Partial Ablation of Postsynaptic Dopamine D2 Receptors in the Central Nucleus of the Amygdala Increases Risk Avoidance in Exploratory Tasks

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Partial ablation of post-synaptic dopamine D2 receptors in the central nucleus of the amygdala increases risk avoidance in exploratory tasks

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Abstract

The central nucleus of the amygdala (CeA) is involved in the expression of fear and has been implicated in several anxiety disorders. This structure is densely innervated by dopaminergic projections that impinge on amygdalar neurons expressing various dopamine receptor subtypes, including D2 receptors (D2Rs). Although various pharmacological approaches have assessed the role of D2Rs in the CeA, the actual participation of post-synaptic D2Rs in the CeA to defensive behaviors remains unclear. Here, we investigated the distribution of D2Rs in the CeA and their role in modifying neuronal activity and fear related behaviors in mice. First, using the mouse reporter strain D2R-EGFP, we verified that D2Rs are present both in neurons of the CeA and in A10dc DAergic neurons that innervate the CeA. Moreover, we showed that pharmacological stimulation of D2Rs increases the activity of PKC δ cells present in the CeA, a type of neuron previously associated with reduced defensive behaviors. Finally, using a molecular genetics approach that discriminates postsynaptic D2Rs from presynaptic D2 autoreceptors, we demonstrated that mice carrying targeted deletions of postsynaptic D2Rs in the CeA display increased risk avoidance in exploratory tasks. Together, our results indicate that postsynaptic D2Rs in the CeA attenuate behavioral reactions to potential environmental threats.

45 Significance Statement

46 The central nucleus of the amygdala (CeA) is a neural hub involved in risk assessment and fear-
47 related behaviors, and its malfunction may trigger anxiety disorders. The CeA is densely
48 innervated by dopamine projections that activate D1 and D2 receptors (D2R). In this study we
49 sought to determine the role that postsynaptic D2Rs in the CeA exert in defensive behaviors.
50 We first showed that pharmacological stimulation of D2Rs increases the activity of neurons
51 known to reduce defensive behaviors. We also showed that mice partially lacking postsynaptic
52 D2Rs in the CeA display increased risk avoidance in exploratory tasks. Together, our results
53 indicate that D2Rs in the CeA attenuate behavioral reactions to potentially aversive
54 environmental stimuli rising new perspectives to manage anxiety disorders.

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59 Introduction

60 Risk assessment, fear, and threat avoidance are highly conserved adaptive behaviors that are
61 essential for fitness and survival. In some cases, however, fear responses appear as exaggerated
62 behavioral reactions to stimuli that do not represent a commensurate threat, underling
63 symptoms of pathological conditions such as anxiety and post-traumatic disorders.
64 Uncontrollable anxiety may also lead to compulsive behaviors commonly observed during
65 withdrawal from major abused drugs such as opioids, cocaine, nicotine, and ethanol (Koob,
66 2008).

67 The central nucleus of the amygdala (CeA) is a neural hub that orchestrates innate and learned
68 fear-related behaviors. The CeA is densely innervated by dopaminergic fibers arriving mainly
69 from A10 dorsocaudal (A10dc) neurons located in the ventral periaqueductal gray (vPAG) and
70 dorsal raphe (DR) (Hasue and Shammah-Lagnado, 2002; Li et al., 2016). The dopamine (DA) D2
71 receptor gene (*DRD2*) is expressed in the CeA of rodents (Scibilia et al., 1992; Kim et al., 2017;
72 McCullough et al., 2018a, 2018b) and humans (Gurevich et al., 1999; Xiang et al., 2008). In
73 mice, DA D2 receptors (D2Rs) present in the CeA have been implicated in impulsive behaviors
74 (Kim et al., 2018) whereas several association human studies have linked particular genetic
75 polymorphisms of *DRD2* with avoidance behavior (Frank and Hutchison, 2009), social phobia
76 (Schneier et al., 2000), social dysfunction (Lawford et al., 2006) and anxiety-driven alcoholism
77 (Joe et al., 2008). Given the involvement of the CeA in defensive behaviors, and its regulation by
78 DA, we hypothesized that D2Rs in the CeA regulate behavioral responses to potential threats,
79 and that ablation of postsynaptic D2Rs in the mouse CeA would increase avoidance behaviors.
80 Although previous studies have used local applications of antagonists to evaluate the function

81 of D2Rs in the CeA (Guarraci *et al.*, 2000; De la Mora *et al.*, 2012; De Bundel *et al.*, 2016),
82 pharmacological approaches are unable to discriminate between the blockade of pre- and post-
83 synaptic D2Rs.

84 In this study, we sought to investigate the role of amygdalar D2Rs in defensive and fear related
85 behaviors. To this end, we first evaluated the distribution of post- and presynaptic D2Rs in the
86 CeA. Then, we determined the pattern of CeA neurons activated by pharmacological
87 stimulation of D2Rs. Finally, we used a molecular genetics approach to partially eliminate
88 postsynaptic D2Rs from the CeA and studied their risk assessment behaviors in
89 approach/avoidance conflict paradigms. Altogether, our results support the hypothesis that
90 postsynaptic D2Rs in the CeA play an active role in threat assessment of environmental cues.

91

92 **Materials and Methods**

93 **Mice husbandry**

94 Mice of both sexes were housed in ventilated cages under controlled temperature and
95 photoperiod (12-h light/12-h dark cycle, lights on from 7:00 AM to 7:00 PM), with tap water
96 and laboratory chow available *ad libitum*, and separated by sex. For behavioral experiments
97 and drug administration 7-16 week-old mice were transferred to an experimental animal room
98 with similar housing conditions and allowed for at least 1 week of habituation prior to
99 experiments. All procedures followed the Guide for the Care and Use of Laboratory Animals,
100 United States Public Health Services (2011) and in agreement with the INGEBI-CONICET
101 Institutional Animal Care and Use Committee. *Drd2*^{-/-} and *Drd2*^{loxP/loxP} mice were generated by
102 crossing a heterozygote male mouse carrying the original floxed exon 2- floxed PGK-neo *Drd2*

103 allele with a B6.FVB-Tg(EIIa-cre)C5379Lmgd/J female (Jackson Laboratory, USA) and then
 104 backcrossed for more than 10 generations to C57BL/6J, as previously described in detail (Bello
 105 et al., 2011). *Drd2*^{loxP/loxP} mice are conditional mutants carrying targeted loxP sites flanking *Drd2*
 106 exon 2 (*Drd2*^{tm1.1Mrub/J}). *Drd2*^{loxP/loxP}, *Drd2*^{-/-}, *Dat*^{+//RES-Cre} (Bäckman et al., 2006), *Drd2*-EGFP
 107 (Gong et al., 2003) and Ai14 (Madisen et al., 2010) mice were all bred in our facility and
 108 maintained in a C57BL/6J background.

109 **Stereotaxic surgeries**

110 Mice were anesthetized with ketamine (100 mg/kg; i.p.) and xylazine hydrochloride (10 mg/kg;
 111 i.p.). A 10 µl Hamilton syringe connected with a 36-gauge metal needle was used to infuse
 112 lentiviral vectors using a microsyringe pump at 0.1 µl/min. Stereotaxic coordinates for the CeA
 113 were in relation to the Bregma (Paxinos and Franklin, 2008): anterior-posterior, -1.5 mm;
 114 medial-lateral, +/-3.0 mm; dorsal-ventral, -4.9 mm. Following infusion, the needle was kept at
 115 the injection site for 5 min, and then slowly withdrawn to half way, kept there for 2 more min
 116 and then slowly withdrawn outside the brain. Skin was sutured, local anesthesia (lidocaine gel)
 117 was applied followed by the analgesic flunixin meglumine (5 mg/kg, s.c.). Mice were maintained
 118 on a regulated warm pad and monitored until recovery from anesthesia.

119 Mice received 0.6 µl of a solution containing 3.3x10⁸ particles/ml directly into the CeA,
 120 bilaterally. CeA*Drd2*KO mice were generated by stereotaxic co-injections of LV:GAD-Cre
 121 (1.65x10⁸ viral particles/ml) and LV:Ub-EGFP (1.65x10⁸ viral particles/ml) into the CeA of
 122 *Drd2*^{loxP/loxP} mice whereas control mice received injections of LV:Ub-EGFP (3.3x10⁸ particles/ml)
 123 alone. The same procedure was performed for LV:GAD-Cre and LV:Ub-EGFP injections in *wild-*
 124 *type* mice (*Drd2*^{+/+}) for control experiments.

125 Lentiviral preparations

126 HEK-293T cells were grown on high glucose DMEM (Invitrogen), supplemented with 10% (v/v)
127 fetal bovine serum (Natocor, Argentina), 0.5 mM L-glutamine, 100 U/ml penicillin and 100
128 µg/ml streptomycin. Cells at 80-85% confluence in 100 mm plates were co-transfected with 3
129 µg of the lentiviral shuttle vector (either GAD-CRE or Ub-EGFP) together with helper vectors
130 encoding packaging and envelope proteins (CMVΔ8.9 and CMV-VSVg, 3 and 1.5 µg
131 respectively), using lipofectamine (Plus reagent, Thermo Fisher). Viral particles were harvested
132 from the culture medium 36 h after transfection, treated with RNase-free DNase I (Invitrogen),
133 filtered and concentrated by ultracentrifugation at 100,000 x g (Ti 90 rotor, Beckman), yielding
134 viral suspensions at a titer of 10^7 TU/ml. LV aliquots were stored at -80°C and thawed on ice
135 before use. LV:GAD-Cre carries Cre recombinase coding sequences driven by the mouse GAD67
136 promoter whereas LV:Ub-EGFP carries EGFP coding sequences driven by the human ubiquitin
137 promoter. Both viral vectors contain a woodchuck hepatitis virus post-transcriptional regulatory
138 element (WPRE); long terminal repeats (LTR); RNA pack and genomic RNA packaging signals; a
139 rev response element (RRE), a central polypurine tract (cPPT), a central termination sequence
140 (CTS), a 3' end polypurine tract (3-PPT) and a ΔU3 400-bp deletion in the 3' LTR.

141 Tissue collection and histology

142 Transcardiac perfusions were performed with phosphate buffered saline (PBS, 0.9% NaCl, 2.7
143 mM KCl, 10 mM K_2HPO_4 , 2 mM KH_2PO_4 , pH 7.5) followed by paraformaldehyde 4% in PBS and
144 brains were removed and post-fixed in the same solution at 4°C for 12-16 h. Brains were
145 sectioned at 40 µm on a vibratome (Leica) and used immediately or stored at -20°C in a solution
146 containing 30% (v/v) ethylene glycol, 30% (v/v) glycerol and PBS, until they were processed for
147 immunofluorescence. Immunolabeling was performed as follows: free-floating sections were

148 rinsed three times for 10 min in PBS. For PKC δ immunofluorescence an antigen-retrieval
149 protocol was applied: following PBS rinse, sections were incubated in citrate buffer (10mM
150 citric acid, 0.05% Tween-20, pH=6.0) at 95°C for 5 min and then rinsed three times for 10 min in
151 PBS. Sections were incubated for 16 h at 4°C in primary antibody solution with normal goat
152 serum 2% (w/v), 0.3% Triton X-100, in PBS. The following primary antibodies were used: rabbit
153 anti-TH (1:2,000; Millipore, AB5935), chicken anti-TH (1:1,000; Abcam, AB76442), rabbit anti-C-
154 FOS (1:500, Santa Cruz Biotechnology, SC-52), mouse anti-PKC δ (1:500, BD Biosciences,
155 610398), chicken anti-EGFP (1:1,000; Aves, GFP-1020). After incubation with a primary
156 antibody, sections were rinsed twice for 20 min in PBS and then incubated for 2 h at room
157 temperature with goat or donkey Alexa-488 or Alexa 555 coupled secondary antibody 1:1,000
158 in 0.3% Triton X-100 in PBS. Finally, sections were rinsed twice for 20 min in PBS and mounted
159 with Vectashield (Vector Labs) for confocal microscopy or glycerol 50% (v/v) in PBS for
160 fluorescence and bright field microscopy.

161 **Microscopy and images analysis**

162 Confocal images for co-expression assays and quantification were obtained using a Leica
163 Confocal TCS-SPE microscope. Images not used for colocalization analysis were obtained by
164 fluorescence microscopy. Images were analyzed with the Fiji platform (Schindelin *et al.*, 2012)
165 of the ImageJ software (Rueden *et al.*, 2017). For c-FOS quantification, cell number was semi-
166 automatically quantified with the tool “Analyze particles” after manually delimitating the region
167 of interest. Colocalization images were obtained by confocal microscopy and was manually
168 quantified using the tool “Cell Counter”.

169 **Experimental design and statistical analyses**

170 Drug administration and c-FOS detection

171 Mice of both sexes older than 8 weeks were used. All drugs were dissolved in NaCl 0.9% to
172 reach a concentration such that the injected volume was 0.1 ml per 10 g of body weight.
173 Experiments evaluating cocaine and quinpirole effects were performed separately, and because
174 of that, they were not analyzed in the same statistical analysis. Vehicle (NaCl 0.9%), cocaine
175 hydrochloride (20 mg/kg; Sigma) or quinpirole (1 mg/kg; Sigma) were injected i.p.. This dose of
176 quinpirole was selected based on a previous report showing increased expression of the
177 neuronal activation marker P-rpS6 in CeA neurons (De Bundel et al., 2016). Mice were left in
178 their home cages and 90 min later were perfused for tissue fixation and histology.

179 The number of c-FOS immunostained cells per hemisphere and coronal section (between 2 and
180 5 per mouse) was obtained. For plotting, the average number of cells between hemispheres
181 and sections of the same mouse was calculated. For statistical analysis, sections corresponding
182 to the same mouse were treated as sub-samples of the same mouse. Colocalization of c-FOS
183 and PKC δ (**Fig. 2B-C**) was analyzed with a two-ways generalized linear mixed model with
184 Poisson distribution (**Fig. 2B**) or Negative Binomial distribution (**Fig. 2C**), with Drug as between-
185 subjects factor with 2 levels (vehicle and quinpirole, or vehicle and cocaine), Neuronal
186 population as intra-subjects factor with 2 levels (PKC δ + and PKC δ -), Mouse as random variable
187 and Section-hemisphere as sub-samples (Neuronal population nested in Mouse, and Section-
188 hemisphere nested in Neuronal population/Mouse). c-FOS in *wild-type* vs *Drd2KO* mice was
189 analyzed with a two-ways generalized linear mixed model with Negative binomial distribution,
190 with Drug as between-subjects factor with 2 levels (vehicle and quinpirole), Genotype as
191 between-subjects factor with 2 levels (*wild-type* and *Drd2KO*), Mouse as random variable and

192 Section-hemisphere as sub-samples (nested in Mouse). Significance was evaluated with the
193 likelihood-ratio test (LTR). Post-hoc Tuckey multiple comparisons were performed.

194 **Behavioral tests**

195 *Drd2*^{loxP/loxP} male mice older than 8 weeks were injected with a combination of LV:GAD-Cre and
196 LV:Ub-EGFP (CeADrd2KO group) or LV:Ub-EGFP alone (control group), and after 2-3 weeks were
197 evaluated in a battery of tests to evaluate avoidance behaviors (open field (OF), dark-light box
198 test (DLBT) and elevated plus maze (EPM)) and fear conditioning (FC). As a control experiment,
199 *wild-type* male mice older than 8 weeks received identical surgeries and behavioral tests except
200 for the fear conditioning (LV:GAD-Cre group and LV:Ub-EGFP group). Two littermate cohorts
201 were used to reach sample size per group (CeADrd2KO= 9, Ctrl= 8; GAD-Cre= 7, Ub-EGFP= 7).

202 Differences in avoidance behavior were evaluated with a Multivariate Analysis of the Variance
203 (MANOVA) including the values obtained from the OF (time in center), the DLBT (time in light,
204 latency to first enter to light and number of entries to light) and the EPM (percentage of time
205 on open arms or normalized time on open arms (see the “Elevated plus maze” section), and
206 entries to open arms). In addition, the results of the MANOVA were confirmed with individual
207 univariate tests (described in the section of each test).

208

209 **Open field exploration test.** Horizontal locomotion and exploration in a novel open field were
210 evaluated in activity boxes (Med Associates) for 30 min for 3 consecutive days. Total distance
211 travelled during the first 5 min of the each day, and the time in the center of the arena, were
212 determined using the software Activity Monitor (Med Associates). Time in center was evaluated

213 by t-test. Distance was evaluated in a 2-ways ANOVA with repeated measures, with group as
 214 between-subjects factor and day as intra-subject factor with 3 levels (day 1, day 2 and day 3).

215

216 **Elevated plus maze.** We used a custom-made apparatus standing 50 cm above the floor and
 217 constructed with black acrylic. Each arm of the maze is 5 cm wide and 30 cm long. The closed
 218 arms have black acrylic walls, 12 cm high. Mice were individually placed in the center of the
 219 maze and allowed to explore for 5 min while being videotaped. Entry to an arm was counted
 220 when more than half of the body of the mouse was inside the arm. Percentage of time on open
 221 arms was calculated with the formula: $100 \times \text{time on open} / (\text{time on open} + \text{time on closed})$;
 222 percentage of entries to open was calculated with the formula: $100 \times \text{entries to open} / (\text{entries}$
 223 $\text{to open} + \text{entries to closed})$. Univariate differences in percentage of time spent on the open
 224 arms were evaluated with a t-test; univariate differences in percentage of entries to open arms
 225 were evaluated with Wald's test in a generalized linear model with quasi-binomial distribution.
 226 Only for the percentage of time spent on the open arms in $Drd2^{loxP/loxP}$ mice, significant effect of
 227 the cohort was detected. In addition, the variance also differed between cohorts, causing a
 228 non-normal distribution of the residuals. Therefore, the data was analyzed in two different
 229 ways: first, using a 2-ways ANOVA with group and cohort as between-subjects' factors with
 230 variance modeling (VarIdent function, library "nlme"); second, dividing each value by the
 231 average of its respective cohort (independently of the group) and analyzing this normalized
 232 data with a t-test. Since the normalization restored the normal distribution of the residuals,
 233 normalized data was used for the MANOVA of $Drd2^{loxP/loxP}$ mice instead of the original
 234 percentage of time spent on the open arms.

235

236 **Light/dark box test.** A custom-made two-chamber shuttle box containing a dividing wall with a
237 4 x 5-cm hole in the center that allows mice free access to both sides was used. Each chamber is
238 20 (w) x 26 (l) x 14 cm (h) with steel walls and floor. Mice were placed on one side of the shuttle
239 box that was then immediately covered with a black acrylic ceiling. The other chamber received
240 ambient illumination. Mice were allowed to explore the chambers for 5 min while videotaped.
241 The time prior to entering into the lit compartment (latency), the time spent on the illuminated
242 side, and the number of entries to the lit compartment were determined. Entry to or exit from
243 the lit compartment was counted when the mouse passed more than half of its body.
244 Univariate differences in latency and time spent on the illuminated chamber were evaluated by
245 t-test. Univariate differences in number of entries were evaluated with Wald's test in a
246 generalized linear model with Poisson (*Drd2^{loxP/loxP}* mice) or quasi-Poisson (*wild-type* mice)
247 distribution.

248

249 **Fear conditioning.** The conditioning chamber consisted in an operant chamber 14.0 (w) x 15.9
250 (l) x 12.7 (h) cm (Med Associates) with steel rods on the floor connected to a scrambler (Med
251 Associates), placed inside a closed ventilated box for sensorial isolation. Mice were introduced
252 into the chamber and light was turned on. After 2 min habituation, 3 consecutive shocks (0.35
253 mA, 2 sec duration) were applied with an inter-trial interval of 90 s. One min after the third
254 shock, light turned off and mice were removed and returned to their home cages. 24 h later
255 mice were reintroduced into the chamber for 5 min to test contextual conditioned freezing.
256 Mice were videotaped during the entire training and testing sessions. Freezing behavior was
257 hand scored by videotape observation and freezing or active avoidance were determined in
258 continuous 4 s bins. Freezing behavior was presented as the percentage of freezing events over

259 total events. Differences in the percentage of time freezing behavior in each stage of the
260 training (baseline, after first shock, after second shock and after third shock) and the testing
261 were evaluated by MANOVA and further validated by univariate tests. Univariate differences in
262 the percentage of time freezing were evaluated by Wald's test in a generalized linear model
263 with quasi-binomial distribution for baseline and test stages, and by likelihood-ratio test in a
264 generalized linear mixed model with quasi-binomial distribution with the Group as between
265 subjects' factor and Shock as within-subject factor with three levels (shock 1, shock 2 and shock
266 3) in the case of the post shocks part of the training.

267

268 **Software and statistics**

269 All data represent the mean $\pm$ SEM and were graphed using GraphPad Prism Software (Version
270 5.01, 2007 GraphPad Software inc., San Diego, USA) and analyzed using R Studio (Version 3.2.3).
271 Data with continuous variables were analyzed by Student's t test, ANOVA or repeated
272 measures ANOVA. Normal distribution and homoscedasticity were verified with Shapiro test,
273 QQ-plot and Levene test (library "car"). When appropriate, variance was modeled with VarIdent
274 function (library "nlme"). Discrete data were analyzed with generalized linear models (GLM) or
275 generalized linear mixed model (GLMM) with Poisson error structure (link:logit). Variables
276 corresponding to rates of success over total trials were analyzed using GLM with Binomial error
277 structure (link:log). For GLM and GLMM, significances were evaluated with "likelihood-ratio
278 test" comparing nested models, or Wald test in the case of Quasi-binomial models.
279 Assumptions were evaluated assessing the absence of patterns in Pearson's residuals graph and
280 calculating the dispersion parameter to assess subdispersion or overdispersion. When
281 assumptions were not achieved, Negative-binomial or quasi-Poisson distributions instead

282 Poisson, or Quasi-binomial instead Binomial, were used. For multivariate analysis of variance,
283 univariate and multivariate normality (Royston's test, library "MVN") and homogeneity of
284 covariance matrices (Box test, library "biotools") and absence of multivariate outliers
285 (Mahalanobis distance) were verified.

286

287 **Code Accessibility**

288 Data analysis and tables can be accessed at <https://github.com/casey-e/Casey-et-al-2021>

289

290 **Results**

291 **Pre- and post-synaptic distribution of D2R in the CeA**

292 To identify neurons expressing D2Rs in the CeA, we performed a comprehensive histological
293 analysis of coronal brain slices of *Drd2*-EGFP BAC transgenic mice (Gong *et al.*, 2003). In
294 agreement with previous reports using *in situ* hybridization (Kim *et al.*, 2017; McCullough *et al.*,
295 2018a), we found a wide distribution of neuronal cell bodies expressing D2Rs along the lateral
296 division of the CeA (CeL) and through the entire antero-posterior axis of this division (**Fig. 1A**).
297 In contrast, D2R-positive cell bodies were only sparsely detected in the medial division (CeM),
298 and mainly located near the boundaries of the CeL (**Fig. 1A**). To analyze if D2R expressing
299 neurons are innervated by DAergic terminals we crossed *Drd2*-EGFP mice with mutant mice
300 carrying a *Dat*^{JRES-Cre} knockin allele (Bäckman *et al.*, 2006) and the Cre-inducible *tdTomato*
301 reporter gene Ai14 (Madisen *et al.*, 2010) that, together, label DAergic neurons with red
302 fluorescence. Using these triple transgenic mice, we found that D2R expressing neurons and
303 DAergic fibers largely overlap in the CeL (**Fig. 1B**), suggesting that D2Rs of the CeA are

functional receptors regulated by DA. In addition, we detected a low density of DAergic fibers in the CeM, a CeA division with sparse D2R expressing neurons (**Fig. 1B**). Then, we investigated whether the major DAergic input to the CeA, originating from the vPAG/DR (Hasue and Shammah-Lagnado, 2002; Li *et al.*, 2016), expresses D2Rs that may function as D2 autoreceptors in the CeA. Using *Drd2*-EGFP mice we found that most DAergic vPAG/DR neurons also express the reporter transgene driven by *Drd2* (81.8 ± 9.7 % of total TH-immunoreactive neurons) (**Fig. 1C-D**), a result that supports prior data suggesting D2 autoreceptor regulation of DA release in the CeA (Bull *et al.*, 1991). Together, these data indicate that D2Rs in the CeA are present both post and pre-synaptically.

D2Rs stimulate PKC δ + neurons in the CeA

The neuronal circuits of the CeA involved in the processing of fear-induced behaviors have been described in detail (Ciocchi *et al.*, 2010; Janak and Tye, 2015; Kim *et al.*, 2017). A group of CeL neurons expressing the molecular marker protein kinase C δ (PKC δ +) is known to reduce the expression of defensive behaviors by inhibiting projection neurons of the CeM (Ciocchi *et al.*, 2010; Haubensak *et al.*, 2010; Tye *et al.*, 2011). Conversely, PKC δ - neurons of the CeL facilitate the expression of fear responses by inhibiting PKC δ + CeL neurons (Ciocchi *et al.*, 2010; Haubensak *et al.*, 2010). To study the participation of D2Rs in the CeA, we investigated whether PKC δ + and PKC δ - neurons are differentially activated by D2R stimulation using an immunofluorescence coexpression analysis of PKC δ and the immediate early gene *c-Fos*. We found that both the DA transporter blocker cocaine (20 mg/kg, i.p.) and the D2R agonist quinpirole (1 mg/kg; i.p.) increased the number of c-FOS+ nuclei in the CeL. Furthermore, the

326 increase in c-FOS expression was significantly greater in PKC δ + neurons than in PKC δ - neurons
 327 (likelihood-ratio test for the interaction between drug and cell type), being 3-fold greater in
 328 cocaine injected mice (**Fig. 2A, B**) and 6-fold greater in quinpirole injected mice (**Fig. 2A, C**), in
 329 agreement with a previous report (De Bundel *et al.*, 2016). The effect of quinpirole was
 330 mediated exclusively by D2Rs, since the number of c-FOS+ nuclei was not increased when given
 331 to *Drd2*^{-/-} (*knockout*) mice (**Fig. 2D-E**). These results indicate that stimulation of D2Rs activate
 332 PKC δ + neurons in the CeA. Because PKC δ + neurons in the CeA have been shown to mediate
 333 anxiolytic effects (Haubensak *et al.*, 2010; Cai *et al.*, 2014), we hypothesized that D2Rs present
 334 in this region regulate risk assessment.

335

336 **Ablation of postsynaptic D2Rs in the CeA increases avoidance in exploratory tasks**

337 To investigate the participation of amygdalar D2Rs in behavioral reactions involved in risk
 338 assessment we generated mice partially lacking postsynaptic D2Rs in the CeA and tested them
 339 in several approach/avoidance conflict paradigms. The molecular strategy used to specifically
 340 ablate postsynaptic D2Rs without affecting D2 autoreceptors was based on the expression of
 341 Cre recombinase directly into the CeA of *Drd2*^{loxP/loxP} mice, a homozygous strain carrying
 342 conditional *Drd2* null alleles (Bello *et al.*, 2011). To this end, we used a lentiviral vector
 343 expressing the Cre recombinase under the control of the GAD67 promoter (Tolu *et al.*, 2010)
 344 (LV:GAD-Cre; **Fig. 3A top**) together with a lentiviral vector expressing EGFP under the control of
 345 the human ubiquitin promoter that allows later visualization of the injection site (LV:Ub-EGFP;
 346 **Fig. 3A bottom**). The use of the GAD67 promoter to drive Cre was based on the fact that CeA
 347 neurons are GABAergic (McDonald, 1982; Ehrlich *et al.*, 2009). We performed bilateral

348 stereotaxic co-injections of LV:GAD-Cre and LV:Ub-EGFP into the CeA of *Drd2*^{loxP/loxP} mice (from
349 now on, CeA*Drd2*KO mice) (**Fig. 3B**), to excise the essential exon 2 of *Drd2* (**Fig. 3C**). Control
350 *Drd2*^{loxP/loxP} mice received LV:Ub-EGFP injections only (**Fig. 3C**). Two to three weeks after this
351 procedure, mice were subjected to a battery of approach/avoidance conflict paradigms where
352 their behaviors were evaluated (**Fig. 3D**). At the conclusion of these tests, the injection sites
353 were determined histologically for each mouse (**Fig. 3E**). Cre-induced deletion of exon 2 from
354 the *floxed Drd2* alleles was verified by PCR using DNA extracted from the CeA (**Fig. 3F**). In
355 addition, to validate the efficacy of the viral vectors to target CeA neurons expressing D2Rs, we
356 injected LV:GAD-Cre into the CeA of compound *Drd2*-EGFP.Ai14 transgenic mice and confirmed
357 that the injections were limited to the CeA inducing *tdTomato* expression in 38±14% (average ±
358 confidence interval, n=5 injections) of *Drd2*-EGFP+ neurons at the injection site (**Fig. 3G**).
359 Noteworthy, we did not detect tdTomato+ neurons in any of the input areas projecting to the
360 CeA, demonstrating that transduction of the LV was restricted to postsynaptic neurons and was
361 not retrogradely transported.

362 A multivariate analysis of variance (MANOVA) for measures of approach/avoidance conflicts
363 demonstrated that CeA*Drd2*KO mice are significantly different from control mice (1 way
364 MANOVA, Pillai= 0.7, approx. $F(1,15) = 3.9$, $p=0.03$, **Fig. 4**). In agreement, using univariate
365 analysis we found that CeA*Drd2*KO mice spent significantly less time than control mice in the lit
366 compartment of the dark/light box (**Fig. 4A, left**), showed greater latencies to enter for the first
367 time into this compartment (**Fig. 4A, middle**), and entered fewer times into the lit box (marginal
368 difference ($p=0.09$), **Fig. 4A, right**). Similarly, CeA*Drd2*KO mice spent significantly less time on
369 the open arms of the elevated plus maze than their control siblings (**Fig. 4B, top-left and right**),
370 and the percentage of entries into the open arms over the total number of arm entries followed

371 the same trend (**Fig. 4B, bottom-left and right**). Differently, the time spent in the center of the
372 open field was similar in CeADrd2KO and control mice (**Fig. 4C**).

373 The increased avoidance behaviors observed in CeADrd2KO mice cannot be attributed to lower
374 motivation for exploring new environments or to lower locomotor activity, because LV:GAD-Cre
375 injections did not affect the distance travelled in the open field during the first day (when the
376 arena is a novel environment) or in the second or third days (when the arena is a known
377 environment) (**Fig. 5**).

378 To verify that the increased avoidance behaviors described in **Fig. 4** were caused by the
379 selective elimination of postsynaptic D2Rs from the CeA, we administered LV:GAD-Cre and
380 LV:Ub-EGFP directly into the CeA of *Drd2*^{+/+} mice (*wild-type* mice without loxP sites flanking
381 exon 2, **Fig. 6A**) and challenged them with the same battery of behavioral tests (**Fig. 6B**). *Drd2*^{+/+}
382 mice receiving LV:GAD-Cre injections did not show any behavioral difference in these tests
383 compared to mice receiving LV:Ub-EGFP only (1 way MANOVA, Pillai= 0.5, approx. F (1, 12)=
384 1.0, p=0.48, **Fig. 6C-E**), indicating that the enhanced avoidance displayed by CeADrd2KO mice
385 was in fact due to the elimination of postsynaptic D2Rs from the CeA. Finally, CeADrd2KO mice
386 did not differ from control mice in the time spent freezing when re-exposed to a chamber
387 previously associated to unavoidable foot-shocks (1 way MANOVA, Pillai= 0.2, approx. F (1, 15)=
388 0.6, p=0.67, **Fig. 7A-D**). Together, these results indicate that partial ablation of postsynaptic D2R
389 in the CeA increases avoidance in exploratory tasks and suggest that amygdalar D2Rs control
390 behavioral responses to riskier environments.

391

392

393 Discussion

394 In this study, we used a conditional site-specific genetic approach in mice and found that partial
395 ablation of postsynaptic D2Rs in the CeA increased avoidance behaviors in approach/avoidance
396 conflict paradigms, suggesting that DA stimulation of amygdalar D2Rs regulates behavioral
397 reactions to potential threats present in novel environments. The enhanced avoidance behavior
398 observed in mice partially lacking D2Rs in the CeA was more profound when mice were studied
399 in the dark-light box test than in the elevated plus maze. This milder, although significant, effect
400 was likely caused by a strong and significant Cohort effect in the time spent on the open arms
401 of the elevated plus maze, probably driven by variable and undetermined environmental
402 conditions existing at the different moments when the two mouse cohorts were studied.
403 Noteworthy, a two-ways ANOVA indicated significant main effects of the Group and the Cohort,
404 indicating that the effect of D2R ablation in the CeA had similar effects in both cohorts.

405 Although D2Rs in the CeA have been previously studied using pharmacological compounds
406 (Guarraci *et al.*, 2000; De la Mora *et al.*, 2012; De Bundel *et al.*, 2016), we believe this is the first
407 report investigating the effects on defensive behaviors elicited by the genetic ablation of post-
408 synaptic D2Rs directly into the CeA. Although the lentiviral-mediated approach used in this
409 study induced only a partial ablation of D2Rs in the injected CeA (**Fig. 3G**), this limited deletion
410 showed to be enough to alter the behavioral reactions of potentially dangerous environments
411 (**Fig. 4**), without affecting spontaneous locomotor activity (**Fig. 5**) or fear memory (**Fig. 7**). In
412 contrast, previous studies based on local applications of D2R antagonists into the CeA had
413 shown no changes in risk avoidance behavior, although elicited impaired reactions to fear
414 conditioning (Guarraci *et al.*, 2000) and unconditioned fear (De la Mora *et al.*, 2012). Such

415 differences may be due to the fact that pharmacological agents not only act on postsynaptic
 416 D2Rs but also on D2 autoreceptors that control DA release (Bello *et al.*, 2011). Indeed, using
 417 *Drd2*-EGFP mice we found that most TH immunoreactive neurons present in the vPAG/DR also
 418 express *Drd2* (**Fig. 1C-D**), a result supported by data collected in the Allen Mouse Brain Atlas
 419 showing intense labeling of *Drd2* mRNA in vPAG/DR cell bodies (Lein *et al.*, 2007). Furthermore,
 420 the D2R antagonist sulpiride has been shown to potentiate DA release elicited by optogenetic
 421 activation of vPAG/DR terminals reaching the bed nucleus of the *stria terminalis* (Xu *et al.*,
 422 2021) whereas an *in-vitro* study demonstrated that D2 autoreceptors regulate DA release in the
 423 CeA (Bull *et al.*, 1991). Thus, functional evidence (Bull *et al.*, 1991; Xu *et al.*, 2021) together with
 424 histological results shown in this work and in Lein *et al.* (2007) indicate that DA release in the
 425 CeA is regulated by presynaptic D2 autoreceptors. Consequently, pharmacological activation or
 426 blockade of D2Rs present in the CeL are likely to alter DA release and affect the activity of CeA
 427 neurons expressing postsynaptic DA receptors.

428 In agreement with a role of D2Rs of the CeA in risk avoidance, our pharmacological results show
 429 D2R-mediated activation of CeL PKC δ + cells (**Fig. 2**), a group of neurons that has been shown to
 430 elicit anxiolytic-like effects in approach/avoidance conflict tests (Cai *et al.*, 2014; Griessner *et*
 431 *al.*, 2018). Considering that D2Rs are generally Gi/o-coupled receptors that decrease neuronal
 432 excitability (Innis and Aghajanian, 1987; Lacey *et al.*, 1987; Uchida *et al.*, 2000), the excitatory
 433 effect on PKC δ + neurons is likely due to polysynaptic transmission, a mechanism that needs to
 434 be further clarified in future studies. Because DA agonists were applied systemically in this
 435 study, we cannot rule out the possibility that the D2R-mediated activation of CeL PKC δ + cells
 436 was indirectly driven by stimulation of D2Rs located in other brain areas. Although specific
 437 subpopulations of PKC δ + neurons drive defensive behaviors (Kim *et al.*, 2018; Cui *et al.*, 2017),

438 the PKC δ ⁺ neurons activated by quinpirole and cocaine most likely reduce anxiety-like
439 behaviors, as c-FOS expression in the CeA elicited by cocaine or amphetamine can be prevented
440 by exposure to stressful environments (Day et al., 2008; Day et al., 2005; Day et al., 2001),
441 suggesting that indirect DA agonists and potential threats drive opposite effects in CeA
442 microcircuits. Our demonstration that partial ablation of D2Rs from the CeA increased
443 avoidance in exploratory tasks (**Fig. 4**) without impairing locomotor activity (**Fig. 5**), together
444 with the finding that D2Rs activate PKC δ ⁺ neurons in the CeL (**Fig. 2**) suggest that amygdalar
445 D2Rs are potential therapeutic targets for anxiolytic compounds. Interestingly, the D2/D3
446 agonist ropinirole has shown anxiolytic effects together with motor improvements in
447 Parkinson's disease patients (Rogers et al., 2000; Rektorova et al., 2008; Mavrikaki et al., 2014).
448 In addition, it has been recently shown that CeL PKC δ ⁺ neurons are necessary and sufficient for
449 the anxiolytic effect of benzodiazepines (Griessner et al., 2018).

450 In summary, here we demonstrate that removing D2Rs in the CeA increases anxiety-like
451 behaviors, as evidenced by reduced exploration of riskier environments. This result is consistent
452 with the work of De Bundel et al., 2017 in which blocking D2Rs in the CeA after a fear
453 conditioning test leads to fear generalization, a clinical marker of anxiety disorders (De Bundel
454 et al., 2016). In contrast, D1R stimulation in the CeA facilitated fear learning (Guarraci et al.,
455 1999b; Groessl et al., 2018) and expression (Guarraci et al., 1999b), without affecting anxiety-
456 like behaviors (Groessl et al., 2018). Thus, we hypothesize that DA in the CeA reduces anxiety-
457 like behaviors via D2Rs and enhances fear learning and memory via D1R. A similar mechanism
458 has been proposed for DA acting in the striatum, where DA regulates behavioral selection by
459 enhancing the contrast between stronger and weaker inputs reaching striatal neurons (for a
460 review see (Nicola et al., 2004)). We propose that in the CeA, DA strengthens the value of

461 aversive stimuli via D1Rs (Guarraci *et al.*, 1999b; Groessl *et al.*, 2018), and attenuates signals
 462 from low risk or neutral stimuli via a D2R-based mechanism, as we found here and in
 463 agreement with a previous report (De Bundel *et al.*, 2016). Further studies will be necessary to
 464 address the participation of other DAergic receptors in amygdalar circuits mediating responses
 465 to fearful, dangerous and high risk situations.

466

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599 **Figure Legends**

600

601 **Figure 1. Post- and pre-synaptic distribution of D2R-expressing neurons. A)** Representative
602 confocal microscopy of coronal sections at two anteroposterior levels of the CeA of a *Drd2*-
603 EGFP mouse. Magnifications of boxed areas are shown in the left bottom corners. **B)**
604 Representative histology of D2R-expressing neurons (green, left) in CeA coronal sections of
605 *Drd2*-EGFP.*Dat*^{+/IRES-Cre}.Ai14 triple transgenic mice. DAergic fibers (reported by Cre-induced
606 tdTomato, in red) and their overlap with D2R-expressing neurons is shown at the center and
607 right, respectively. **C-D)** Analysis of *Drd2* expression in DAergic neurons of the PAG/DR. **C)**
608 Representative confocal microscopy of TH immunofluorescence on a PAG/DR coronal section of
609 a *Drd2*-EGFP mouse. Magnifications of boxed areas are shown. **D)** Percentage of D2R+ and
610 D2R- neurons from total TH+ neurons in the vPAG/DR (mean $\pm$ SEM, n=3 mice). CeL, central
611 nucleus of the amygdala, lateral part; CeM, central nucleus of the amygdala, medial part. Scale
612 bars: 200 μ m.

613

614

615 **Figure 2. D2R stimulation activates CeA PKC δ neurons.** **A)** Representative histology of PKC δ
616 (green, top) and c-FOS (red, center) double immunofluorescence in coronal sections of the CeA
617 of WT mice receiving vehicle, cocaine (20 mg/kg, i.p.) or quinpirole (1 mg/kg, i.p.). Insets are
618 magnifications of the boxed areas. **B)** Quantification of c-FOS activation induced by cocaine;
619 lines connect data from the same mouse. Two ways generalized linear mixed model with
620 Negative Binomial family (link: log); likelihood-ratio test: Type of neuron, $\chi^2(1)= 66.1$, $p<0.0001$;
621 Drug, $\chi^2(1)= 15.4$, $p<0.0001$; Drug*Type of neuron, $\chi^2(1)= 3.5$, $p=0.06$; vehicle, $n= 9$; cocaine, $n=$
622 7 ; post hoc Tuckey test shown. **C)** Quantification of c-FOS activation induced by quinpirole;
623 lines connect data from the same mouse. Two ways generalized linear mixed model with
624 Poisson family (link: log); likelihood-ratio test: Drug*Type of neuron, $\chi^2(1)= 9.3$, $p=0.03$; vehicle,
625 $n= 4$; quinpirole, $n= 4$; post hoc Tuckey test shown. **D)** Representative double
626 immunofluorescence for c-FOS (cyan) and TH (red) in the CeA and **(E)** quantification of c-FOS
627 expression in the CeA induced by saline (Veh) or quinpirole (Quin) (1 mg/kg, i.p.) given to *wild-*
628 *type* (wt) or *Drd2*KO (KO) mice. Two ways generalized linear mixed model with Negative
629 Binomial family (link: log); likelihood-ratio test: Drug*Genotype, $\chi^2(1)< 8.1$, $p<0.01$; post hoc
630 Tuckey test shown. Veh: WT, $n=5$; KO, $n=3$; Quin: WT, $n=5$; KO, $n=3$. CeL, CeA, lateral part;
631 CeM, CeA, medial part. Scale bars: 100 μ m. *** $p<0.001$, ** $p < 0.01$, n.s. $p>0.05$.

632

633

634 **Figure 3. Strategy for selective genetic ablation of D2Rs in the CeA.** **A)** Schematic of the
635 lentiviral vectors used to express Cre driven by the GAD67 promoter and EGFP driven by the
636 ubiquitin promoter. **B)** Schematic of lentiviral vectors bilateral injections. **C)** Strategy for
637 generating CeADrd2KO and control mice. **D)** Experimental timeline. OF, exploratory activity in
638 an open field; EPM, elevated plus maze; DLBT, dark/light box test; FC, fear conditioning. Each
639 vertical bar indicates one day. **E)** Representative histology of a coronal section showing co-
640 injections of LV:GAD-Cre and LV:Ub-EGFP into the CeA. **F)** LV:GAD-Cre-induced recombination
641 in $Drd2^{loxP/loxP}$ mice was verified by PCR with primers detecting deleted ($Drd2^-$), floxed ($Drd2^{loxP}$)
642 and *wild-type* ($Drd2^+$) alleles from biopsies containing the CeA or thalamus -as negative control-
643 of CeADrd2KO (n=4) and control (n=4) mice. Result of a mouse per group are shown, followed
644 by a negative control (water) and 2 positive controls. **G)** Representative histology of a coronal
645 brain section of a $Drd2$ -EGFP.Ai14 double transgenic mouse receiving a stereotaxic injection of
646 LV:GAD-Cre into the CeA. D2R+ (green), Cre-induced tomato (red).

647

648

649 **Figure 4. D2R ablation from the CeA increases avoidance in exploratory tasks.** The result of a
 650 MANOVA including every measure is shown at the center, right. **A) Dark/light box. Top:** time
 651 spent in the lit chamber. Student's t test, $t(15)=3.4$; $p = 0.004$. **Middle:** latency to first entry into
 652 lit chamber in a dark/light box test. Student's t test; $t(15)=-2.3$; $p=0.034$. **Bottom:** number of
 653 entries into the lit chamber in a dark/light box test. Generalized linear model with Poisson
 654 family (link: log); Wald test, $z=-1.7$, $p= 0.092$. **B) Elevated plus maze. Top:** Percentage of time on
 655 open arms. Only in this measure, the cohort of mice had a significant effect. Therefore, the
 656 percentage of time on open arms was analyzed in 2 different ways (see the "Elevated plus
 657 maze" section in **Materials and Methods**). **Top-left:** Percentage of time in open arms, 2 way
 658 (group x cohort) ANOVA with variance modeling ("VarIdent" function, applied to Cohort factor),
 659 Group, $F(1,13)=8.9$, $p=0.011$; Cohort, $F(1,13)=19.9$, $p < 0.001$; Group*Cohort, $F(1,13) = 0.16$,
 660 $p=0.69$. **Top-right:** Percentage of time in open arms normalized to the cohort average.
 661 Student's t Test; $t(15)=2.1$; $p=0.049$. **Bottom:** Percentage of entries to open arms over total
 662 entries (open+closed). Since statistical differences between cohorts were not detected,
 663 normalization to cohort averages was not performed. **Bottom-left:** Percentage of entries to
 664 open arms over entries to open and closed arms in an elevated plus maze, with data separated
 665 according to the cohort. Two-way Generalized linear mixed model with Quasi-binomial family
 666 (link: logit); Wald test; Group, $t= -0.31$, $p = 0.76$; Cohort, $t=1.86$, $p=0.09$; Group*Cohort, $t= 0.1$, p
 667 0.92 . **Bottom-right)** Percentage of entries to open arms, with data of both cohorts pulled.
 668 Generalized linear mixed model with Quasi-binomial family (link: logit); Wald test, $t= -0.37$, $p =$
 669 0.72 **C) Open field.** Time in center of the arena during the first 5 min of exposure. Student's t
 670 test, $t(15)= 0.35$, $p =0.73$. CeADrd2KO, $n= 9$; Ctrl, $n= 8$ in all experiments. ** $p<0.01$, * $p < 0.05$,
 671 n.s. $p>0.05$.

672

673 **Figure 5. D2R ablation from the CeA does not affect locomotion.** Daily distance travelled by
 674 CeADrd2KO and control mice during 30 min sessions of open field exploration; 2 way ANOVA
 675 with repeated measures; Group, $F(1,45)=0.001$, $p>0.1$; Days, $F(2,45)=76.3$, $p < 0.001$;
 676 Group*Days, $F(2,45) = 2$, $p > 0.1$. CeADrd2KO, $n= 9$ and Ctrl, $n= 8$. n.s., $p>0.05$.

677 **Figure 6. LV:GAD-Cre injections into the CeA of *Drd2*^{+/+} mice does not alter behaviors in**
 678 **exploratory tasks. A)** Experimental design with viral injections. **B)** Experimental timeline. OF,
 679 exploratory activity in an open field; EPM, elevated plus maze; DLBT, dark/light box test. Each
 680 vertical bar indicates one day. **C-E)** Avoidance behavior in exploratory tasks. The result of a
 681 MANOVA including every measure is shown in the center. **C) Dark/light box. Top:** time in the lit
 682 chamber. Student's t test, $t(12)=1.3$; $p=0.22$. **Middle:** latency to first entry to light chamber;
 683 Student's t test; $t(12)=1.1$, $p=0.29$. **Bottom:** number of entries to light chamber; Generalized
 684 linear model with quasi-Poisson family (link: log); Wald test, $z=1.51$, $p=0.16$. **D) Elevated plus**
 685 **maze. Top:** percentage of time on the open arms over the time on open and closed arms;
 686 Student's t test; $t(12)=0.21$; $p=0.83$. **Bottom:** Percentage of entries to open arms over total.
 687 Generalized linear mixed model with Quasi-binomial family (link: logit); Wald test, $z=-0.49$, $p=$
 688 0.64 . **E) Open field.** Time in center of the arena during the first 5 min of exposure; Student's t
 689 test, $t(12)=1.1$, $p=0.29$. LV:GAD-Cre, $n=7$; LV:Ub-EGFP, $n=7$ in all the experiments. ** $p<0.01$, *
 690 $p<0.05$, n.s. $p>0.05$.

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692

693 **Figure 7. Mice lacking D2R in the CeA express unaltered fear-conditioning memory. A)**
 694 Contextual fear conditioning training protocol. **B)** Percentage of freezing during the first 2 min
 695 of chamber habituation during the training (Pre-test, Generalized linear model with quasi-
 696 binomial family (link: log); Wald test, $z=1.23$, $p=0.23$). **C)** Percentage of freezing after each foot
 697 shock (Training session, Generalized linear mixed model with quasi-binomial family (link: log);
 698 LRT test; Group*Shock, $X^2(1)=2.8$, $p=0.24$; Group, $X^2(1)=0.2$, $p=0.65$; Shock, $X^2(1)=21.7$,
 699 $p<0.001$). **D)** Percentage of freezing during re-exposition to the conditioning chamber, 24 h
 700 after the training (testing session, Generalized linear model with quasi-binomial family (link:
 701 log); Wald test, $z=0.15$, $p=0.88$). Result of MANOVA including all variables is shown.
 702 CeADrd2KO, $n=9$ and Ctrl, $n=8$. n.s., $p>0.05$.

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Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7