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RESEARCH PAPER

The orphan receptor GPR3 modulates the early phases of cocaine reinforcement

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Gpr3 receptor-deficient mice; orphan receptor; adenylyl cyclase; cocaine; reinforcement; intravenous self-administration

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BACKGROUND AND PURPOSE
The modulatory activity of the orphan receptor GPR3 in the brain has been related to the control of emotional behaviours. Limbic structures that express GPR3 have been associated with the effects of drug abuse.

EXPERIMENTAL APPROACH
The role of GPR3 in different cocaine-elicited behaviours including locomotor activity, behavioural sensitization, conditioned place preference (CPP) and intravenous self-administration was evaluated in *Gpr3*–/– mice and their *Gpr3*+/+ littermates. Cocaine-induced dopamine release in the nucleus accumbens was also evaluated to elucidate the effect of *Gpr3* deletion on extracellular levels of dopamine.

KEY RESULTS
Gpr3–/– mice exhibited higher rewarding responses in the CPP paradigm. *Gpr3*–/– mice self-administered more cocaine, especially during the first days of training. No differences were found between genotypes regarding behavioural sensitization and the maximal effort required to obtain a cocaine infusion. Non-contingent priming injections of cocaine before operant training eliminated enhanced cocaine self-administration in *Gpr3*–/– mice. Extracellular levels of dopamine in the nucleus accumbens induced by cocaine did not differ between genotypes.

CONCLUSIONS AND IMPLICATIONS
The increased responsiveness of *Gpr3*–/– mice to the acute locomotor effects of cocaine and the inconsistency to further increase this effect reflected an 'already maximally sensitized' basal state. Enhanced responsiveness of *Gpr3*–/– mice to cocaine reward and to early phases of reinforcement suggests that an initial alteration increased vulnerability to this type of drug abuse. Overall, altered signalling pathways of GPR3 could contribute to the neurobiological substrate involved in developing addiction to cocaine.

Abbreviations
CPP, conditioned place preference; FR1, fixed ratio 1; PR, progressive ratio schedule of response

Introduction

GPR3 is an orphan GPCR that causes strong constitutive activation of adenylyl cyclase in various mammalian cell lines (Eggerickx *et al.*, 1995; receptor nomenclature follows Alexander *et al.*, 2011). Whether cAMP accumulation is the result of a true constitutive activity of the receptor or the consequence of the chronic stimulation by a ubiquitous ligand is not known. Sphingosine-1-phosphate has been proposed as an agonist of the rat GPR3 homologue (Uhlenbrock *et al.*, 2002; Kostenis, 2004; Hinckley *et al.*, 2005), but this has not been confirmed yet (Valverde *et al.*, 2009).

In mouse oocytes, GPR3 contributes to the maintenance of cAMP concentrations at a level required to ensure meiotic arrest in prophase I until the luteinizing hormone surge (Mehlmann *et al.*, 2004; Ledent *et al.*, 2005). In addition, GPR3 plays a key role in the establishment of embryonic developmental competence and the maintenance of fertility in aging female mice (Ledent *et al.*, 2005). Findings in this system argue against the existence of an extracellular GPR3 agonist (Freudzon *et al.*, 2005). However, inhibition of GPR3 by an endogenous inverse agonist, as described for the melanocortin-4 receptor with which it shares some homology, cannot be excluded (Nijenhuis *et al.*, 2001; Joost and Methner, 2002).

GPR3 is widely expressed in the brain (Saeki *et al.*, 1993). It promotes neurite outgrowth (Tanaka *et al.*, 2009), modulates emotional behaviours (Valverde *et al.*, 2009) and stimulates amyloid-beta peptide generation in neurons (Thathiah *et al.*, 2009). However, little is known about the role of this receptor in cognitive functions. Structures involved in emotional behaviours such as habenula, hippocampus, amygdala and cortex all express GPR3 transcripts (Valverde *et al.*, 2009). Thus, these limbic regions and cortical areas have received most attention because they represent the neuroanatomical network involved in drug addiction. In this regard, a current hypothesis proposes that drugs of abuse abnormally recruit neuronal pathways and transmitter systems responding to natural reinforcement and progressively alter their function (Everitt and Robbins, 2005; Nestler, 2005; Hyman *et al.*, 2006). Therefore, the presence of GPR3 in these brain areas suggests that this system participates in the control of different phases in the development of drug addiction.

Cocaine is a psychostimulant drug with high addictive potential, acting by blocking uptake by neuronal plasma membrane transporter for dopamine, 5-HT and noradrenaline reuptake (Sora *et al.*, 2001; 2010). Cocaine reward and reinforcement have been linked to increased extracellular levels of dopamine and 5-HT in limbic and cortical areas (DiChiara *et al.*, 1992; Kaddis *et al.*, 1993). Dopamine activates members of a family of GPCRs that are divided into two major groups: the D_1 and D_2 types. Dopamine D_1-like receptors are coupled to $G\alpha_s/G\alpha_{olf}$ proteins and stimulate cAMP production, while D_2-type receptors are coupled to $G\alpha_i/G\alpha_o$ proteins and inhibit adenylyl cyclase activity (Jaber *et al.*, 1996). Activation of the adenylyl cyclase is required to elicit the hyperlocomotor responses to cocaine (DiRocco *et al.*, 2009). As GPR3 is also densely expressed in many limbic structures (Valverde *et al.*, 2009), this receptor may play a role in locomotion and rewarding effects of cocaine by modulating the adenylyl cyclase activity in these regions, particularly the nucleus accumbens which is densely innervated by dopaminergic neurons.

To address the physiological importance of this receptor in mediating responses to cocaine, we used mice lacking *Gpr3* (*Gpr3*–/–; Ledent *et al.*, 2005) to explore the behavioural consequences of GPR3 deficiency in locomotion, behavioural sensitization, reward and reinforcing responses. Neurochemical changes induced by cocaine were also evaluated in these mice. Extracellular levels of dopamine in the nucleus accumbens were measured after cocaine administration to *Gpr3*-deficient mice to assess the consequences of GPR3 receptor deletion in this brain structure related to reward and motivation. This study shows the important role played by GPR3 on the reinforcement induced by cocaine.

Methods

Animals

All animal care and experimental procedures were conducted according to the guidelines of the European Communities Directive 86/609/EEC regulating animal research, and approved by the local ethical committees, CEEA-PRBB and by the French Agriculture and Forestry Ministry (decree 87849, license B75-05-22). The results of all studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010). A total of 250 animals were used in the experiments described here.

Gpr3–/– mice and their *Gpr3*+/+ littermates, from the Université Libre de Bruxelles animal facility were used in our experiments. The generation of *Gpr3*–/– mice and their genotyping by polymerase chain reaction amplification of tail DNA were described previously (Ledent *et al.*, 2005). Heterozygous progeny were backcrossed with CD1 females at least 20 generations before generating the *Gpr3*–/– and *Gpr3*+/+ founders. Experimental Gpr3–/– and Gpr3+/+ mice were generated by syngenic crosses between founders.

All data were recorded without knowledge of the treatments given to the different experimental groups. Knockout and wild-type male mice weighing 30–35 g at the beginning of the experiment were housed in a controlled temperature (21 ± 1°C) and humidity (55 ± 10%) room with a 12 h light/dark cycle (lights on at 8:00 AM and off at 8:00 PM). Food and water were available *ad libitum* during all the procedures except for the food self-administration experiments where animals were food restricted, keeping body weight to 85 ± 5% of the original weight. For behavioural experiments, animals were habituated to the testing room and handled for 3 days before starting the experiment. At the end of the experiments, mice were quickly killed by decapitation.

Locomotor activity and locomotor sensitization

Locomotor activity was evaluated in a circular corridor with four infrared beams placed at every 90° (Imetronic, Pessac, France) in low luminosity and measured as the number of beam breaks on horizontal movements. Locomotor activity was first recorded for 60 min in *Gpr3*–/– and *Gpr3*+/+ mice for

3 consecutive days. On the fourth day, locomotion was measured in *Gpr3*–/– and *Gpr3*+/+ mice immediately after cocaine administration (7.5, 15 and 30 mg·kg^{-1} i.p.) or saline injection (0.1 mL per 10 g body weight).

To evaluate cocaine-induced locomotor sensitization, cocaine (15 mg·kg^{-1}, i.p.) was administered to *Gpr3*–/– and +/+ mice for 5 consecutive days. This treatment was followed by 9 days of withdrawal, and, on day 14, the animals received a challenge injection of saline or cocaine (15 mg·kg^{-1}, i.p.). Locomotor activity was measured immediately after injection for 15 min on days 1, 5 and 14. The locomotor effects of cocaine were measured in a context-specific environment by administering the drug in the locomotor activity chamber.

Conditioned place preference (CPP)

The rewarding effects of cocaine in *Gpr3*–/– and *Gpr3*+/+ mice were evaluated in the CPP paradigm as previously reported (Martin *et al.*, 2000). Briefly, this paradigm was carried out in a two-compartment conditioning box (Martin *et al.*, 2000), and was conducted in three phases: preconditioning, conditioning and testing or post-conditioning phase, respectively. During the preconditioning phase, drug-naive mice were placed in the middle of the central area of the box and had free access to both compartments of the apparatus for 20 min. The time spent in each compartment was recorded by computerized monitoring software (Smart; Panlab, Barcelona, Spain). During the conditioning phase, mice received daily alternating injections of cocaine (7.5, 15, or 30 mg·kg^{-1}) or saline, and were immediately placed into one of the two conditioning compartments for 30 min. Control animals received saline every day. Four pairings were performed with cocaine and three pairings with vehicle on alternate days. Treatments were counterbalanced between compartments. The post-conditioning phase was conducted exactly as the preconditioning phase, that is, free access to each compartment for 20 min. A preference score was calculated for each animal as the difference between the time spent in the drug-paired compartment during the testing and preconditioning phases (Martin *et al.*, 2000).

Operant self-administration

Mice trained for food self-administration were food restricted (keeping weight up to 85 ± 5% of the original, using a 15% weight reduction limit) for the whole acquisition period of food-maintained operant behaviour. Mice were trained to nose-poke in order to receive a food pellet under a fixed ratio 1 (FR1) schedule of reinforcement for 7 days.

Mice trained to self-administer cocaine were anaesthetized under a ketamine/xylazine solution and then implanted with indwelling i.v. silastic catheters. Briefly, a 6 cm length of silastic tubing (0.3 mm inner diameter, 0.6 mm outer diameter) (silastic, Dow Corning, Houdeng-Goegnies, Belgium) was fitted to a 22-gauge steel cannula (Semat, Herts, England) that was bent at a right angle and then embedded in a cement disk (Dentalon Plus, Heraeus Kulzer, Germany) with an underlying nylon mesh. The catheter tubing was inserted 1.3 cm into the right jugular vein and anchored with a suture. The remaining tubing ran subcutaneously to the cannula, which exited at the mid-scapular region. All incisions were sutured and coated with antibiotic ointment (Bactroban, GlaxoSmithKline, Spain). After surgery, animals were allowed to recover for 3 days prior to the initiation of self-administration sessions. The catheter was flushed daily with a saline solution containing heparin (30 UI·mL^{-1}) in order to maintain its patency. The patency of intravenous catheters was evaluated periodically (approximately every 6 days) by the infusion of 0.1 mL of thiobarbital (5 mg·mL^{-1}; B. Braun Medical S.A., Barcelona, Spain) through the catheter. If prominent signs of anaesthesia were not apparent within 3 s of the infusion, the animal was removed from the experiment. Mice were trained to nose-poke in order to receive a cocaine infusion (0.3 or 1 mg·kg^{-1}) under a FR1 schedule of reinforcement for 10 days. The volume of injection was 0.23 µL per infusion delivered over 2 s. Self-administration sessions (1 h daily) started by priming the mice with a reinforcer. The number of reinforcers was limited to 50 per session and each reinforcer was followed by a 30 s time-out period where active nose-poking had no consequences. The stimulus light signalled delivery of the reinforcer. A mouse was considered to acquire self-administration when it followed these criteria for at least three consecutive sessions: (i) the number of responses on the active hole exceeded 75% of that on the inactive hole; (ii) the number of responses on the active hole was higher than 10; and (iii) the mean deviation of the total number of reinforcers earned in three consecutive sessions was less than 20% (80% stability). After 10 days of training and once the acquisition criteria were achieved, the reinforcement schedule was changed to progressive ratio schedule of response (PR), in which the response requirement to earn an injection escalated throughout the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. The PR session lasted for 2 h or until the mice did not complete the ratio for delivery of one reinforcer within 1 h, and was performed only once. The breaking point to extinguish self-administration behaviour was determined in each animal. After each session, the mice were returned to their home cages.

In order to confirm that GPR3 modulated the effects of acute cocaine on naive mice but not in mice that were previously exposed to a cocaine administration, a priming injection of cocaine (20 mg·kg^{-1}, i.p.) was administered to *Gpr3*–/– mice and their *Gpr3*+/+ littermates 24 h before the first cocaine self-administration (0.3 mg·kg^{-1}) training day.

In vivo *microdialysis*

Microdialysis studies were performed as previously described (Soria *et al.*, 2005; Touriño *et al.*, 2008). A cannula guide was implanted 1 mm above the nucleus accumbens (antero-posterior, +1.60 mm; medio-lateral, –0.9 mm; dorso-ventral, –3.60 mm from bregma) (Figure 1). Three days after the guide cannula implantation, the analytical probe (CMA/7/1 mm, CMA microdialysis, Stockolm, Sweden) was inserted into the guide cannula. Forty-eight hours after the probe implantation, animals were habituated to the experimental environment overnight as previously reported (Soria *et al.*, 2005; Touriño *et al.*, 2008; Aso *et al.*, 2009). The following morning, Ringer's solution was pumped through the dialysis probe at a constant rate of 1 µL·min^{-1}. Five consecutive 20 min dialysis samples were collected for the determination of baseline dopamine levels. Then, mice were injected with either saline or cocaine (10 or 20 mg·kg^{-1}, i.p.) and samples were collected every 20 min for 4 h. Dialysate samples (15 µL) were injected

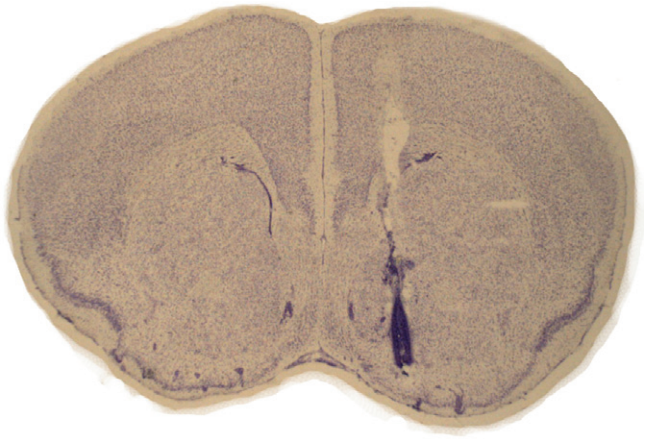

Figure 1

Representation of coronal sections (20 µm) of the mouse brain stained with cresyl violet illustrating the placement of the probe in the nucleus accumbens (bregma + 1.34 mm). Probes were successfully placed in the nucleus accumbens in 87% of the experimental animals.

without any purification into an HPLC system to quantify dopamine. At the end of the experiments, animals were killed, and brains were quickly removed and stored at –80°C in order to check the probe placement. Brains were sectioned using a cryostat in 20 µm serial coronal sections, which were then processed with cresyl violet and observed under a microscope. Only those animals that had been implanted correctly were included in the analysis.

Dopamine determination

Dialysate samples (15 µL) were injected without any purification into an HPLC system that consisted of a pump linked to an automatic injector (Agilent 1100; Agilent Technologies, Palo Alto, CA, USA), a reverse-phase column (Zorbax SB C18, 3.5 µm, 150 × 4.6 mm; Agilent Technologies) and a coulometric detector (Coulochem II; CMA Microdialysis, North Chelmsford, MA, USA) with a 5011 analytical cell to quantify dopamine. The first electrode was fixed at –100 mV and the second electrode at +300 mV. The gain of the detector was set at 10–20 nA. The signal of the second electrode was connected to a HP Chemstation for HPLC. The composition of the mobile phase was 50 mM NaH_2PO_4, 0.1 mM Na_2EDTA, 0.65 mM octyl sodium sulphate and 14% (v/v) methanol, pH 3.5. The flow rate was set at 0.9–1 mL·min^{-1}.

Statistical analysis

The effects of cocaine on locomotor activity and sensitization in *Gpr3*–/– and *Gpr3*+/+ mice were compared by using two-way ANOVA. For the locomotor activity experiments, the factors of variation were treatment and genotype. For locomotor sensitization, the factors of variation were day and genotype. Subsequent *post hoc* analyses were calculated when required. For genotype effects, a Bonferroni *post hoc* test was calculated. For treatment effect, a Newman–Keuls test was calculated. Cocaine-induced CPP data were analysed by using a two-way ANOVA with treatment and genotype as factors of variation. For treatment effect, a Dunnett's *t*-test was calculated when required. Cocaine infusions in self-administration procedure between *Gpr3*–/– and *Gpr3*+/+ were analysed by using two-way ANOVA with day and genotype as factors of variation. For all the experiments, subsequent one-way ANOVA and *post hoc* analysis (Dunnett's test) were calculated when required. Average cocaine infusions, cocaine self-administration acquisition day and breaking point between genotypes were compared by using one-way ANOVA. Percentage of acquisition between genotypes was compared by using Fisher's exact test. Cocaine-induced increase in dopamine extracellular levels in the nucleus accumbens of *Gpr3*–/– mice was compared with *Gpr3*+/+ by using three-way ANOVA (time, treatment and genotype as factors of variation).

Materials

Cocaine hydrochloride (Ministerio de Sanidad, Spain and Cooper, France) was dissolved in 0.9% physiological saline, and injected i.p. in a volume of injection of 0.1 mL per 10 g body weight. Ketamine hydrochloride (100 mg·kg^{-1}; Imalgène 1000®, Rhône Mérieux, Lyon, France) and xylazine hydrochloride (20 mg·kg^{-1}; Sigma Chemical Co., Madrid, Spain) were mixed and dissolved in ethanol and water (1:9). This mixture was injected i.p. in a volume of 0.2 mL per 10 g and used as anaesthetic during surgery.

Results

Acute cocaine treatment increases locomotor activity but repeated cocaine administration failed to increase the locomotor responses in Gpr3–/– mice

Acute cocaine-induced hyperlocomotion and behaviour sensitization were evaluated in *Gpr3*-deficient mice (Figure 2). Regarding locomotion effects, two-way ANOVA revealed significant effects of acute cocaine treatment, genotype and interaction (Table 1). One-way ANOVA showed no differences between genotypes when spontaneous locomotor activity was evaluated, but cocaine administered at the doses of 15 and 30 mg·kg^{-1}, i.p., induced a significant increase in locomotor activity in *Gpr3*–/– mice ($P < 0.01$) (Bonferroni *post hoc* test) (Figure 2A).

Sensitization to repeated cocaine (15 mg·kg^{-1}, i.p.) administration showed significant effects of days and genotypes (two-way ANOVA; Table 1). *Post hoc* analysis (Bonferroni *post hoc* test for genotype effects and Newman–Keuls for treatments) revealed that following 5 consecutive days of cocaine injections, *Gpr3+/+* mice developed a sensitized response to cocaine as shown by the enhanced locomotor activity on day 5 ($P < 0.01$) and on day 14 ($P < 0.05$) when compared to the day 1 (Figure 2B). In contrast, following repeated treatment with cocaine (15 mg·kg^{-1}, i.p.), *Gpr3*–/– mice did not show any significant increase in the locomotor response on days 5 and 14 when compared with that on day 1. However, the effect of the first cocaine injection (day 1) was more intense in *Gpr3*–/– mice than in the *Gpr3+/+* mice ($P < 0.01$ genotype comparison) (Figure 2B). However, because the responses to cocaine increased in *Gpr3+/+* and remained unchanged in *Gpr3*–/– mice after repeated treatments, the differences

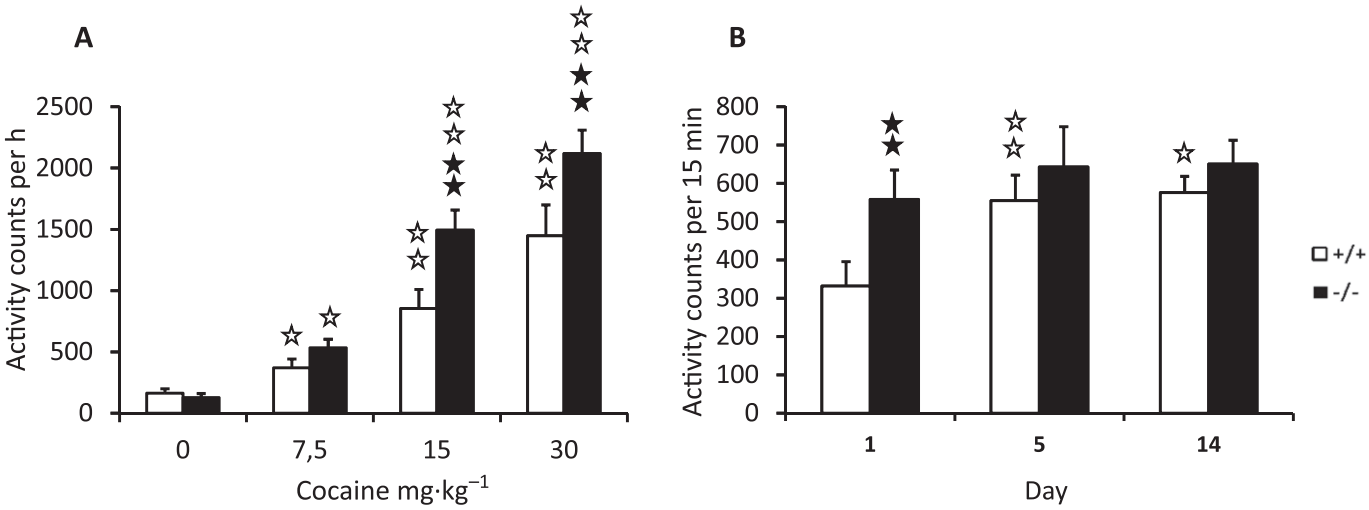

Figure 2

Effects of acute (A) and repeated (B) administration of cocaine on locomotor activity in *Gpr3–/–* ($n = 7$) and *Gpr3+/+* ($n = 9$) mice, over a range of doses of cocaine. (A) Locomotor activity was recorded for 1 h in animals treated with cocaine (7.5, 15 or 30 mg·kg^{-1}, i.p.) or saline. (B) Locomotor activity was recorded on day 1, 5 and 14 for 15 min in animals treated daily with repeated cocaine (15 mg·kg^{-1}, i.p.). Locomotor activity data are expressed as mean ± SEM of activity counts per hour (A) or per 15 min (B). One star, $P < 0.05$; two stars, $P < 0.01$; black stars represent genotype comparisons (Bonferroni *post hoc* test); white stars represent treatment comparisons (Newman–Keuls test).

Table 1

Data for acute hyperlocomotion, locomotor sensitization, conditioned place preference induced by cocaine in Gpr3+/+ and Gpr3-/- mice, analysed by two-way and one-way ANOVA.

	Two-way ANOVA			One-way ANOVA	
Locomotor activity	Treatment	Genotype	Interaction		Treatment
	$F_{(3, 63)} = 48.097$***	$F_{(1, 63)} = 11.615$**	$F_{(3, 63)} = 2.832$*	GPR3+/+	$F_{(3, 33)} = 14.360$***
				GPR3–/–	$F_{(3, 28)} = 38.230$***
Locomotor sensitization	Day	Genotype	Interaction		Day
	$F_{(2, 54)} = 3.878$*	$F_{(1, 54)} = 10.126$**	$F_{(2, 54)} = 0.780$	GPR3+/+	$F_{(2, 26)} = 16.630$***
				GPR3–/–	$F_{(2, 26)} = 2.013$***
Conditioned place preference	Treatment	Genotype	Interaction		Treatment
	$F_{(3, 89)} = 7.428$**	$F_{(1, 89)} = 4.371$*	$F_{(3, 89)} = 0.459$	GPR3+/+	$F_{(3, 44)} = 5.090$**
				GPR3–/–	$F_{(3, 44)} = 3.287$*
Self-administration	Day	Genotype	Interaction		Day
Food	$F_{(6, 90)} = 9.656$***	$F_{(1, 15)} = 0.407$	$F_{(6, 90)} = 2.293$	GPR3+/+	$F_{(6, 55)} = 3.404$**
				GPR3–/–	$F_{(6, 62)} = 9.650$***
Cocaine (mg·kg^{-1} per infusion):					
1 mg·kg^{-1}	$F_{(9, 189)} = 5.269$***	$F_{(1, 21)} = 6.811$*	$F_{(9, 189)} = 1.409$	GPR3+/+	$F_{(9, 119)} = 4.142$***
				GPR3–/–	$F_{(9, 119)} = 4.119$***
0.3 mg·kg^{-1}	$F_{(9, 198)} = 5.480$***	$F_{(1, 22)} = 9.774$**	$F_{(9, 198)} = 2.772$**	GPR3+/+	$F_{(9, 79)} = 4.172$***
				GPR3–/–	$F_{(9, 89)} = 1.002$
0.3 mg·kg^{-1} (priming)	$F_{(9, 135)} = 3.697$***	$F_{(1, 15)} = 0.567$	$F_{(9, 135)} = 1.474$	GPR3+/+	$F_{(9, 99)} = 2.614$*
				GPR3–/–	$F_{(9, 129)} = 6.171$***

Two-way ANOVA with genotype and treatment as between-subject, and day as within-subject factors of variation. One-way ANOVA with treatment as between-subject, and day as within-subject factors of variation. One-way ANOVA calculated only for factors with three or more groups. See Materials and Methods for details.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

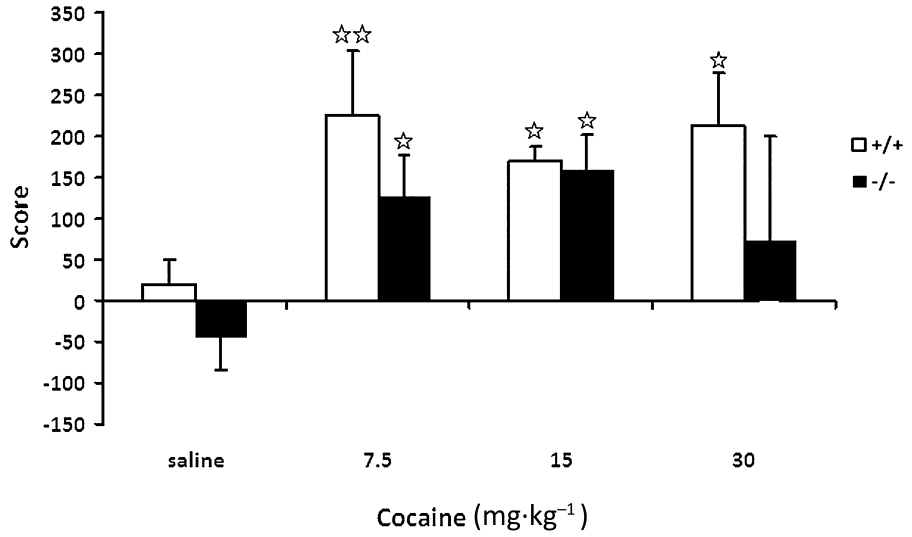

Figure 3

Cocaine-induced conditioned place preference in *Gpr3*–/– (n = 7–19) and *Gpr3*+/+ (n = 8–17) mice. Four pairings were carried out with cocaine (7.5, 15 and 30 mg·kg^{-1}, i.p.) and four pairings with saline on alternate days. Cocaine and saline were administered before each conditioning session. Data are expressed as mean ± SEM of score values (test–pretest). One star, $P < 0.05$; two stars, $P < 0.01$; white stars represent treatment comparisons (Dunnett's t-test).

between the cocaine responses in the two genotypes were not significant on days 5 and 14 (Figure 2B).

Rewarding effects of cocaine on CPP was enhanced in Gpr3–/– mice

Rewarding effects of cocaine (7.5, 15 and 30 mg·kg^{-1}) were evaluated in *Gpr3*–/– mice and their wild-type littermates using cocaine-induced CPP. Two-way ANOVA (treatment and genotype) revealed a significant effect of treatment and genotype without interaction between these two factors (Table 1). One-way ANOVA calculated for treatment revealed a rewarding effect of cocaine in both genotypes (Table 1). *Post hoc* analysis showed significant effects in *Gpr3*+/+ mice for the three doses of cocaine (7.5 mg·kg^{-1}, $P < 0.01$; 15 mg·kg^{-1}, $P < 0.05$; $P < 0.05$), and in *Gpr3*–/– at the doses of 7.5 mg·kg^{-1} ($P < 0.05$) and 15 mg·kg^{-1} ($P < 0.05$) (Figure 3). *Gpr3*–/– mice appeared to be more sensitive to cocaine effects than *Gpr3*+/+, because at the higher dose (30 mg·kg^{-1}), four animals died during the CPP procedure.

Food-maintained operant behaviour is similar in the Gpr3–/– and Gpr3+/+ mice

Food operant self-administration was evaluated in both *Gpr3*–/– and their *Gpr3*+/+ littermates (Figure 4). Two-way ANOVA revealed an effect of training day, without genotype effect and without interaction between these two factors (Table 1). No differences were observed in food self-administration between *Gpr3*–/– and *Gpr3*+/+ mice during the 7 days of training period (Figure 4A), indicating that GPR3 did not modulate the reinforcing properties of standard food in mice. We calculated the average of food pellets consumed in the last 3 days of acquisition, and no significant differences were found between genotypes (Figure 4B). In addition, 90% of *Gpr3*–/– and 75% of *Gpr3*+/+ mice achieved the acquisition criteria, showing no differences between genotypes (under FR1 schedule of reinforcement) (Figure 4C). Finally, at the end of the training period, we exposed the animals to a PR schedule of response in order to determine the reinforcing efficacy of standard food. Similar breaking point values were obtained on the PR schedule for both genotypes (Figure 4D).

Cocaine self-administration is enhanced in Gpr3–/– mice

Cocaine (0.3 or 1 mg·kg^{-1} per infusion) self-administration was assessed in both *Gpr3*–/– and their *Gpr3*+/+ littermates (Figure 5 and 6). *Gpr3*–/– mice self-administered significantly more cocaine at the dose of 1 mg·kg^{-1} per infusion, as revealed by statistical analysis. Two-way ANOVA calculated for animals treated with cocaine 1 mg·kg^{-1} per infusion showed a significant effect for training day and for genotype, but no interaction between these two factors (Table 1). Intake of cocaine (1 mg·kg^{-1}) infusions was on average higher in *Gpr3*–/– than in *Gpr3*+/+ mice, in particular between days 1 and 7 of the training (under FR1 schedule of reinforcement). However, we only found significant differences between genotypes on days 3 ($P < 0.05$), 6 ($P < 0.05$) and 7 ($P < 0.05$), respectively (Figure 5A). Significant differences were found between genotypes in the percentage of animals that achieved the acquisition criteria across genotypes ($P < 0.01$, one-way ANOVA). Thus, 100% of *Gpr3*–/– mice achieved the criteria, whereas only the 40% of wild-type mice achieved the acquisition criteria. Furthermore, the average of infusions of the last 3 days of the acquisition period was significantly higher for *Gpr3*–/– mice, reflecting a greater amount of cocaine intake for *Gpr3*–/– mice ($P < 0.05$; one-way ANOVA) (Figure 5B). A significant difference between genotypes was also found for the day of acquisition ($P < 0.05$; one-way

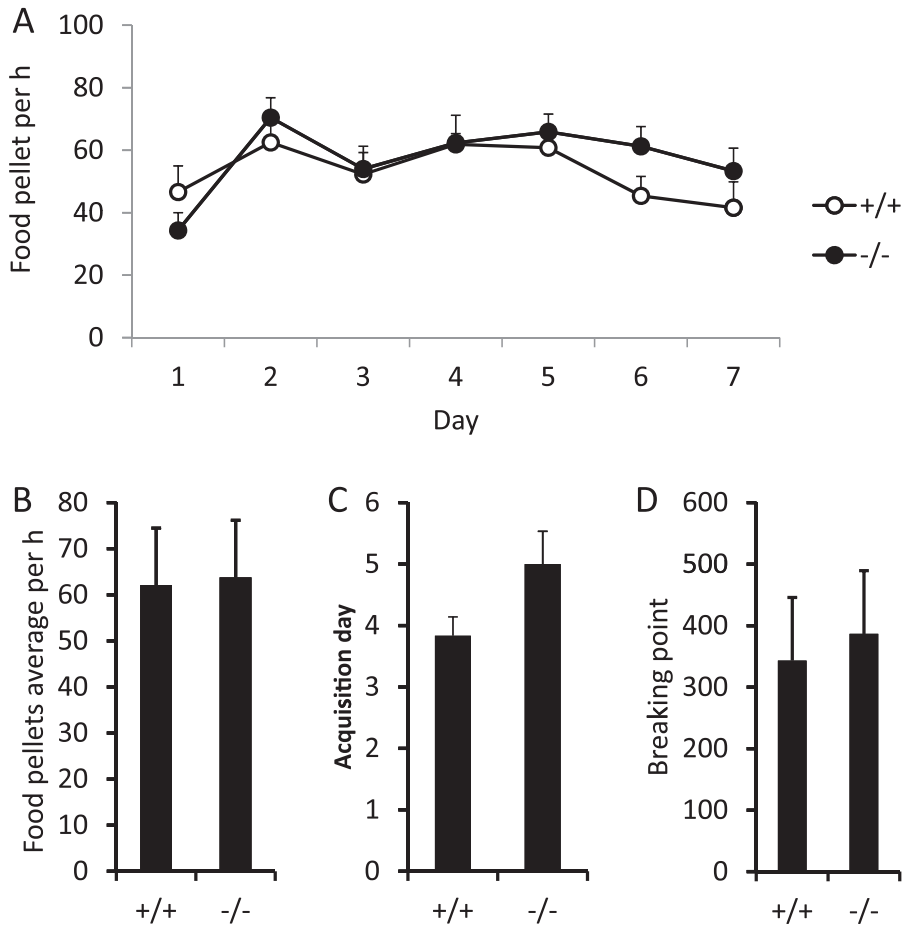

Figure 4

Food self-administration in *Gpr3*–/– ($n = 9$) and *Gpr3*+/+ ($n = 8$) mice. (A) Data are expressed as mean ± SEM of the number of nose pokes in the active hole in 1 h sessions performed during 10 days. (B) Data are expressed as mean ± SEM of the average number of infusions of the last 3 days of administration. (C) Data are expressed as mean ± SEM of the day when animals achieved the acquisition criteria (see materials and methods). (D) Data are expressed as mean ± SEM of the breaking point achieved by animals under a progressive ratio schedule of reinforcement.

ANOVA) (Figure 5C), whereas no differences were observed for the breaking point achieved when the mice were exposed to a PR schedule of reinforcement (Figure 5D).

In a second set of experiments, mice were exposed to a lower dose of cocaine (0.3 mg·kg^{-1} per infusion (Table 1, Figure 6). *Gpr3*–/– mice still self-administered significantly more cocaine than *Gpr3*+/+ mice (two-way ANOVA). Indeed, a significant effect was revealed for genotype and day without interaction between these two factors (Table 1). Cocaine intake was significantly higher in *Gpr3*–/– than in *Gpr3*+/+ mice from day 2 to 7 (Figure 6A). Moreover, 92% of *Gpr3*–/– mice achieved the acquisition criteria compared to only 42% of *Gpr3*+/+ mice ($P < 0$, 05). The average of infusions of the last 3 days of acquisition period revealed significant differences between genotypes ($P < 0.01$, one-way ANOVA) with a higher number of infusions for the *Gpr3*–/– mice (Figure 6B). However, the acquisition day (Figure 6C) and the breaking point obtained under a PR schedule (Figure 6D) were similar between genotypes.

The effect of a priming injection of cocaine (20 mg·kg^{-1}, i.p.) on cocaine (0.3 mg·kg^{-1} infusion) self-administration was also evaluated (Figure 7). This experiment was designed to test if previous acute exposure to cocaine suppresses the differences observed between genotypes for cocaine self-administration. Animals pre-exposed to acute cocaine (20 mg·kg^{-1}, i.p.) and trained to self-administered cocaine (0.3 mg·kg^{-1} per infusion) revealed no differences in cocaine self-administration between genotypes (Table 1; Figure 7A). Two-way ANOVA showed a significant effect of the day, but neither genotype effect nor interaction between factors (Table 1). In addition, the percentage of mice that achieved the acquisition criteria (*Gpr3*+/+, 62%; *Gpr3*–/–, 55%) was in this case similar for both genotypes. Moreover, no differences between genotypes were seen for the infusion average of the last 3 days of acquisition (Figure 7B), the acquisition day (Figure 7C) and the breaking point obtained in a PR schedule (Figure 7D).

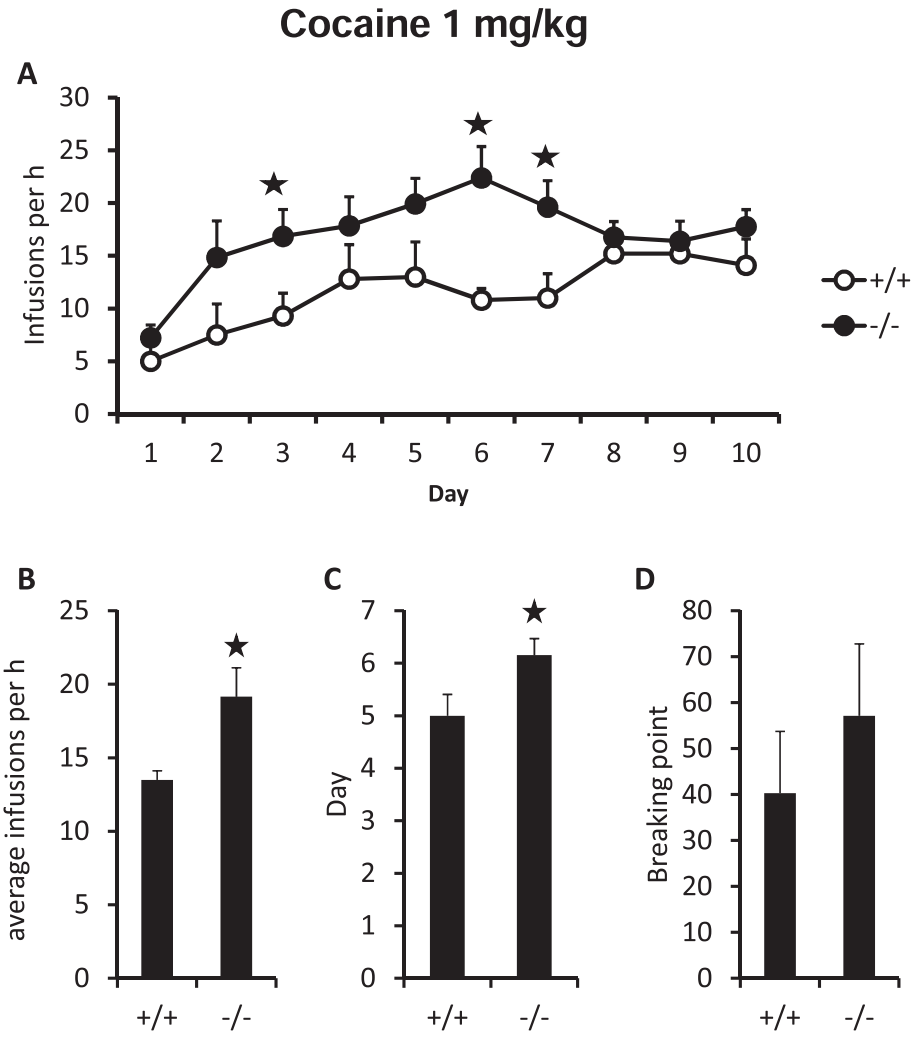

Figure 5

Cocaine (1 mg·kg^{-1} infusion) self-administration in *Gpr3*–/– (n = 13) and *Gpr3*+/+ (n = 10) mice. (A) Data are expressed as mean ± SEM of the number of nose pokes in the active hole in 1 h sessions performed during 10 days. (B) Data are expressed as mean ± SEM of the average number of infusions of the last 3 days of administration. (C) Data are expressed as mean ± SEM of the day when animals achieved the acquisition criteria. (D) Data are expressed as mean ± SEM of the breaking point achieved by animals under a progressive ratio schedule of reinforcement. One star, $P < 0.05$; black stars represent genotype comparisons (one-way ANOVA).

Cocaine-induced dopamine release in the nucleus accumbens is similar between Gpr3-/- and Gpr3+/+ mice

The effects of two doses of cocaine (10 and 20 mg·kg^{-1}) on extracellular dopamine levels were evaluated in the nucleus accumbens of *Gpr3*–/– and their *Gpr3*+/+ littermates (Figure 8). Both doses of cocaine increased extracellular dopamine levels independently of *Gpr3* genotype and no differences between genotypes were observed at basal levels. Three-way ANOVA (time, treatment and genotype as factor of variations) showed an effect of the time ($F_{(6, 192)} = 14.127$, $P < 0.001$) and the treatment ($F_{(2, 32)} = 4.424$, $P < 0.05$) but no effect of the genotype ($F_{(1, 32)} = 0.001$, n.s.). The calculation of interactions between the different factors indicated an interaction between time and treatment ($F_{(12, 192)} = 5.105$, $P < 0.01$) with no interactions between time and genotype, genotype and treatment and time or genotype and treatment.

Discussion

The present study reveals that GPR3 modulates the acute effects of cocaine as well as the rewarding and reinforcing effects of cocaine evaluated in the CPP and the self-administration paradigms, respectively. Compared with the wild-type *Gpr3*+/+ strain, the *Gpr3*–/– mice were more sensitive to the stimulant and rewarding effects of cocaine, but extracellular levels of dopamine induced by cocaine administration in the nucleus accumbens were similar between genotypes. Reinforcing effect produced by a natural reward as non-palatable food, evaluated with self-administration paradigm, showed similar data between genotypes, indicating that the modulator effect of GPR3 in acute reward is specific for cocaine.

The locomotor activity exhibited by *Gpr3*–/– mice after acute injection of cocaine is comparable to that observed for

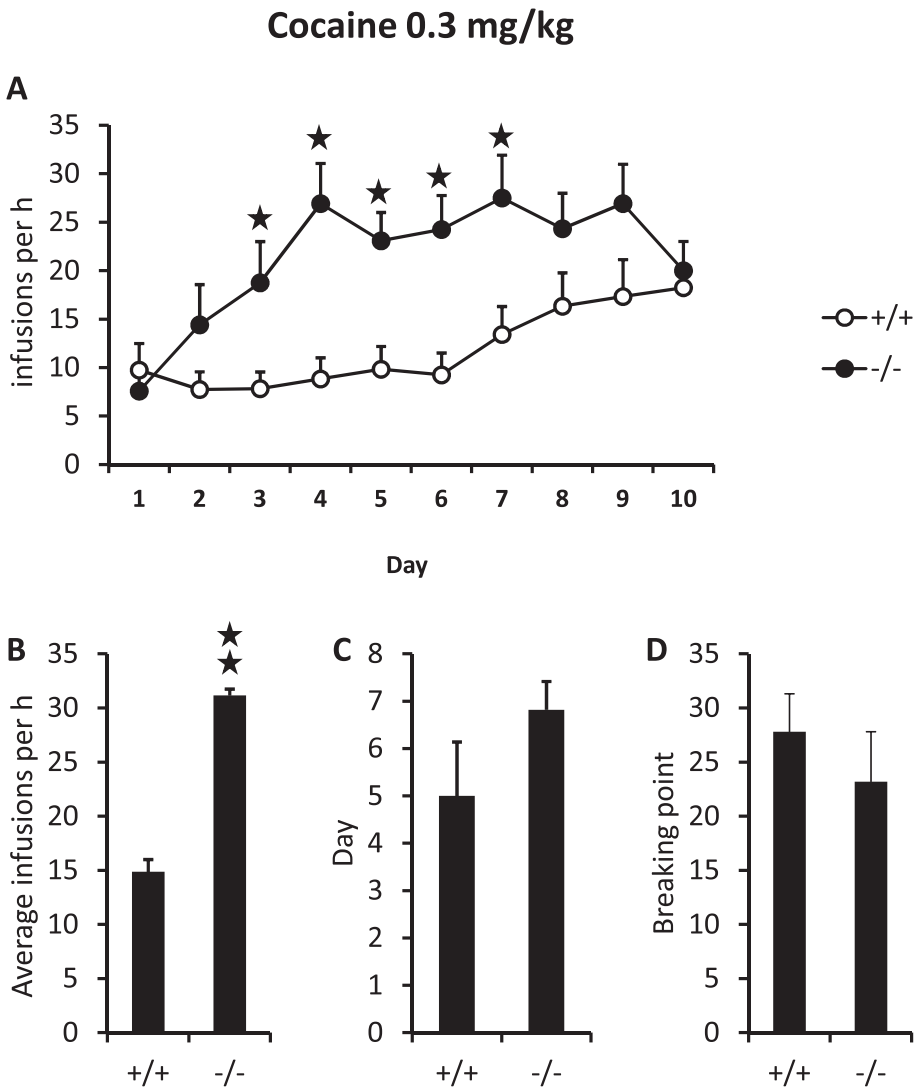

Figure 6

Cocaine (0.3 mg·kg^{-1} infusion) self-administration in *Gpr3*–/– (n = 12) and *Gpr3*+/+ (n = 12) mice. (A) Data are expressed as mean ± SEM of the number of nose pokes in the active hole in 1 h sessions performed during 10 days. (B) Data are expressed as mean ± SEM of the average number of infusions of the last 3 days of administration. (C) Data are expressed as mean ± SEM of the day when animals achieved the acquisition criteria. (D) Data are expressed as mean ± SEM of the breaking point achieved by animals under a PR schedule of reinforcement. One star, $P < 0.05$; two stars, $P < 0.01$; black stars represent genotype comparisons (one-way ANOVA).

Gpr3+/+ mice after chronic injection. This indicated that a single cocaine (15 mg·kg^{-1}, i.p.) administration was sufficient to induce the maximal locomotor response in *Gpr3*–/– mice, which normally developed after chronic cocaine administration in *Gpr+/+* mice. This could reflect a 'basal maximally sensitized state' in *Gpr3*–/– mice which would render them more responsive to cocaine. Indeed, it has been recently reported that *Gpr3*–/– mice exhibited a higher response to stressful conditions (Valverde *et al.*, 2009) and repeated stress could sensitize the mesocorticolimbic dopamine system to induce an increased response after psychostimulant administration (Antelman *et al.* 1980). The 'absence of sensitization' observed in *Gpr3*–/– mice could be explained by a ceiling effect since a maximal response to cocaine was already observed after a single injection of the psychostimulant. Independent data support that cAMP is an important mediator of cocaine action in the nucleus accumbens. The inhibition of CREB function, a mediator in the cAMP signal transduction pathway, made animals more sensitive to the locomotor effects of cocaine (Miserendino and Nestler, 1995; Green *et al.*, 2006).

The striatum is one of the brain areas with higher density of GPR3 (Valverde *et al.*, 2009) and this receptor causes strong constitutive activation of adenylyl cyclase (Eggerickx *et al.*, 1995). Therefore, by its ability to activate the cAMP system, GPR3 might modulate the activity of these neurons and be one of the regulators preventing the development of an exaggerated behavioural sensitivity to the effects of cocaine on locomotor activity in mice.

Cocaine induced CPP in both genotypes. However, at the higher dose (30 mg·kg^{-1}), a significant effect was only

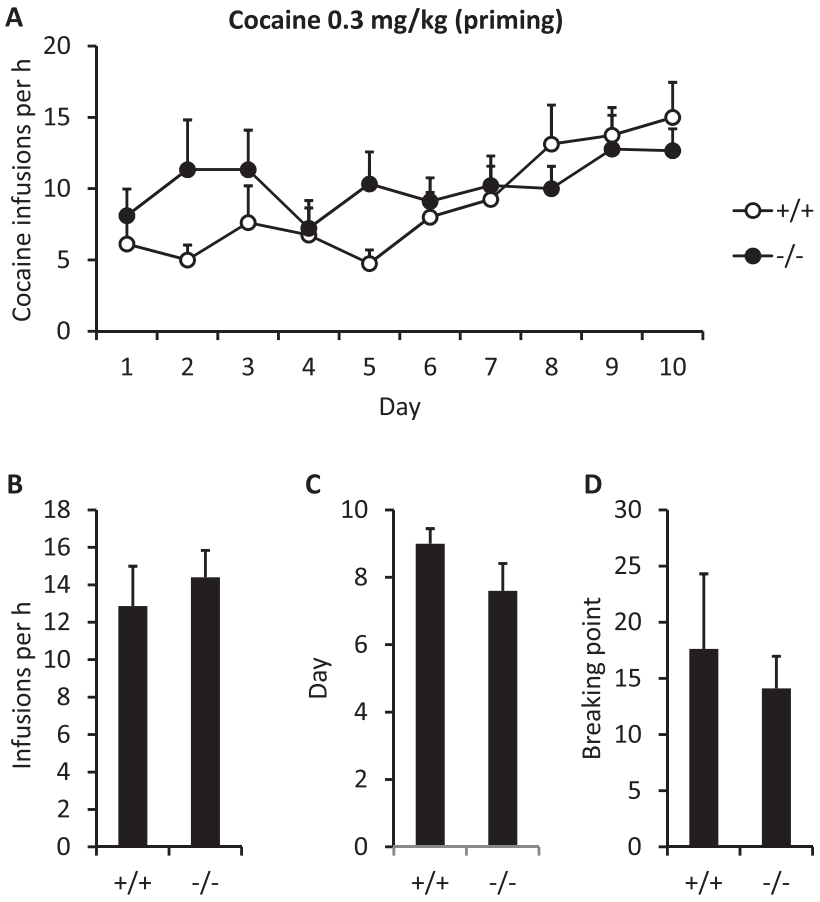

Figure 7

Cocaine (0.3 mg·kg^{-1} infusion) self-administration in *Gpr3–/–* (n = 8) and *Gpr3+/+* (n = 9) mice after a priming injection of cocaine (20 mg·kg^{-1}, i.p.) 24 h before the first session of self-administration. (A) Data are expressed as mean ± SEM of the number of nose pokes in the active hole in 1 h sessions performed during 10 days. (B) Data are expressed as mean ± SEM of the average number of infusions of the last 3 days of administration. (C) Data are expressed as mean ± SEM of the day when animals achieved the acquisition criteria. (D) Data are expressed as mean ± SEM of the breaking point achieved by animals under a PR schedule of reinforcement.

observed in wild-type mice supporting the hypothesis that there is a possible shift to the left of the dose–response curve for cocaine effects in this model. Moreover, four *Gpr3–/–* mice died before the end of the experiments. Toxic effects may result from overdose, as it is well-established that cocaine dose-dependently induces lethality. The dose–response curve using the CPP paradigm frequently exhibits a bell-shaped curve pattern, in which lowest and highest doses are ineffective (Martin *et al.*, 2000; Roux *et al.*, 2003). In *Gpr3–/–* mice, a shift to the left in the dose–response curve would result in a toxic effect at highest doses, as we have observed in our experiments. Previous studies showed that Gnal+/- mice exhibit normal behavioural sensitization and CPP induced by cocaine (Corvol *et al.*, 2007). In our experimental conditions, the expression of cocaine-induced CPP is consistent with the fact that GPR3-induced changes in the adenylyl cyclase-cAMP pathway might modulate the effects of acute cocaine administration and the earliest phases of reward.

We have conducted experiments in order to evaluate the reinforcing responses of non-palatable food using the self-administration paradigm. No differences were found between genotypes during all training sessions leading us to discount a possible learning deficit due to the absence of GPR3. Cocaine self-administration studies were conducted to evaluate the role of GPR3 in the reinforcing effects of cocaine from the first day of exposure. *Gpr3–/–* mice self-administered higher amounts of cocaine (1 mg·kg^{-1} per infusion) during the first days, and the average of infusions for cocaine self-administration was higher for *Gpr3–/–* mice in the last days of training (0.3 and 1 mg·kg^{-1} per infusion) (Figures 5B and 6B). In addition, knockout mice achieved the acquisition criteria before wild-type, when the dose of cocaine was 1 mg·kg^{-1} per infusion (Figure 5C). However, when *Gpr3–/–* and *Gpr3+/+* mice were primed with cocaine 24 h before starting the self-administration training, both groups of mice self-administered similar amounts of cocaine. These results are consistent with previous data reporting tolerance to cAMP-dependent protein kinase A activation in the nucleus accumbens following repeated cocaine self-administration (Edwards *et al.*, 2007). Our data support the hypothesis that

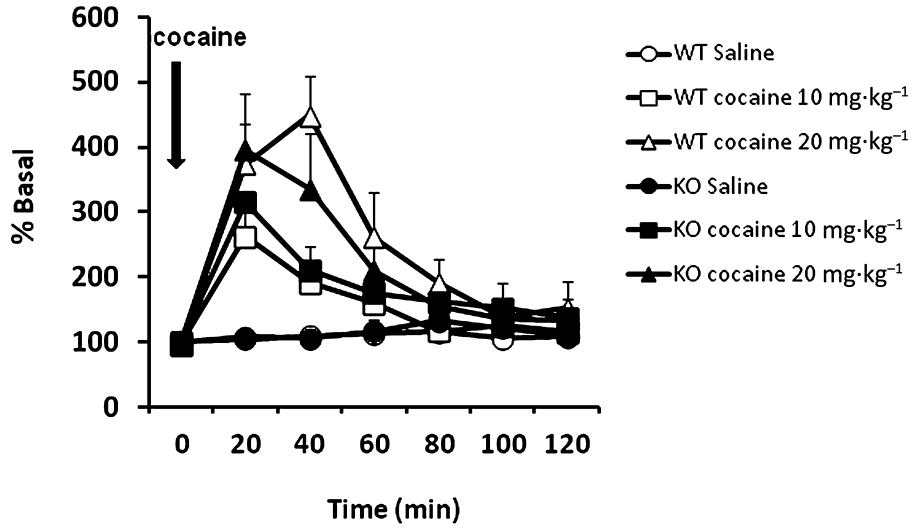

Figure 8

Effects of cocaine (10 and 20 mg·kg^{-1}, i.p.) and saline on dopamine concentrations in dialysates obtained by *in vivo* microdialysis from the nucleus accumbens of *Gpr3*–/– (KO; n = 4 –10) and *Gpr3*+/+ (WT; n = 4–7) mice. The arrow indicates cocaine or saline administration at time 0. Dialysate samples were taken every 20 min during 1 h before and 120 min after injection. Data are expressed as percentage of the basal values.

GPR3 modulates only the acute effects of cocaine and the early stages of reinforcement and reward, without playing a key role in the neuroadaptive phenomena underlying addiction which develops following prolonged exposure to the drug and that is characteristic of addiction. Both genotypes self-administered similar amounts of standard non-palatable food, suggesting that GPR3 does not play an important role in modulating the reinforcing properties of natural rewards. This was in agreement with earlier studies showing that dopamine D_1 and D_2 receptor antagonists decreased cocaine self-administration without altering the self-administration of food (Bari and Pierce, 2005). However, we cannot exclude a possible influence of GPR3 receptors when a more palatable food is consumed. Taken together, these results suggest that the modulation of the adenylyl cyclase-cAMP pathway by GPR3 is important for cocaine but not for standard non-palatable food self-administration.

Cocaine enhances locomotor activity by increasing the amount of dopamine that binds to dopamine receptors in discrete brain areas, particularly in the nucleus accumbens (Kaddis *et al.*, 1993). Dopamine release in the nucleus accumbens after acute administration of cocaine was evaluated by *in vivo* microdialysis. *Gpr3*–/– and *Gpr3*+/+ mice showed a similar increase in dopamine levels at all tested doses. The absence of differences between genotypes even at the lower dose of cocaine (10 mg·kg^{-1}, i.p.), in microdialysis experiments, leads us to reject the hypothesis that lack of GPR3 facilitates the release of dopamine. Thus, the behavioural alteration in reward and reinforcing effects of cocaine in *Gpr3*–/– mice does not seem to be directly related to an enhancement of the dopamine levels in the limbic areas. We hypothesized that GPR3 by tuning the adenylyl cyclase activity of the medium spiny neurons of the striatum, modulated the effects of cocaine in a dopamine-independent way. However, we cannot exclude the possibility that other mechanisms compensate for the lack of a potential function of GPR3 on dopamine neurotransmission. There is experimental and clinical evidence suggesting that dopaminergic mechanisms alone cannot fully explain the behavioural effect of cocaine (Kiyatkin and Rebec, 2000). Indeed, the reinforcing effects of cocaine self-administration and cocaine-induced CPP have been established in mice lacking the dopamine transporter (Rocha *et al.*, 1998; Sora *et al.*, 1998). Moreover, dopamine-deficient mice were able to express a CPP for cocaine, independently of dopamine release (Hnasko *et al.*, 2007). Blockade of membrane transporters for 5-HT and noradrenaline and the release of other neuromodulators by dopaminergic neurons are capable of modulating behavioural responses to drug abuse (Sora *et al.*, 2001; 2010; Rothman and Baumann, 2003; Chuhma *et al.*, 2004). Further studies are required to clarify the participation of GPR3 in dopamine-independent mechanisms of the actions of cocaine and to exclude the existence of mechanisms compensating for some of the missing receptor functions.

In conclusion, *Gpr3*–/– mice showed differences in the locomotor, rewarding and reinforcing effects of cocaine mainly after acute administration of cocaine, compared to *Gpr3*+/+ mice. Our results suggest that *Gpr3*–/– mice may have developed a 'basal sensitized state' characteristic of animals repeatedly exposed to cocaine. This would explain why acute locomotor effects of low doses of cocaine in *Gpr3*–/– mice are similar to the effects of repeated cocaine administration in *Gpr3*+/+ mice. It also would explain the higher response elicited by mutant mice in the CPP and cocaine-induced self-administration. Taken together, our data support the proposition that GPR3 controls acute responses of cocaine and suggest that a deregulation of GPR3 signalling pathways may alter in, a dopamine-independent way, specific responses related to psychostimulant addictive behaviours.

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Conflict of Interest

The authors declare no conflict of interest.

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