Rs) exhibit equal levels of freezing during exposure to a novel context as to a context that was
82 previously paired with shock[16]. In addition, using differential cued fear conditioning procedures in
83 mice, it has been shown that differential responding between CS+ and CS- can be increased by
84 optogenetic stimulation of dopaminergic neurons during conditioning[14] or by systemic injection of a
85 D2R agonist immediately after conditioning[13]. The latter study, by De Bundel and colleagues, found
86 the dopaminergic effect to be bidirectional, with post-learning injection or intra-amygdala infusion of
87 a D2R antagonist impairing differential responding. A PET imaging study in humans[20] found evidence
88 consistent with these findings in mice. Likewise, recent rat studies (often with more elaborate
89 behavioral procedures that also included reward cues, or using compound or contextual stimuli, rather

90 than simple discrimination learning with a tone CS+ and CS-[21–24]) have provided accumulating evidence
91 for the involvement of dopamine signaling in the discrimination between danger and the absence of
92 danger. The pharmacological and recording techniques that were used in these rat studies did,
93 however, not allow for conclusions regarding the specific involvement of D2 receptors.

94 The abovementioned neurobiological studies have proposed a range of different mechanisms
95 that may underlie dopamine's effect on the spreading of fear towards safe situations. For example, it
96 has been suggested that the role of dopamine in the encoding of novel perceptual information may
97 lie at the basis of its effects on fear generalization[16]. Alternatively, it has been proposed that the
98 generalized fear observed in knockout mice with impaired dopaminergic signaling can be attributed
99 to inadequate learning of CS-US associations due to aberrant salience detection[17] and the erratic
100 assignment of negative valence to both the CS+ and CS- during learning[14]. Yet another possible way in
101 which dopamine may affect differential fear responding between stimuli that predict danger versus
102 safety is via enhancement of the stabilization or 'consolidation' of acquired fear memory[13]. Related to
103 this idea, it has been suggested that ensuring adequate stabilization of the fear memory trace may be
104 required for the establishment of a sufficiently precise memory to prevent generalization to non-
105 threatening situations[25]. Overall, dopamine's effects on fear memory generalization have thus been
106 attributed to several aspects of learning (i.e., encoding of perceptual information, association
107 formation, differential learning), at different points in time (i.e., during versus shortly after learning),
108 and via various hypothetical mechanisms (i.e., by coding salience or valence, or by enhancing memory
109 stabilization).

110 Our ultimate goal was to study, in rats rather than mice, to what extent dopamine D2 signaling
111 after differential fear learning plays a role in subsequent differential fear responding and, if so, which
112 aspect(s) of learning (perceptual, associative, and/or differential) would be involved. To this aim, we
113 first embarked on a conceptual replication of published findings in mice by De Bundel and colleagues[13],
114 in which it was shown that systemic injection of a D2R agonist or antagonist immediately after
115 differential cued fear conditioning can attenuate or enhance subsequent fear generalization (i.e.,

116 enhanced fear responding to a safe cue in a new context), respectively. One advantage of
117 administering dopaminergic drugs after learning rather than before is that it allows to distinguish
118 between the importance of dopaminergic signaling during versus after learning. It may thus provide
119 useful insight into the role of dopamine during the memory stabilization phase specifically.

120 While most prior evidence in non-human animals regarding the role of dopamine D2Rs in CS+
121 versus CS- discrimination has been obtained in mice, the translation of those findings to rats remains
122 unclear. Despite the many similarities between both species, rats and mice are characterized by
123 important differences in functional brain anatomy and physiology, including neurochemistry[26]. Rats
124 are an important model organism for behavioral neuroscience, in particular in the study of fear
125 learning and interventions to influence fear memory specificity.

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

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136 ## 2. Materials and Methods

137

138 ### 2.1. Protocol registration

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

141

142 **2.2. Availability of data and materials**

143 All datasets generated and analyzed during the current study, the analysis scripts, and results
144 of all preregistered statistical analyses are also available at https://osf.io/kzcyt. This study is reported
145 in accordance with ARRIVE guidelines (https://arriveguidelines.org).

146

147 **2.3. Subjects**

148 Male Wistar rats (Janvier Labs, Le Genest-Saint-Isle, France) were housed in groups of 3 or 4
149 rats per cage on a 12 h/12 h day-night cycle (lights on at 8 am). Cage enrichment, in the form of a
150 tunnel hanging from the top grid, was provided and with food and water available ad libitum.
151 Experiments were carried out between 9 am and 5 pm. All experiments were approved by the KU
152 Leuven animal ethics committee, and conducted in accordance with the Belgian Royal Decree of
153 29/05/2013 and European Directive 2010/63/EU.

154

155 **2.4. Drug administration**

156 Depending on the experiment (see **Table 1**), raclopride HCl (RACLO; 0.3 mg/kg; Santa Cruz
157 Biotechnology), (-)-quinpirole HCl (QUIN; 0.05 mg/kg, 1 mg/kg or 5 mg/kg; Tocris), or saline was
158 administered immediately after conditioning. Both drugs were dissolved in saline (0.9%, w/v) and
159 injected intraperitoneally at a volume of 1 ml/kg. Drug doses were based on the study in mice by De
160 Bundel and colleagues[13]. Similar doses have previously been used in rats[27,28].

161

162 **2.5. Differential cued fear conditioning**

163 Four Med Associates test chambers were used to train and test the animals. Details can be
164 found in the **Supplement**. Fear conditioning in context A (CTX-A) consisted of presentations of a
165 dangerous tone (5 x CS+, always co-terminated with a foot shock (US)) and a safe tone (5 x CS-, never
166 paired with the shock) in a semi-random order (see **Table 1** and **Fig. 1**). Next, rats received an
167 intraperitoneal injection of saline, quinpirole (0.05, 1 or 5 mg/kg) or raclopride (0.3 mg/kg) at a volume

168 of 1 ml/kg. Twenty-four hours after training, rats underwent a drug-free test in a novel context (CTX-
169 B), during which the CS+ and CS- were repeatedly presented (4 times each) in a semi-random order.
170 Depending on the experiment, additional drug-free test sessions were performed, in either CTX-A or
171 CTX-B.
172
173 **2.6. Statistical analyses**
174 Percentage freezing during tones was scored by an experienced observer, blinded to
175 treatment condition, tone frequency and CS type (CS- or CS+). Average % freezing during CS+ and CS-
176 was calculated for each rat and for each test session. According to a preregistered exclusion criterion,
177 rats showing less than 10% freezing during CS+ presentations on average at Test 1 were excluded from
178 the analysis, as these low freezing scores may indicate a lack of acquisition of the CS-US association.
179 Analyses were performed with and without those rats. R was used for frequentist and Bayesian
180 analyses. For Bayesian analyses, we used a default Cauchy prior on the standardized effect size with a
181 scale of 0.707 in the BayesFactor package. Bayes factors indicate how likely the obtained data are
182 under H_A, relative to H_0 (i.e., BF_{10}).
183 Primary analyses for Experiments 1-5 (secondary analyses are described in the **Supplement**)
184 included mixed ANOVAs with between-subjects factor Treatment (saline or drug) and within-subjects
185 factor CS type (CS- or CS+) to investigate whether treatment affected differential fear responding
186 during tests. Mixed ANOVAs were performed for each drug (versus saline) separately. The Bayes factor
187 (BF) quantifying the evidence for including the Treatment x CS type interaction was calculated by
188 dividing the BF for the model with interaction and main effects by the BF for the model with only main
189 effects, (see doi 10.31234/osf.io/spreb; referred to as 'BFinclusion across matched models'). One-
190 sided paired t-tests assessed whether freezing during CS+ was higher compared to CS- for each
191 treatment condition.
192 Primary statistical analyses for Experiment 5 were the same as for Exp. 1-4, but in contrast
193 with the previous experiments, this study used a sequential design, for which the total sample size

194 depended on the value of the Bayes factor for the CS type x Treatment interaction observed during
195 interim assessments (see **Table 2**). The first assessment took place after testing a total of 40 rats (batch
196 1; n = 20 rats/treatment condition) and the second assessment after testing a total of 64 rats (batch
197 2; 24 additional rats; n = 12 rats/treatment condition). The maximum sample size (N = 88) was
198 determined based on frequentist power calculations using the results obtained in Experiment 4 (CS
199 Type x Treatment interaction; power = 92%, alpha = .05, partial eta squared = .13).
200
201
202 **3. Results**
203
204 All details regarding the behavioral protocol optimization studies (Experiments A-B-C) that preceded
205 Experiments 1-5 can be found in the **Supplement**.
206
207 **3.1. Experiment 1: No evidence for an effect of post-training injection of raclopride (0.3 mg/kg) or**
208 **quinpirole (1 mg/kg) on subsequent differential fear responding**
209 Based on the results of De Bundel and colleagues in mice[13], we hypothesized that 0.3 mg/kg
210 raclopride or 1 mg/kg quinpirole would decrease or increase differential fear responding at test,
211 respectively.
212 Primary analyses provided no evidence for Treatment (dopaminergic drug versus saline) x CS
213 type (CS- versus CS+) interactions at Test 1 (RACLO: $F(1, 22) = .04$, $p = .847$, $\eta 2p < .01$, BF = .38; QUIN1:
214 $F(1, 22) = .20$, $p = .662$, $\eta 2p = .01$, BF = .38). One-sided paired t-tests suggested higher freezing during
215 CS+ than CS- presentations at test in saline control rats ($t(11) = -2.42$, $p = .017$, $d = -.64$, BF = 4.35), and
216 raclopride rats ($t(11) = -2.6$, $p = .012$, $d = -.67$, BF = 5.66), but not in quinpirole rats ($t(11) = -1.38$, $p =$
217 $.098$, $d = -.33$, BF = 1.09).
218 Although freezing during CS+ presentations at Test 1 was on average lower in quinpirole rats
219 than in saline control rats (see **Fig. 2a**), two-sided t-tests (preregistered secondary analysis, see OSF)

220 suggested no evidence for differences in freezing during CS+ presentations between treatment
221 conditions (i.e., QUIN1 versus SAL or RACLO versus SAL). In addition, there was no evidence for
222 differences in baseline freezing (i.e., before onset of the first tone, see OSF) or the proportion of
223 generalizers between treatment conditions.

224 During Test 2, which took place in the training context on Day 3 (**Fig. 2b**), there was again no
225 evidence for Treatment x CS type interactions (RACLO: $F(1, 22) = .67$, $p = .421$, $\eta 2p = .03$, BF = .45;
226 QUIN1: $F(1, 22) = 1.25$, $p = .276$, $\eta 2p = .05$, BF = .63) and one-sided paired t-tests suggested higher
227 freezing during CS+ than CS- presentations at test in saline control rats ($t(11) = -2.09$, $p = .03$, $d = -.60$,
228 BF = 2.75), raclopride rats ($t(11) = -2.31$, $p = .021$, $d = -.29$, BF = 3.75), and in quinpirole rats ($t(11) = -$
229 3.75, $p = .002$, $d = -1.14$, BF = 30.08). Secondary analyses suggested no evidence for differences in
230 freezing during CS+ presentations between treatment conditions (i.e., QUIN1 versus SAL or RACLO
231 versus SAL). In addition, there was no evidence for differences in baseline freezing (i.e., before onset
232 of the first tone), average freezing during CS+ presentations, or the proportion of generalizers
233 between treatment conditions.

234

235 **3.2. Experiments 2 & 3: No evidence for an effect of post-training injection of quinpirole (0.05 or 5**
236 **mg/kg) on subsequent differential fear responding**

237 Locomotor activity studies in rats suggest that quinpirole differentially affects presynaptic
238 versus postsynaptic D2 receptors depending on the applied dose[29–32]. Experiments 2 and 3 aimed to
239 assess how different doses of quinpirole influence differential fear responding. We hypothesized that
240 a low dose of quinpirole (0.05 mg/kg) would decrease differential responding (due to the assumed
241 activation of presynaptic autoreceptors), whereas a higher dose of 5 mg/kg would increase differential
242 responding (due to the assumed activation of postsynaptic D2Rs). Experiments 2 and 3 adopted the
243 exact same behavioral procedures and drug doses (apart from additional tests 2 and 3), and are
244 therefore presented together.

In Experiment 2 (**Fig. 3a-b**), three rats (1 SAL and 2 QUIN5 rats) were excluded due to the preregistered exclusion criterion of showing less than 10% freezing on average during CS+ presentations at Test 1, so all preregistered analyses were performed with and without those three rats. Statistical results are shown here for the sample in which the exclusion criterion was applied, and results for both samples (i.e., with and without exclusions) are provided only when both analyses reached different overall conclusions. All results can be found at https://osf.io/kzcyt.

Primary analyses suggested no evidence for Treatment (dopaminergic drug versus saline) x CS type (CS- versus CS+) interactions (QUIN (0.05 mg/kg): $F(1, 21) = 2.01$, $p = .171$, $\eta 2p = .09$, 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.

270 During Test 2, which took place in the training context on Day 27 (**Fig. 3b**), there was again no
271 evidence for Treatment x CS type interactions (QUIN (0.05 mg/kg): $F(1, 21) = .06$, $p = .801$, $\eta 2p < .01$,
272 BF = .37; QUIN (5 mg/kg): $F(1, 19) < .01$, $p = .945$, $\eta 2p < .01$, BF = .38). Furthermore, both QUIN0.05
273 and saline rats now showed significantly higher freezing during CS+ than CS- ($t(10) = -2.96$, $p = .007$, d
274 $= -.77$, BF = 8.98; $t(11) = -3.85$, $p = .001$, $d = -1.20$, BF = 34.85, respectively), whereas QUIN5 rats did
275 not show statistically significant differential responding ($t(9) = -1.61$, $p = .071$, $d = -.63$, BF = 1.51). In
276 addition, secondary analyses suggested no evidence for differences in baseline freezing (i.e., before
277 onset of the first tone), average freezing during CS+ presentations, or the proportion of generalizers
278 between treatment conditions.
279
280 In Experiment 3 (**Fig. 3c-e**), eight rats (2 SAL, 2 QUIN0.05, and 4 QUIN5 rats) were excluded
281 due to the preregistered exclusion criterion of showing less than 10% freezing on average during CS+
282 presentations at Test 1, so all preregistered analyses were performed with (N = 36) and without (N =
283 28) those eight rats.
284 Primary analyses suggested no evidence for Treatment (dopaminergic drug versus saline) x CS
285 type (CS- versus CS+) interactions (QUIN (0.05 mg/kg): $F(1, 18) = .55$, $p = .468$, $\eta 2p = .03$, BF = .47;
286 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
287 suggested higher freezing during CS+ than CS- presentations at test in all treatment conditions (saline
288 control rats: ($t(9) = -3.64$, $p = .003$, $d = -.42$, BF = 20.35; QUIN (0.05 mg/kg): $t(9) = -2.82$, $p = .01$, $d = -$
289 $.76$, BF = 7.03; QUIN (5 mg/kg): $t(7) = -3.52$, $p = .005$, $d = -.98$, BF = 13.27). However, differential
290 responding between tones was smaller (and non-significant) when including all QUIN5 rats ($t(22) =$
291 $.08$, $p = .933$, $d = .03$, BF = .37), probably due to the fact that two rats showed no freezing during any
292 of the tones, and thus, no differential responding.
293 During Test 2 (**Fig. 3d**), which took place in the testing context (CTX-B) on Day 28, there was
294 again no evidence for Treatment x CS type interactions (QUIN0.05: $F(1, 18) = .57$, $p = .458$, $\eta 2p = .03$,
295 BF = .47; QUIN5: $F(1, 16) = .03$, $p = .861$, $\eta 2p < .01$, BF = .39). Saline control rats ($t(9) = -1.25$, $p = .121$,

296 d = -.24, BF = .99) and QUIN5 rats (t(7) = -1.37, p = .107, d = -.77, BF = 1.18) did no longer show
297 statistically significant differential responding between CS+ and CS-, whereas QUIN0.05 rats did show
298 significant differential responding between tones still (t(9) = -2.76, p = .011, d = -.78, BF = 6.47).
299 However, when including all rats, all groups showed statistically significant differential responding
300 (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;
301 QUIN5: t(11) = -2.11, p = .029, d = -.65, BF = 2.83).

302 During Test 3 (**Fig. 3e**), which took place in the training context (CTX-A) on Day 29, there was
303 no evidence for Treatment x CS type interactions (QUIN0.05: F(1, 18) < .01, p = .982, η2p < .01, BF =
304 .41; QUIN5: F(1, 16) = .74, p = .402, η2p = .04, BF = .58) and there was no statistical evidence for
305 differential responding between tones in any of the treatment conditions (SAL: t(9) = -1.45, p = .09, d
306 = -.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,
307 BF = .32).

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

311

312 **3.3. Experiment 4: No evidence for an effect of post-training injection of quinpirole (0.05 or 1 mg/kg)**
313 **on differential fear responding when using higher shock intensity during training**

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

321 Saline control rats showed differential responding between tones at test ($t(15) = -1.8$, $p = .046$,
322 $d = -.35$, BF = 1.78). Numerically even higher levels of differential responding were observed in
323 quinpirole rats, especially in rats that received the dose of 1 mg/kg (QUIN0.05: $t(15) = -2.34$, $p = .017$,
324 $d = -.44$, BF = 4.01; QUIN1: $t(15) = -5.04$, $p < .001$, $d = -.86$, BF = 410.27) (**Fig. 4a**). There was, however,
325 no reliable evidence for Treatment (dopaminergic drug versus saline) x CS type (CS- versus CS+)
326 interactions (QUIN (0.05 mg/kg): $F(1, 30) = .25$, $p = .622$, $\eta 2p = .01$, BF = .36; QUIN (1 mg/kg): $F(1, 30)$
327 $= 3.28$, $p = .08$, $\eta 2p = .1$, BF = 1.08).
328 Secondary analyses suggested no evidence for between-group differences in average freezing
329 during CS+ presentations, baseline freezing, (i.e., before onset of the first tone), or the proportion of
330 generalizers.
331
332 **3.4. Experiment 5: No evidence for an effect of post-training injection of quinpirole (1 mg/kg) on**
333 **differential fear responding when using higher shock intensity during training and large sample size**
334 In Experiment 4 we observed numerically higher levels of differential responding in rats that
335 received 1 mg/kg quinpirole. Therefore, we aimed to investigate this effect further by taking into
336 account the size of the effect observed in Exp. 4 and using a sample size that is required to detect such
337 an effect with a power of more than 90%. Experiment 5 followed the procedures from Experiment 4,
338 but only included two of the groups from Experiment 4 (i.e., quinpirole 1 mg/kg versus saline). The
339 experiment had a sequential design with a predetermined maximum sample size, and was performed
340 in three separate batches. Given that we did not observe the predefined Bayes factor for the CS type
341 x Treatment interaction after testing batch 1 or 2, the maximum sample size of 88 rats was tested.
342 One saline rat was excluded from analyses because it showed no freezing at all during testing
343 (i.e., 0% freezing; exclusion due to preregistered criterion). In addition, the excluded rat did not show
344 any freezing during training either, and a lack of responding at the time the shocks should have been
345 given, so we decided not to perform analyses with this rat included. Note that we hereby deviate from
346 the preregistration because we planned to perform the analyses with all subjects included, apart from

347 applying the preregistered exclusion criterion. A total of 87 rats was thus included in the analyses
348 described below.

349 There was no evidence for a CS type x Treatment interaction ($F(1, 85) = .17$, $p = .68$, $\eta 2p < .01$,
350 BF = .23), and the obtained Bayes factor suggested substantial evidence for the absence of an
351 interaction[33] (**Fig. 4b**). One-sided paired t-tests suggested statistically significant differential
352 responding between CS+ and CS- in saline control rats ($t(42) = -4.93$, $p < .001$, $d = -.62$, BF = 3032.43)
353 and quinpirole rats ($t(43) = -4.31$, $p < .001$, $d = -0.56$, BF = 502.73).

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

357

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 [32], we found that 1 mg/kg quinpirole first suppressed locomotor activity,
362 followed by a statistically significant increase in locomotor activity compared to saline (**Supplement,**
363 **Fig. S4**). This indicates, that in a different task than the differential fear conditioning procedure, the
364 drug did produce the expected acute behavioral effect at the applied dose in male Wistar rats.

365

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[13]. 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)[13], a number of differences between these two studies should
386 be acknowledged. First and foremost is of course the difference in species, given the reported species-
387 dependent variations in ventral tegmental area dopamine signaling[34]. The fact that DA neurons in mice
388 and rats show different sensitivity to D2-mediated inhibition could have important implications for
389 presynaptic or postsynaptic effects of D2R modulation. Similarly, differences in DA agonist-induced
390 locomotor effects, prepulse inhibition, or yawning between rats and mice have been described[35–37].
391 Another relevant question is whether mice and rats express fear in the same manner (e.g., comparable
392 levels of freezing versus escape behavior) during presentations of tones with different frequencies. If
393 existent, between-species differences in prototypical fear behavior may obviously complicate the
394 comparison of studies in rats versus mice when differential freezing responses are used as a measure
395 of generalization.

396 Furthermore, and as expected, pilot studies in our lab indicated the need for different
397 behavioral parameters than those used in the mouse studies[13] in order to allow for the study of
398 differential fear responding in rats (e.g., tones of 5 and 10 kHz rather than 2.5 and 7.5 kHz, shocks of

399 .5 s rather than 2 s, and intermixed rather than blocked testing). In addition, we opted to leave out
400 the habituation session prior to conditioning, and used a semi-random rather than a fixed order of
401 CS+ and CS- presentations during learning. Finally, our saline control animals showed, on average, a
402 different behavioral pattern than those in the De Bundel study. The control rats in most of our
403 dopamine studies (except for Experiment 2) showed statistically significant higher freezing during CS+
404 presentations than during CS-, whereas the control mice in the prior quinpirole study showed a lack
405 of differential responding (see Fig. 1C of De Bundel et al.[13]). The observed variability in freezing
406 behavior of the control animals between some of our experiments could be perceived as a limitation,
407 but is probably a logical consequence of our attempt to tread a fine line, i.e., to create a learning
408 experience that still contained a degree of ambiguity (with the control animals of Experiment 2
409 showing statistically insignificant discrimination, and thus flipping to the other side than in
410 Experiments A, 1 and 3, where the effect was significant). As mentioned before, we did not want to
411 overtrain the animals in their discrimination between the CS+ and CS- (which could, for example, have
412 been achieved with multiple training days[38]), nor did we want them to have inadequate learning
413 opportunities to discriminate between the CS+ and CS- (in such scenario, full generalization between
414 the CS+ and CS- might just reflect a perceptual discrimination deficit). Overall, the observed behavioral
415 variability between experiments is most likely not a major issue, given that each of our experiments
416 had its own appropriate saline control group.

417 As far as the effects of raclopride concern, our conclusions rely on a single study with a sample
418 size of 12 rats per treatment condition, yielding only anecdotal evidence for the absence of a CS type
419 x Treatment interaction effect. In contrast, we performed five studies with quinpirole, in which we
420 used three different doses of the drug (0.05, 1, or 5 mg/kg), two different shock intensities (.4 or 1
421 mA), and including a final study with a large sample size yielding a power of over 90% to detect an
422 effect if any. Moreover, Experiment 3 (N = 36) was a replication of Experiment 2 (N = 36) (except for
423 additional tests), and Experiment 5 (N = 88) was a replication of Experiment 4 (N = 48) (except for the
424 omission of the 0.05-mg/kg quinpirole group in Exp. 5). The final experiment provided substantial

425 evidence (Bayesian analyses) for the absence of an effect of quinpirole (1 mg/kg) on differential fear
426 responding. In other words, we have gathered evidence against an effect of quinpirole on differential
427 fear responding under the applied conditions in rats.

428 It should be noted that our studies are limited to the use of systemic drug injection and that
429 we did not directly target a particular brain region via, for example, drug infusion or optogenetic
430 manipulation. Given the apparent lack of generalizability to rats of the results of De Bundel et al. [13] of
431 systemic application of dopaminergic drugs, a possible follow-up study to investigate the involvement
432 of post-learning D2R signaling in differential fear responding could include the use of intracerebral
433 drug administration. In this case, a replication in rats of previous experiments in which the D2R
434 antagonist raclopride was infused into the central or basolateral amygdala (see Fig. 2I and 3I in De
435 Bundel et al.[13]) would be optimal to inform about the replicability of the effect specifically and the
436 role of post-learning D2R signaling in differential fear responding in rats more generally.

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528

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	/	/

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 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 consecutive days of handling preceded the fear conditioning protocol). Rats underwent differential cued fear conditioning in context A (CTX-A), after which 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 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+).

538

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

539 **Table 2. Overview of the sequential design used for Experiment 5.** The reported power was
540 calculated based on the effect size for the CS Type x Treatment interaction observed in Experiment 4.
541 Interim assessments – performed after collecting data for batch 1 and 2 – included the computation
542 of the Bayes factor for the CS type x Treatment interaction (testing stopped if the obtained BF was
543 larger than 10 or smaller than 1/10 at interim assessment).
544

545

Figures

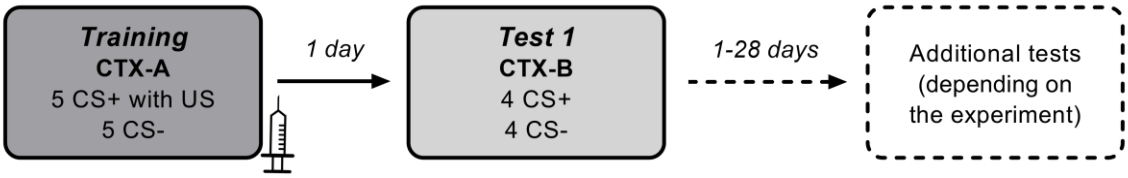
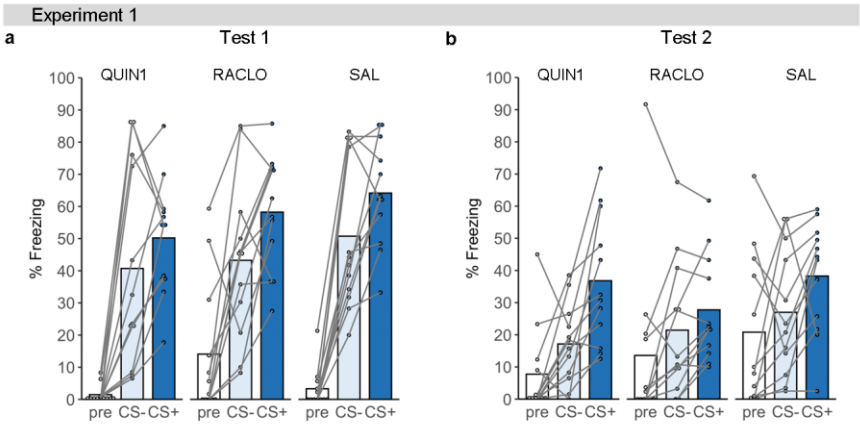


Fig. 1. Experimental procedure. Immediately after differential cued fear conditioning in context A (CTX-A), rats were systemically injected with saline, raclopride, or quinpirole (see **Table 1** for details). Twenty-four hours later, behavioral differentiation between CS+ and CS- was assessed in a novel 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.



558

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

560 a) and Test 2 (Day 3; CTX-A; panel b), shown for the 180-s baseline period (pre), CS- and CS+. QUIN1 =

561 quinpirole (1 mg/kg), RACLO = raclopride (0.3 mg/kg), SAL = saline.

562

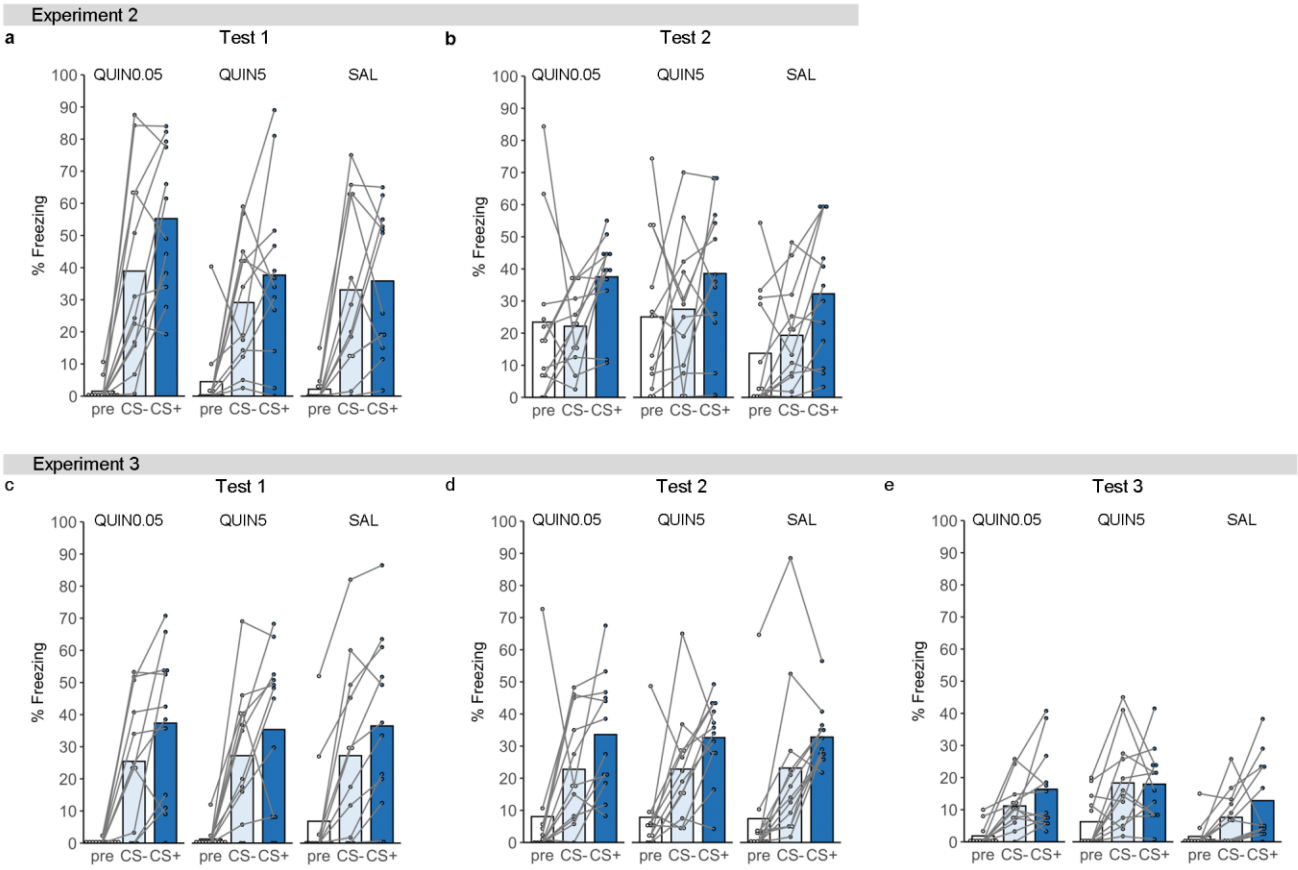


Fig. 3. Experiments 2 & 3. Average % freezing per treatment condition during Test 1 (Day 2; CTX-B; panel a) and Test 2 (Day 27; CTX-A; panel b) of Experiment 2, and during Test 1 (Day 2; CTX-B; panel c), Test 2 (Day 28; CTX-B; panel d), and Test 3 (Day 29; CTX-A; panel e) of Experiment 3, shown for the 180-s baseline period (pre), CS- and CS+. QUIN0.05 = quinpirole (0.05 mg/kg), QUIN5 = quinpirole (5 mg/kg), SAL = saline.

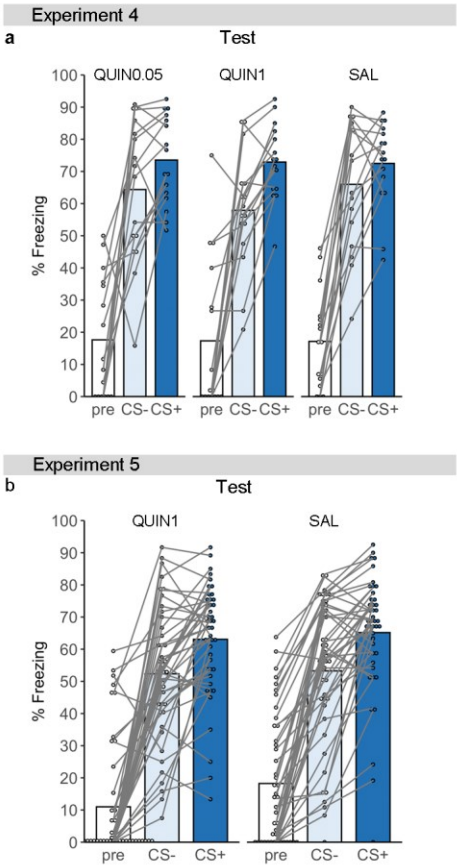


Fig. 4. Experiments 4 & 5. Average % freezing per treatment condition during testing (Day 2; CTX-B) in Experiment 4 (panel a) and Experiment 5 (panel b), shown for the 180-s baseline period (pre), CS- and CS+. QUIN0.05 = quinpirole (0.05 mg/kg), QUIN1 = quinpirole (1 mg/kg), SAL = saline.

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

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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[1–6]. 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[5]. 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.

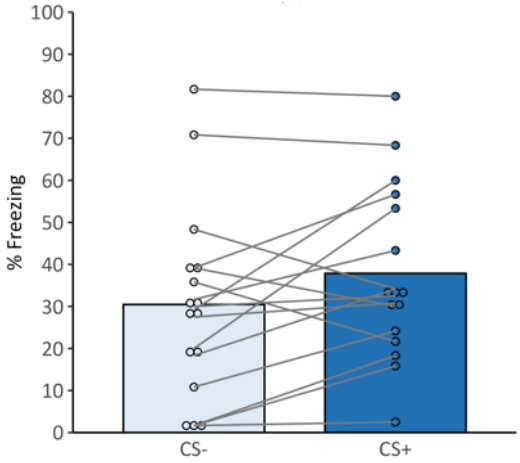

Figure S1. Average % freezing during testing (Day 2; CTX-B) of Experiment A.

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.[7] 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).

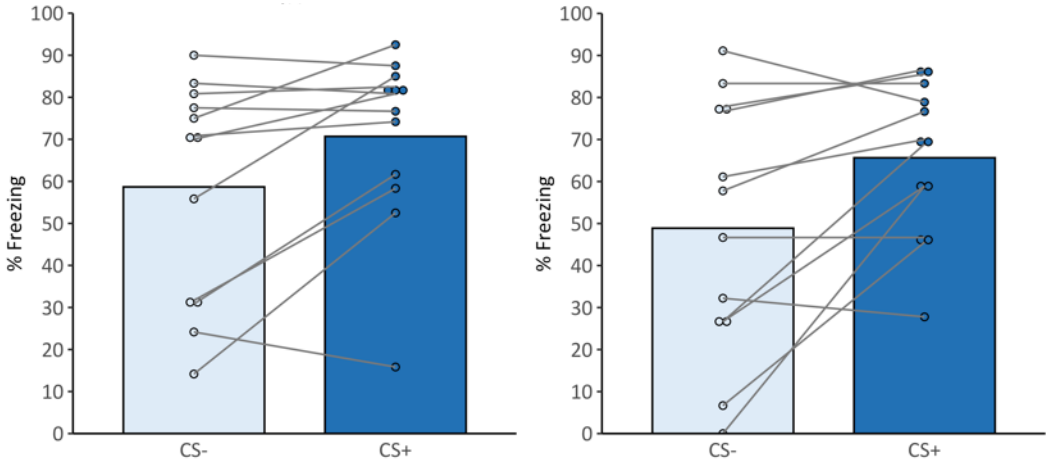

Figure S2. Average % freezing during intermixed testing (Day 2; CTX-B; left panel) and blocked testing (Day 2 and 3; CTX-B; right panel) of Experiment B.

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.

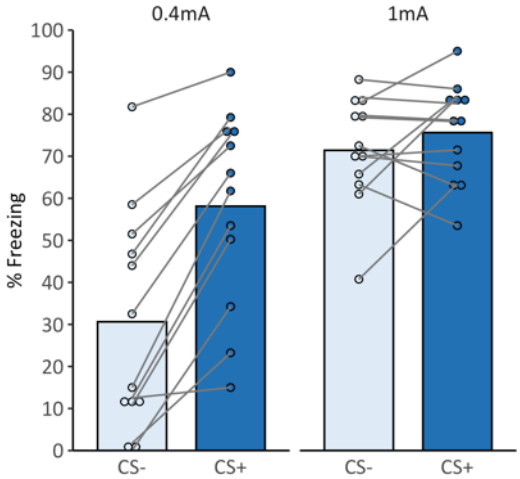

Figure S3. Average % freezing scores during testing (Day 2; CTX-B) of Experiment C illustrate that rats receiving 1-mA shocks during training show smaller differential fear responding between CS- and CS+ than rats that received .4-mA shocks.

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.

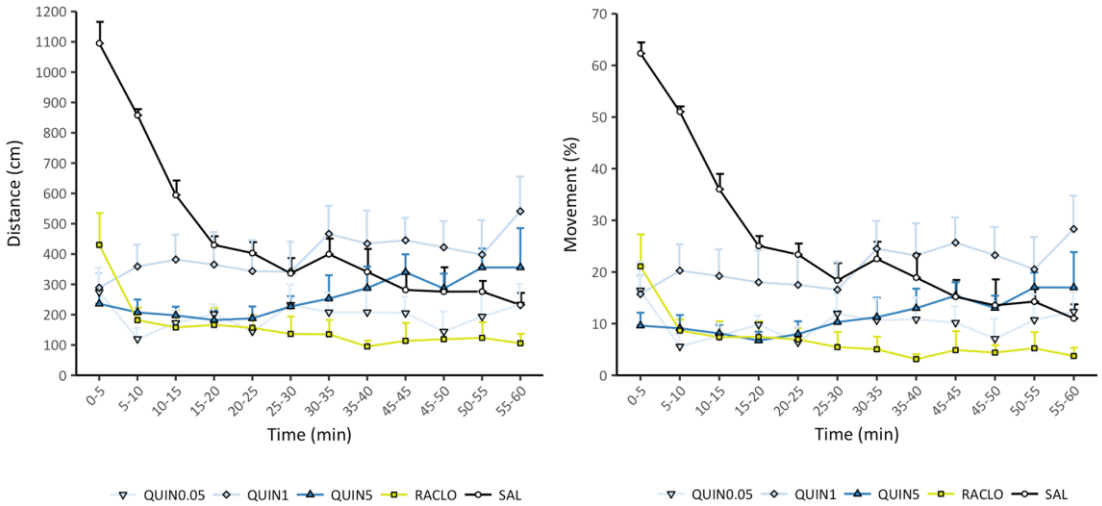

Figure S4. Acute effects of raclopride (RACLO; 0.3 mg/kg) and quinpirole (QUIN, different doses in mg/kg) on locomotor activity (means and SEMs) in Experiment 6. Distance travelled (cm) (left panel) and % movement (right panel) were measured to assess locomotor activity.

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, η2p = .81, p < .001, BF = 187.45	F(4.68, 56.19) = 27.67, η2p = .70, p < .001, BF = 3.85*10^18	F(4.68, 56.19) = 8.48, η2p = .41, p < .001, BF = 2.71*10^8
QUIN0.05, QUIN1, QUIN5, SAL	F(3, 24) = 6.30, η2p = .44, p = .003, BF = 14.83	F(5.29, 127.04) = 4.71, η2p = .16, p < .001, BF = 3.91	F(15.88, 127.04) = 8.64, η2p = .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 = 2049.88	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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