A Novel Anxiogenic Role for the Delta Opioid Receptor Expressed in Gamma-Aminobutyric Acidergic Forebrain Neurons

Supplemental Information

Supplemental Methods and Materials

Animals

Mice with a floxed delta opioid receptor (DOR) gene ($Oprd1^{fl/fl}$) were previously generated in the laboratory (1). Briefly, a loxP site 5' and 3' from exon 2 of *Oprd1* gene is inserted by homologous recombination. It has been verified that $Oprd1^{fl/fl}$ show intact DOR activity by using a delta agonist-stimulated [^{35}S]-GTPγS binding assay on brain membrane preparations. The total KO mice (CMV-KO) were previously generated by interbreeding $Oprd1^{fl/fl}$ mice with CMV-Cre mice that expressed Cre recombinase under the cytomegalovirus promoter (2), leading to germ-line deletion of Oprd1 exon 2. The total excision has been verified by agonist-stimulated [^{35}S]-GTPγS binding assay. These mice were on a 75% C57BL/6J–25% 129SvPas background and were used as controls for behavioral and molecular studies. Additionally, in the present study wild-type mice (WT) were also used for agonist-stimulated [^{35}S]-GTPγS binding assay.

We used a Cre-LoxP strategy in order to delete DOR specifically from GABAergic neurons of the forebrain. The $Oprd1^{fl/fl}$ mice were interbred with Dlx5/6-Cre mice that expressed Cre recombinase in interneurons of the forebrain (obtained from Beat Lutz laboratory, Institute of Physiological Chemistry, Johannes Gutenberg University, Germany). The driver Dlx5/6-Cre line had been used successfully to produce a conditional knockout of cannabinoid 1 receptor in GABAergic neurons of the forebrain (3). We obtained $DOR^{fl/fl;\ Dlx5/6\text{-}Cre}$ (Dlx-DOR) and $DOR^{fl/fl}$ (Ctrl) littermate control with the same mixed genetic background (53.5% C57BL/6J; 45.5% 129SvPas; 1% unknown).

All mice were generated at Institut Clinique de la Souris-Institut de Genetique et Biologie Moleculaire et Cellulaire. $Oprd^{fl/fl}$ (Ctrl), conditional knockout mice (Dlx-DOR) and full knockout mice (CMV-DOR) used in behavioral tests were first habituated to their new experimental environment and handled for 1 week before starting the

experiments. An independent cohort of naïve animals was used for each behavioral paradigm, except for the elevated plus maze that was performed in the same cohort as open field test. All behavioral testing was performed with the observer blinded to the genotype and/or treatment of the animals.

Genomic DNA Analysis

Polymerase chain reaction (PCR) analysis on genomic DNA allows us to detect 1) the coding sequence of the Cre recombinase and 2) the exon 2 of Oprd1 gene flanked by loxP sites. PCR was performed on DNA from digit sample from $Oprd1^{fl/fl}$, $Dlx5/6\text{-}Oprd1^{-}$ and $CMV\text{-}Oprd^{-}$ mice using primers sequences GATCGCTGCCAGGATATACG (Cre recombinase forward), CATCGCCATCTTCCAGCAG (Cre recombinase reverse), CCTGGCCAGCCAGTTCACAATCT (Oprd1 forward) and GGTTAGCCTTCTGAGGGCTGGG (Oprd1 reverse).

Quantitative Real Time-PCR

The olfactory bulb (OB), prefrontal cortex, caudate-putamen nucleus (CPu), nucleus accumbens (NAc), amygdala (Amy) were bilaterally punched and ventral (vHipp), dorsal hippocampus (dHipp) and spinal cord (SC) were dissected from 4 animals per genotype, and RNA samples processed for quantitative real time PCR, as detailed previously (4). Briefly, total RNA was extracted by TriZol (Invitrogen, Cergy Pontoise, France). Quality and quantity of the RNA was evaluated by a ND-1000 Nanodrop spectrophotometer and gel electrophoresis. Total RNA (1 μg) from each bilaterally pooled brain region was reverse transcribed in a final volume of 20 μl. Real-time PCR was performed on cDNA in triplicate on a Light-Cycler-480 instrument (Roche). Primer sequences were GACGGCCAGGTCATCACTAT (β-actin forward), CCACCGATCCACACAGAGTA (β-actin reverse), TGAGATTCGGGATATGCTGTTG (36B4 forward), TTCAATGGTGCCTCTGGAGAT (36B4 reverse), GCTCGTCATGTTTGGCATC (Oprd1 forward) and AAGTACTTGGCGCTCTGGAA (Oprd1 reverse). Relative expression ratios were normalized to level of the 36B4

reference gene, and the 2-$\Delta\Delta C_t$ method was used to evaluate differential expression levels (5).

Autoradiographic Binding Assay

Following decapitation, intact brains were removed, snap frozen at -20°C in isopentane and then stored at -80°C until sectioned. Brains were sectioned in a cryostat (Zeiss Hyrax C 25, Carl Zeiss MicroImaging GmbH, Germany), with an internal temperature of -21°C. SCs were mounted in OCT medium before sectioning. Twenty µm coronal sections were cut at 300 µm intervals, from rostral to caudal levels and thaw-mounted onto gelatine coated ice-cold microscope slides and processed for autoradiography. Adjacent sections were cut from wild-type, Dlx-DOR and full CMV-DOR brains (*n* = 3) for determination of total binding for DOR using [^{3}H] deltorphin-1. Sections were stored at -20°C for radioligand binding.

On the day of the experiment, sections were thawed and processed according to established protocols (6,7), with minor modifications. Sections for analysis were derived from four to six brains from each of the six treatment groups (*n* = 3-4 per group). Multiple, adjacent sections from all groups were processed together in a paired binding protocol.

For binding, slides were pre-incubated for 30 min in 50 mM Tris-HCl pre-incubation buffer, containing 0.9% w/v NaOH, pH 7.4 at room temperature. The slides were then incubated in 50 mM Tris-HCl buffer, pH 7.4 at room temperature in the presence of 7 nM [^{3}H]deltorphin-1 for 60 min. Non-specific binding (NSB) was determined in adjacent sections in the presence of 10 µM naloxone. Incubation was terminated by rapid rinses (3 x 5 min) in ice-cold 50 mM Tris-HCl buffer, pH 7.4 at room temperature and distilled water (3 x 5 min). Slides were then rapidly cool-air dried.

Film exposure and development. Following binding, sections were rapidly dried under cold air for 2 hours, and dried for up to 7 days using anhydrous calcium sulphate (BDH Chemicals, Poole, UK). Adjacent total and non-specific labeled sections were apposed to Kodak BioMax MR-1 film alongside autoradiographic microscale standards of known concentration. [^{3}H]-bound sections were exposed to film with ^{3}H microscale standards for a period of 10 weeks for opioid receptors.

For development, films were covered with an aqueous solution of 50 % v/v Kodak D19 developer for 1 min. The reaction was stopped by 1 min rinse in distilled water containing a drop of glacial acetic acid. Images were fixed by submersion in Kodak rapid fix solution for 5 min. Films were then rinsed in distilled water and dried overnight in a fume cupboard.

Quantitative analysis. Films were analyzed by video-based densitometry using an MCID image analyzer (Imaging Research, Canada) as previously described by Kitchen *et al.* (7). In brief, fmol/mg tissue equivalents for receptor binding were derived from [^{3}H] microscale standards, and the relationship between tissue radioactivity and optical density was calculated using MCID software, with appropriate adjustments to allow for radioactive decay of both the standards and the radioligands. Specific receptor binding was derived by subtraction of NSB from total binding. NSB was homogenous across each film.

For each region quantified measures were taken from both left and right hemispheres, therefore receptor binding represents a duplicate determination for each brain region and the *n* values listed refer to the number of animals analyzed. The following structures were analyzed by sampling 5 – 20 times with a box tool: cortex (8 x 8 mm), olfactory tubercle (6 x 6 mm) and Hipp (5 x 5 mm). All other regions were analyzed by free-hand drawing. Brain structures were identified by reference to the mouse atlas of Franklin and Paxinos (8).

Agonist-Stimulated [^{35}S]-GTPγS Binding Assays

Brain areas obtained by mechanical punches on 1 mm thickness sections (OB, Hipp and CPu) and SC from Ctrl, Dlx-DOR and CMV-DOR mice were processed for membrane preparation as described (9). Samples were incubated with and without the DOR agonist ARM390 (10^{-4} to 10^{-11} M) for 1 h at 25°C in assay buffer containing 30 μM GDP and 0.1 nM [^{35}S]GTPγS. Non specific binding was defined as binding in the presence of 10 μM GTPγS, and basal binding indicates binding in the absence of agonist (Table S1).

Behavioral Assays

The behavioral tests were performed as previously described (10-12).

Olfactory discrimination

This test has been adapted from a previously described procedure (13) and is used to assess odor detection (discrimination of novel attractive odors from neutral odor) and odor habituation (reduced preference for novel odor following repeated exposure) in two successive sessions. The procedure exploits the innate preference of mice to novel stimuli and therefore does not require prior training or reward that could influence discrimination performance. A cohort of Ctrl and Dlx-DOR mice was assessed for water versus social odor discrimination, and a second cohort for water versus non-social odors (lemon) discrimination. Social odor was obtained from Balbc mice of the same sex by swiping the cage bottom with the cotton-tipped applicator. The odor source cages were kept in an adjacent room. Lemon odor was selected based on preliminary experiments showing that wildtype mice display a clear-cut preference (approaches behavior and olfactory investigation) for the former odor over water (no odor). Odor exploration was measured in a clean cage with fresh bedding. Odors were presented on two cotton-tipped wooden applicators. Animals were first habituated to the novel cage for 3 min, and then tested during 3 consecutive sessions of 3 min each with 2 min inter-trial interval. During each session, two applicators are presented: (“Hab” session) water-water, (“Odor 1” session) water-odor and (“Odor 2” session) odor-water. The position of the odor for the sessions (“Odor 1”) and (“Odor 2”) was randomized. Two stopwatches are used to record the cumulative time spent sniffing each tip.

Depressive-like behavior

Forced swim test. This test allows the detection of despair behaviors by forcing mice to swim in a narrow cylinder from which they cannot escape. Each mouse was placed in a Plexiglas cylinder containing water to a depth of 15 cm (21°C–23°C) as previously described (14). After a brief period of vigorous activity, the mice adopt a characteristic immobile posture that is reversed by the administration of compounds with antidepressant activity. Each animal was submitted to a forced swim session of 6 min, and the total duration of immobility, swimming and climbing behaviors were measured.

Tail suspension test. This test allows assessment of depressive-like behaviors and was performed as previously described (15). Mice were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. The total duration of immobility and the latency for the first immobility period of at least 2 s are automatically recorded during a 6-min period as previously described.

Locomotion

Locomotor activity boxes. Mice were placed individually in actimetry boxes consisting of a plastic square area (25 x 25 x 25 cm, 100 lux). The distance covered by the mouse was recorded by a videotracking system for periods of 5 min, over 24 h, with water and food pellet ad libitum.

DOR or dopamine D1/D3 receptor agonists-induced hyperlocomotion. Animals were tested in actimetry boxes as described above. Mice freely explored the box during 2 h (habituation session), then received an injection of saline or SNC80 (10 mg/kg, intraperitoneal) or D1/D3 dopamine receptor agonist SKF-81297 (1 or 2.5 mg/kg, subcutaneous injection) and were placed back in the same boxes for further recording (90 min).

Anxiety-related behavior

Open field. The apparatus is composed of a black ground square (45 x 45 cm) limited by transparent Plexiglas walls (18 cm) and under indirect illumination (50 lux). Test was performed as previously described (11). Movements are detected by infrared beams and sensors so that distance (cm) and time spent (s) in periphery and center parts of the apparatus were automatically recorded (Viewpoint software) each period of 5 min. Sessions lasted for 30 min starting with the mouse positioned in a corner.

Elevated plus-maze test (EPM). The EPM consisted of four arms (30 x 5 cm) in black Plexiglas set in cross from a neutral central square (5 x 5 cm) (Imetronic). Two opposite arms were delimited by vertical walls (closed arms) and the two other opposite arms had unprotected edges (3 mm) (open arms). The maze is elevated 60 cm above the ground and placed in indirect light (50 lux). Test was performed as previously described (16;11). Movements are detected by infrared beams and sensors so that locomotor activity, time spent, number of entries and number of attempts to enter in

open or closed arms were automatically recorded (Viewpoint software). Sessions lasted for 5 min starting with the mouse in the central square.

Novelty suppressed feeding (NSF). The NSF is a conflict test based on opposite behaviors: the motivation to obtain the food pellet versus the natural avoidance of an aversive environment. The NSF was carried out as previously described (17). The testing box consisted of an open field box (50 x 50 x 35 cm) with 5 cm of fresh sawdust on the floor. Two or three food pellets of ordinary lab chow were placed on a white paper positioned at the center of the apparatus. After 24 h of food deprivation and water *ad libitum*, mice were placed in a corner of the testing apparatus. Sessions lasted for a maximum of 15 min. We counted the number of approaches to the food pellets and the latency to feed that was scored as the time when the mice began biting the food. Immediately after that, the mice were transferred to their home cage for 5 min, and food intake amount over this time was measured. The weight of the home-cage food was measured before and after the 5 min, and the difference was calculated (home-cage food intake).

Chocolate Food-Pellets Self-Administration

Animals. All experiments were carried out with Ctrl and Dlx-DOR 2-4 months old male littermates. Mice were housed individually in controlled laboratory conditions with the temperature maintained at 21 ± 1ºC and humidity at 55 ± 10%. Mice were tested during the first hours of the dark phase of a reversed light/dark cycle (lights off at 8.00 h and on at 20.00 h). Mice were food-deprived (85% of the initial weight) and water was available *ad libitum*. Animal procedures were conducted in strict accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and were approved by the local ethical committee (CEEA-PRBB).

Food self-administration apparatus. Operant responding maintained by food was performed in mouse operant chambers (Model ENV-307A-CT, Med Associates, Georgia, VT, USA) equipped with two holes, one randomly selected as the active hole and the other as the inactive. Stimuli lights (cues), one located inside the active hole and the other above it were paired with the delivery of the reinforcer. Nose-poking on the active hole resulted in a pellet delivery together with a stimulus-light named

conditioned stimulus (CS), located above the active hole and inside the hole while pressing on the inactive lever had no consequences. The chambers were made of aluminum and acrylic, and were housed in sound- and light-attenuated boxes equipped with fans to provide ventilation and white noise. A food dispenser equidistant between the two levers permitted delivery of food pellets when required.

Acquisition of operant responding maintained by chocolate. Ctrl and Dlx-DOR male mice (*n* = 39) were trained during 1 h for 10 consecutive days to nose-poke for chocolate-flavored food-pellets as reward, paired with the presentation of a cue-light serving as CS, on a fixed ratio (FR) 1 schedule of reinforcement followed by 5 sessions under FR5. Each chocolate-flavored pellet (TestDiet, Richmond, IN, USA) of 20 mg (20.5% protein, 12.7% fat, 66.8% carbohydrate, with a caloric value of 3.48 kcal/g) contained the addition of chocolate flavor (2% pure unsweetened cocoa), and the proportion of sugars within the carbohydrate part included a sucrose content of 50.11%. The criteria for acquisition of operant responding were achieved when mice maintained a stable responding with less than 20% deviation from the mean of the total number of food-pellets earned in three consecutive sessions, with at least 75% responding on the reinforced lever, and a minimum of 10 reinforcements per session (18;19). Mice were food-deprived during the whole experiment at 85% of their *ad libitum* initial weight adjusted for growth. After the 15 FR sessions, animals were tested in a progressive ratio (PR) schedule during one session where the response requirement to earn the reinforcer escalated according to the following series: 1-5-12-21-33-51-75-90-120-155-180-225-260-300-350-410-465-540-630-730-850-1000-1200-1500-1800-2100-2400-2700-3000-3400-3800-4200-4600-5000-5500. The maximum duration of the PR session was 5 h or until mice did not respond on any lever within 1 h. After each session, mice were returned to their home-cages. Each chamber was cleaned at the end of each session to prevent the presence of odor of the previous mouse. After PR session, mice were moved to the extinction phase.

Extinction of operant responding maintained by chocolate. The experimental conditions during the extinction phase were similar to the acquisition of operant responding sessions except that chocolate were not available and stimuli lights (environmental cues) were not presented after nose-poking in the active hole. Mice

were given 1-h daily sessions (7 days per week) until reaching the extinction criterion. The criterion for extinction was achieved when mice made during 3 consecutive sessions a mean number of nose-poking in the active hole of less than 30% of the responses obtained during the mean of the three days of achievement of the acquisition criteria of chocolate self-administration training. All animals were run during 10 consecutive daily sessions. Then after, all mice were test under reinstatement induced by cue.

Cue-induced reinstatement. The presentation of conditioned environmental cue was performed to evaluate the reinstatement of food-seeking behavior. Test for cue-induced reinstatement was conducted under the same conditions used in the training phase except that chocolate-flavored pellets were not available. Each nose-poke in the active hole led to the presentation of both stimuli lights for 2 sec. The reinstatement criterion was achieved when nose-pokes in the active hole were double than nose-pokes in the active hole during the three 3 consecutive days that mice acquired extinction criteria or a minimum of 10 nose-pokes in the active hole.

Statistical analysis. Analysis of the data of number of pellets obtained during the acquisition phase was conducted using two-way analysis of variance (ANOVA) of repeated measures with day as within-subjects factors and genotype as between-subjects factor. Post-hoc analysis (Newman-Keuls) was performed when required. Data of active and inactive nose-poking responses during the FR1, FR5 or extinction was conducted using three-way ANOVA of repeated measures with day and hole (active/inactive) as within-subjects factors and genotype as between-subjects factor. Post-hoc analysis (Newman-Keuls) was also performed when required. Data of the breaking point achieved during the PR session was analyzed with one-way ANOVA with genotype as between-subjects factor. To evaluate the extinction and cue-induced reinstatement, three-way ANOVA of repeated measures was performed with experimental phase and hole as within-subjects factors, and genotype as between-subject factor. Post-hoc analysis (Newman-Keuls) was performed when required. All results are expressed as mean ± SEM. Differences were considered significant at $p < 0.05$. The statistical analysis was performed using the Statistical Package for Social Science program SPSS® 15.0 (SPSS Inc, Chicago, USA).

c-Fos Immunohistochemistry

Animals were deeply anesthetized with an overdose of ketamine and xylazine (10 ml/kg, intraperitoneal; ketamine 1 g/kg; xylazine 100 mg/kg) 90 min after the beginning of the behavioral test (NSF), and perfused transcardially with 50 ml of 0.1 M phosphate buffer (PB, pH 7.4) followed by 50 ml of cold 4% paraformaldehyde prepared in 0.1 M PB. Brains were dissected, post-fixed for 48 hours in the same fixative and cryoprotected in 30% sucrose/PB overnight at 4°C. Frozen brains were stored at -80°C until 50 μm coronal sections were cut on a cryostat.

Immunohistochemistry was performed on free-floating sections using a standard avidin-biotin (ABC) peroxidase method (Elite Vectastain Kit, Vector Laboratories, Burlingame, CA, USA) as previously (20). The peroxidase was detected with diaminobenzidine (Sigma-Aldrich, Saint-Quentin, France) as chromogen. The primary antibody was a rabbit polyclonal antibody (Ab-5, Calbiochem®, Merck, Darmstadt, Germany, 1:2,000) rose against a synthetic peptide derived from amino acid sequences 4-17 of the Fos protein. The secondary antibody was a biotinylated goat anti-rabbit IgG (Jackson Immunoresearch, West Baltimore Pike, PA, USA, 1:2,000).

Slides were acquired using a Hamamatsu Nanozoomer 2-HT whole slide scanner (Hamamatsu Photonics, Hamamatsu, Japan) at 20x magnification. Frames focused on each structure of interest were acquired using NDP View software, and Fos-positive nuclei were counted using ImageJ software (NIH). Data were expressed as the number of Fos-positive nuclei per mm^2. The number of Fos-immunoreactive neurons in each brain region was assessed bilaterally using 6 to 12 sections for each animal (6 to 8 mice per genotype). Fos immunostaining was evaluated in 8 cerebral regions (the basolateral, central and basomedial nuclei of the Amy; the CPu nucleus; the cingulate cortex; the insular cortex; the NAc core and shell; the ventral tegmental area) according to the mouse brain atlas (8). Brain regions of interest were selected as involved in anxiety and reward/approach processes.

Drugs

The SNC80 (Tocris Bioscience, Bristol, UK) was prepared as previously described (9). The powder was dissolved in NaCl 0.9% and was administered at a dose of 10 mg/kg. The solutions were prepared before the experiments.

The dopamine D1 receptor agonist SKF-81297 (2,3,4,5-tetrahydro-6-chloro-7,8-dihydroxy-phenyl-1H-3-benzazepine; Tocris Bioscience, Bristol, UK) was prepared as previously described (12). The SKF-81297 was dissolved in NaCl 0.9% and was administered at a dose of 1 or 2.5 mg/kg. The SNC80 was dissolved by probe sonication in acidic saline solution (pH 4-5) and acidic saline solution (pH 4-5) was used as a control.

Both compounds were administered intraperitoneally before the experiments in a volume of 10 ml/kg.

Statistical Analyses

Statistical differences were determined by ANOVA (StatView 5, SAS Institute Inc., Cary, North Carolina) followed by Bonferroni/Dunn post hoc analysis. The *F* values and experimental degrees of freedom are included in the Results section. For experiments with two groups, a Student *t* test was used. The level of statistical significance was set at $p < 0.05$. For the behavioral tests during which data were obtained on several periods during the same session (locomotor tests, the open field test and despair-like behavior paradigms), the analysis of variance repeated measures was used.

Supplemental Results

Dlx-DOR Mice Show Normal Olfaction and Despair Behavior

We previously found that constitutive DOR KO mice display a depressive-like phenotype (11). In this study, Dlx-DOR mice show a major DOR loss in OB and NAc, two areas associated with altered mood. For example, olfactory bulbectomy is classically used to induce depressive-like symptoms in rodents (21) and deep brain stimulation of the NAc is being investigated in patients suffering from treatment-resistant depression (22). We therefore examined whether Dlx-DOR mice show a phenotype similar to constitutive KO mice.

Because lack of DORs in OB could perturb basal olfactory perception, we first tested Dlx-DOR mice and their control littermates for discrimination between neutral (water) and attractive odors (social or lemon odor (Figure S1A). During the first exposure, Dlx-DOR and control mice showed high preference for social compared to neutral odor ($p < 0.001$, vs. habituation and chance level, Student *t*-test). After re-exposure to the same odor, both genotypes showed a clear decline in preference (habituation) and there was no difference between genotypes during the first or second exposure to social odor ($p > 0.05$, two-way ANOVA). Similar results were obtained upon repeated animal exposure to non-social odor (lemon odor, data not shown). Thus, Dlx-DOR mice show no gross alteration in olfactory skills that may confound behavioral testing for emotional responses.

Dlx-DOR and control littermates were then submitted to forced swim and tail suspension tests (Figure S1B) classically used to assess despair behaviors in rodents (14,15). Dlx-DOR mice did not significantly differ from controls for either forced swim (time of floating, $p > 0.05$, one-way ANOVA and Student *t*-test) or tail suspension (time of immobility, $p > 0.05$, one-way ANOVA and Student t-test). DOR deletion in forebrain GABAergic neurons, therefore, does not alter despair behavior in those tests.

Table S1. DOR coupling to G proteins using ARM390-stimulated [^{35}S]-GTPγS binding assay in control (Ctrl), conditional (Dlx-DOR) and total knockout (CMV-DOR) mice. E_{max} (%) corresponds to the maximal percentage of activation above basal [^{35}S]-GTPγS, and reflects receptor activity. CMV-DOR mice samples showed E_{max} values close to 100%, reflecting no detectable G-protein activation in any regions. Lower E_{max} values in samples from olfactory bulb and caudate-putamen in Dlx-DOR mice compared to Ctrl indicate reduced G-protein signaling, reflecting lower receptor density. A trend for reduced activity was observed in the hippocampus of Dlx-DOR mice, whereas DOR signaling was unchanged in prefrontal cortex and spinal cord. *n* = 3-4. One-way ANOVA revealed significant effect of Genotype for each region, Olfactory Bulb ($F_{(2,6)}$ = 107.37; p < 0.001), Frontal cortex ($F_{(2,6)}$ = 80.76; p < 0.001), Hippocampus ($F_{(2,9)}$ = 21.25; p < 0.001), Caudate-putamen ($F_{(2,8)}$ = 64.32; p < 0.001) and Spinal Cord ($F_{(2,9)}$ = 5.20; p = 0.032). Post-hoc comparisons revealed significant differences compared to control: ***p < 0.001.

E_{max} (%)

Region	Ctrl	Dlx-DOR	CMV-DOR
Olfactory Bulb	245.3 ± 10.2	121.7 ± 2.8***	107.8 ± 2.9***
Frontal Cortex	156.8 ± 2.8	144.3 ± 3.6	111.8 ± 3.1***
Hippocampus	163.1 ± 3.6	144.0 ± 4.2	119.1 ± 6.2***
Caudate-Putamen	177.8 ± 5.6	116.5 ± 4.9***	107.5 ± 4.4***
Spinal Cord	136.0 ± 1.1	140.4 ± 6.6	119.0 ± 5.3

Table S2. Chocolate flavored pellets self-administration. Operant responding maintained by chocolate-flavored food-pellets during acquisition, extinction and cue-induced reinstatement. Statistical analysis of chocolate-flavored food pellets self-administration experiment. Ctrl and Dlx-DOR mice were tested in fixed ratio sessions (FR1 and FR5), then under a progressive ratio schedule (PR) as an index of the motivation and under extinction as well as cue-induced reinstatement sessions. Genotype effect corresponds to the comparison between Ctrl (n = 23) and Dlx-DOR mice (n = 16). Hole effect refers to the comparison between the active and inactive nosepoke. Three-way ANOVA with genotype as between-subjects factor and repeated measures in the factors day/experimental phase and hole (active/inactive). See materials and methods for details.

	Three-way ANOVA			
	Acquisition FR1		**Acquisition FR5**	
	***F*-value**	***p*-value**	***F*-value**	***p*-value**
Genotype	$F_{(1,37)} = 0.77$	*n.s.*	$F_{(1,37)} = 0.22$	*n.s.*
Hole	$F_{(1,37)} = 1043.17$	$p < 0.001$	$F_{(1,37)} = 1772.36$	$p < 0.001$
Day	$F_{(9,333)} = 50.54$	$p < 0.001$	$F_{(4,148)} = 3.64$	$p < 0.01$
Genotype × Hole	$F_{(1,37)} = 0.43$	*n.s.*	$F_{(1,37)} = 1.26$	*n.s.*
Genotype × Day	$F_{(9,333)} = 0.50$	*n.s.*	$F_{(4,148)} = 0.11$	*n.s.*
Hole × Day	$F_{(9,333)} = 78.15$	$p < 0.001$	$F_{(4,148)} = 4.38$	$p < 0.01$
Genotype × Hole × Day	$F_{(9,333)} = 0.79$	*n.s.*	$F_{(4,148)} = 0.57$	*n.s.*

	Extinction		**Cue-induced reinstatement**	
	***F*-value**	***p*-value**	***F*-value**	***p*-value**
Genotype	$F_{(1,37)} = 1.92$	*n.s.*	$F_{(1,37)} = 0.02$	*n.s.*
Hole	$F_{(1,37)} = 73.78$	$p < 0.001$	$F_{(1,37)} = 618.76$	$p < 0.001$
Day	$F_{(9,333)} = 25.50$	$p < 0.001$	$F_{(3,111)} = 281.92$	$p < 0.001$
Genotype × Hole	$F_{(1,37)} = 1.94$	*n.s.*	$F_{(1,37)} = 0.01$	*n.s.*
Genotype × Day	$F_{(9,333)} = 1.80$	*n.s.*	$F_{(3,111)} = 0.66$	*n.s.*
Hole × Day	$F_{(9,333)} = 24.30$	$p < 0.001$	$F_{(3,111)} = 292.03$	$p < 0.001$
Genotype × Hole × Day	$F_{(9,333)} = 1.91$	$p < 0.05$	$F_{(3,111)} = 0.55$	*n.s.*

n.s.: non significant

Table S3. Quantification of c-fos immunoreactivity in control (Ctrl) and conditional (Dlx-DOR) mice under basal conditions and upon food deprivation. Under standard housing conditions with food and water *ad libitum*, Ctrl and Dlx-DOR mice show similar levels of c-fos protein expression in all regions considered. Ctrl and Dlx-DOR mice were also exposed to 24 h of food deprivation in the standard housing cages. Dlx-DOR mice showed decrease c-fos positive cells in the nucleus accumbens, insular cortex and VTA. No differences between the two genotypes were detected in the other regions. *$p < 0.05$; **$p < 0.01$ (Student *t*-test).

Region		Bregma coordinates	c-fos positive cells / mm^2		Stats
			Ctrl	Dlx-DOR	
Basal conditions					
Nucleus accumbens	NAc	1.18	138.3 ± 56.3	118.5 ± 1	$p>0.05$
Cingulate cortex	Cg Cx	1.10	246.9 ± 138.1	83.6 ± 1.4	$p>0.05$
Insular cortex	Ins Cx	1.10	131.8 ± 42.5	76.5 ± 14.1	$p>0.05$
Striatum		1.10			
Dorsomedial	DM		155.0 ± 105.0	45.8 ± 7.5	p>0.05
Dorsolateral	DL		123.8 ± 86.3	35 ± 5.8	p>0.05
Ventromedial	VM		124.2 ± 65.8	51.7 ± 5.0	p>0.05
Ventrolateral	VL		75.4 ± 34.6	35.8 ± 0.0	p>0.05
Amygdala		-1.46			
Basolateral nucleus	BLA		118.8 ± 66.4	103.2 ± 49.5	$p>0.05$
Basomedial nucleus	BMA		130.7 ± 58.7	133.8 ± 48.5	$p>0.05$
Central nucleus	CeA		165.6 ± 71.7	121.3 ± 18.7	$p>0.05$
Ventral tegmental area	VTA	-2.54	98.8 ± 13.8	62.5 ± 1	$p>0.05$
24h food deprivation					
Nucleus accumbens	NAc	1.18	230.0 ± 9.5	70.8 ± 16.3*	$p<0.05$
Cingulate cortex	Cg Cx	1.10	215.3 ± 58.1	71.7 ± 19.4	$p>0.05$
Insular cortex	Ins Cx	1.10	209.3 ± 21.3	61.3 ± 8.7*	$p<0.05$
Striatum		1.10			
Dorsomedial	DM		71.7 ± 23.3	77.5 ± 12.5	$p>0.05$
Dorsolateral	DL		67.1 ± 33.8	60.4 ± 13.8	$p>0.05$
Ventromedial	VM		80.0 ± 10.0	39.2 ± 2.5	$p>0.05$
Ventrolateral	VL		69.2 ± 2.5	26.7 ± 1.7	$p>0.05$
Amygdala		-1.46			
Basolateral nucleus	BLA		138.1 ± 11.0	49.7 ± 21.6	$p>0.05$
Basomedial nucleus	BMA		201.8 ± 21.7	155.6 ± 15.6	$p>0.05$
Central nucleus	CeA		182.1 ± 18.6	102.3 ± 38.4	$p>0.05$
Ventral tegmental area	VTA	-2.54	93.8 ± 1.3	75.0 ± 0.0**	$p<0.01$

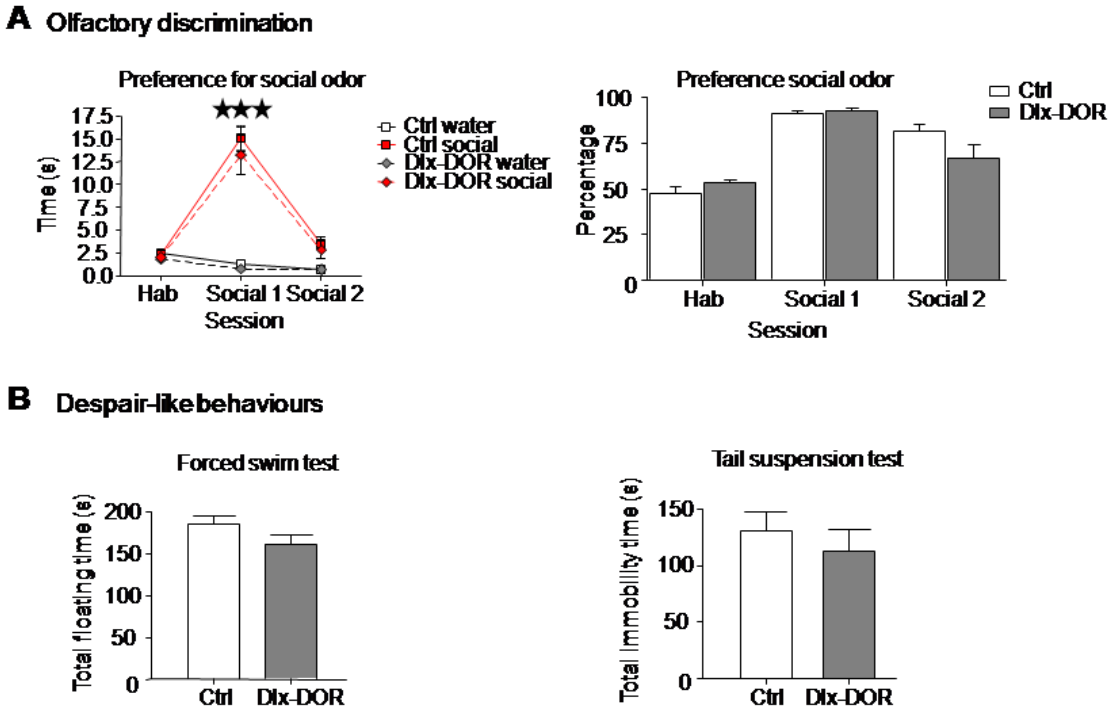

Figure S1. Olfactory discrimination skills and despair behavior. Ctrl and Dlx-DOR mice were tested for olfactory discriminative skills. Hab, habituation session with two neutral odors presented; Social 1, first exposure to social versus neutral odor (water); Social 2, exposure to same odors as Social 1 but in inverted positions **(A)** Time spent sniffing the social and neutral odor (left panel) and preference for the social odor (right). Dlx-DOR and Ctrl mice showed comparable increased exploration time for the social odor at first exposure and showed similar preference above chance level when exposed to the social odor (*n* = 11-16 per genotype). **(B)** Forced swim test (left) and tail suspension test (right). A slight decrease in immobility time was observed in Dlx-DOR mice (gray bars) compared with Ctrl (white bars), however no significant difference was found across genotypes (*n* = 16 per genotype). For all the tests, filled stars indicate significant differences between treatments. ***$p < 0.001$ (one-way ANOVA).

Supplemental References

1. Gaveriaux-Ruff C, Nozaki C, Nadal X, Hever XC, Weibel R, Matifas A, *et al.* (2011). Genetic ablation of delta opioid receptors in nociceptive sensory neurons increases chronic pain and abolishes opioid analgesia. Pain 152(6):1238-1248.
2. Metzger D, Chambon P (2001). Site- and time-specific gene targeting in the mouse. *Methods* 24(1):71-80.
3. Monory K, Massa F, Egertová M, Eder M, Blaudzun H, Westenbroek R, *et al.* (2006). The endocannabinoid system controls key epileptogenic circuits in the hippocampus. *Neuron* 51(4):455-466.
4. Befort K, Filliol D, Ghate A, Darcq E, Matifas A, Muller J, *et al.* (2008). Mu-opioid receptor activation induces transcriptional plasticity in the central extended amygdala. *Eur J Neurosci* 27(11):2973-2984.
5. Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25(4):402-408.
6. Kitchen I, Leslie FM, Kelly M, Barnes R, Crook TJ, Hill RG, *et al.* (1995). Development of delta-opioid receptor subtypes and the regulatory role of weaning: radioligand binding, autoradiography and in situ hybridization studies. *J Pharmacol Exp Ther* 275(3):1597-1607.
7. Kitchen I, Slowe SJ, Matthes HW, Kieffer B (1997). Quantitative autoradiographic mapping of mu-, delta- and kappa-opioid receptors in knockout mice lacking the mu-opioid receptor gene. *Brain Res* 778(1):73-88.
8. Franklin KBJ, Paxinos G (1997). *The Mouse Brain in Stereotaxic Coordinates.* San Diego, CA: Academic Press.
9. Pradhan AA, Becker JA, Scherrer G, Tryoen-Toth P, Filliol D, Matifas A, *et al.* (2009). In vivo delta opioid receptor internalization controls behavioral effects of agonists. *PLoS One* 4(5):e5425.
10. Goeldner C, Lutz PE, Darcq E, Halter T, Clesse D, Ouagazzal AM, Kieffer BL (2011). Impaired emotional-like behavior and serotonergic function during protracted abstinence from chronic morphine. *Biol Psychiatry* 69(3):236-244.
11. Filliol D, Ghozland S, Chluba J, Martin M, Matthes HW, Simonin F, *et al.* (2000). Mice deficient for delta- and mu-opioid receptors exhibit opposing alterations of emotional responses. *Nat Genet* 25(2):195-200.
12. Le Merrer J, Rezai X, Scherrer G, Becker JA, Kieffer BL. (2013). Impaired hippocampus-dependent and facilitated striatum-dependent behaviors in mice lacking the delta opioid receptor. *Neuropsychopharmacology* 38(6):1050-9.
13. Yang M, Crawley JN (2009). Simple behavioral assessment of mouse olfaction. *Curr Protoc Neurosci* Chapter 8: Unit 8 24.
14. Porsolt RD, Anton G, Blavet N, Jalfre M. (1978). Behavioural despair in rats: a new model sensitive to antidepressant treatments. *Eur J Pharmacol* 47(4):379-391.
15. Steru L, Chermat R, Thierry B, Simon P. (1985). The tail suspension test: a new method for screening antidepressants in mice. *Psychopharmacology (Berl)* 85(3):367-370.

16. Pellow S, Chopin P, File SE, Briley M. (1985). Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J Neurosci Methods* 14(3):149-167.
17. Santarelli L, Gobbi G, Debs PC, Sibille ET, Blier P, Hen R, Heath MJ. (2001). Genetic and pharmacological disruption of neurokinin 1 receptor function decreases anxiety-related behaviors and increases serotonergic function. *Proc Natl Acad Sci U S A* 98(4):1912-1917.
18. Martín-García E, Burokas A, Kostrzewa E, Gieryk A, Korostynski M, Ziolkowska B, *et al.* (2011). New operant model of reinstatement of food-seeking behavior in mice. *Psychopharmacology (Berl)* 215(1):49-70.
19. Burokas A, Gutiérrez-Cuesta J, Martín-García E, Maldonado R. (2012). Operant model of frustrated expected reward in mice. *Addict Biol* 17(4):770-782.
20. Le Merrer J, Gavello-Baudy S, Galey D, Cazala P. (2007). Morphine self-administration into the lateral septum depends on dopaminergic mechanisms: Evidence from pharmacology and Fos neuroimaging. *Behav Brain Res* 180(2):203-217.
21. Song C, Leonard BE (2005): The olfactory bulbectomised rat as a model of depression. *Neurosci Biobehav Rev*. 29:627-647.
22. Bewernick BH, Kayser S, Sturm V, Schlaepfer TE (2012): Long-term effects of nucleus accumbens deep brain stimulation in treatment-resistant depression: evidence for sustained efficacy. *Neuropsychopharmacology*. 37:1975-1985.