GPR88 in A2AR Neurons Enhances Anxiety-Like Behaviors

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Abstract

GPR88 is an orphan G-protein-coupled receptor highly expressed in striatal dopamine D1 (receptor) R- and D2R-expressing medium spiny neurons. This receptor is involved in activity and motor responses, and we previously showed that this receptor also regulates anxiety-like behaviors. To determine whether GPR88 in D2R-expressing neurons contributes to this emotional phenotype, we generated conditional Gpr88 knock-out mice using adenosine A2AR (A2AR)-Cre-driven recombination, and compared anxiety-related responses in both total and A2AR-Gpr88 KO mice. A2AR-Gpr88 KO mice showed a selective reduction of Gpr88 mRNA in D2R-expressing, but not D1R-expressing, neurons. These mutant mice showed increased locomotor activity and decreased anxiety-like behaviors in light/dark and elevated plus maze tests. These phenotypes were superimposable on those observed in total Gpr88 KO mice, demonstrating that the previously reported anxiogenic activity of GPR88 operates at the level of A2AR-expressing neurons. Further, A2AR-Gpr88 KO mice showed no change in novelty preference and novelty-suppressed feeding, while these responses were increased and decreased, respectively, in the total Gpr88 KO mice. Also, A2AR-Gpr88 KO mice showed intact fear conditioning, while the fear responses were decreased in total Gpr88 KO. We therefore also show for the first time that GPR88 activity regulates approach behaviors and conditional fear; however, these behaviors do not seem mediated by receptors in A2AR neurons. We conclude that Gpr88 expressed in A2AR neurons enhances ethological anxiety-like behaviors without affecting conflict anxiety and fear responses.

Key words: amygdale; anxiety-like behavior; D2R-medium spiny neurons; G-protein-coupled receptors; striatum

Significance Statement

GPR88, a striatal enriched orphan G-protein-coupled receptor has been implicated in the regulation of anxiety-like behaviors. In the striatum, GPR88 is most abundant in both medium spiny neurons expressing dopamine D1 and D2 receptors (Rs). To evaluate the contribution of GPR88 in D2R-neurons, we compared anxiety-like and fear-related behavioral responses of newly generated conditional A2AR-Gpr88 mice with those of total Gpr88 knock-out animals. Our data show that GPR88 expressed in A2AR neurons increases ethological anxiety-like behaviors without affecting conflict anxiety and fear responses. These results represent a first step toward understanding the circuit mechanisms underlying GPR88 function in the brain. Future studies will evaluate the role of GPR88 in D1R neurons.
Introduction

G-protein-coupled receptors (GPCRs) are the target for ~40% of marketed drugs, and are major players in biomedicine (Rask-Andersen et al., 2011). Orphan GPCRs, whose ligands remain unknown and functions have been little studied, offer great promise (Leyvo et al., 2006; Rask-Andersen et al., 2011; Ghanem, 2015). The orphan GPCR GPR88 has been implicated in a number of behaviors related to psychiatric disorders. Mice lacking GPR88 present a complex behavioral phenotype that includes motor coordination deficits, reduced prepulse inhibition, stereotypies, and altered cue-based learning (Logue et al., 2009; Massart et al., 2009; Quintana et al., 2012; Ingallinesi et al., 2015). These behaviors all can be related to the strong enrichment of GPR88 in the striatum (Swerdlov et al., 2001; Lewis and Kim, 2009; Liljeholm and O’Doherty, 2012). In humans, the GPR88 gene was associated with bipolar disorders and schizophrenia (Del Zompo et al., 2014). Recently, we found that Gpr88 deletion in mice also decreases anxiety-like behavior (Meirsm et al., 2016), implicating this receptor in emotional processing and in the evaluation of environmental stimuli value. Concordant with this finding, Gpr88 expression was shown regulated by antidepressant and mood-stabilizer treatments in both rodent models and humans (Ogden et al., 2004; Brandish et al., 2005; Böhm et al., 2006; Conti et al., 2007).

Several lines of evidence suggest that GPR88 alters behavior by modulating striatal transmission. In the striatum (dorsal and ventral), GPR88 is most abundant in medium spiny neurons (MSNs) expressing dopamine D1 receptors (Rs; D1-R-MSNs coexpressing substance P) and dopamine D2R MSNs coexpressing adenosine A2A-R (A2A(R)) and regulates the excitability of these neurons, possibly by acting on glutamatergic, GABAergic, and dopaminergic receptors activity (Logue et al., 2009; Quintana et al., 2012). Conversely, glutamatergic and dopaminergic depletion differentially alters Gpr88 expression in these distinct MSN subpopulations (Logue et al., 2009). However, the precise mechanism by which GPR88 regulates the transmission of MSNs to alter behavior remains unknown. In recent years, research on MSNs subtype function has revealed that these two neuronal populations differentially regulate not only motor behaviors but also responses to rewarding and aversive stimuli (Durieux et al., 2009; Lobo et al., 2010; Kravitz et al., 2012). For instance, it has been suggested that altered D2R MSN transmission may disrupt inhibitory controls and avoidance in a decision conflict task (Hikida et al., 2010, 2013). Moreover, studies in humans and rodents suggest that the D2R modulates reward and emotional processing (Hranilovic et al., 2008; Peciña et al., 2013; Brandão et al., 2015), while the activation of D2R neurons in mice induced depressive-like behavior (Francis et al., 2015).

To gain a better understanding of how GPR88 in D2R MSNs regulates emotional processing, we generated a conditional knockout (KO) of Gpr88 in neurons expressing A2A-R (A2A-R-Gpr88 KO) known to be selectively expressed in D2R MSNs (Schiffmann et al., 1991; Schiffmann and Vanderhaeghen, 1993). To evaluate the contribution of Gpr88 in D2R MSNs, we compared behavioral responses of A2A-R-Gpr88 KO mice with those of total Gpr88 KO animals using behavioral tests measuring anxiety-like behaviors and fear responses. We show that Gpr88 expressed in these neurons is responsible for ethological anxiety-like behaviors, but does not regulate conflict anxiety behaviors.

Materials and Methods

Subjects

Mice (male and female) 9–15 weeks of age were bred in-house in a group house of three to five animals per cage. Animals where maintained on a 12 h light/dark cycle at a controlled temperature (22 ± 1°C). Food and water were available ad libitum throughout all experiments except for the novelty suppressed feeding test. All experiments where approved by the local ethics committee (COMETH 2014-029). For all experiments, Gpr88A2A-Cre mice were compared with their littermates (Gpr88flx/flx), and Gpr88−/− mice were compared with Gpr88+/+ mice. An independent cohort of naïve animals was used for each behavioral paradigm, except for the fear conditioning that was performed in the same cohort as the light/dark test 48 h after the latter. All behavioral testing was performed and analyzed blind to genotypes.

Generation of Gpr88−/− and Gpr88A2A-Cre mice

Gpr88-floxed mice (Gpr88flx/flx), total Gpr88 KO mice (Gpr88−/−; Meirsm et al., 2016), and Adora2a-Cre mice (Durieux et al., 2009) were produced as previously described. Briefly, to generate a total KO, Gpr88flx/flx mice, in which exon 2 is flanked by a loxP site (upstream) and a Lox-FRT neomycin-resistance cassette (downstream) were crossed with cytomegalovirus (CMV)-Cre mice ex-
pressing Cre recombinase under the cytomegalovirus promoter. This led to germline deletion of Gpr88 exon 2 under a mixed background (13.96%, C57BL/6; 60.94%, C57BL/6J; 0.05%, FVB/N; 25%, 129/SvPas; 0.05%, SJL/J).

To generate a conditional KO of Gpr88 in A2A-R-expressing neurons (Gpr88\textsuperscript{A2A-Cre}, Adora2a-Cre) mice were crossed with Gpr88\textsuperscript{lox/lox} mice. The introduction of loxP sites in the mouse Gpr88 gene had no effect on the agonist-induced activation of GPR88 receptor in homozygous floxed mice (Gpr88\textsuperscript{lox/lox}), compared with wild-type animals (Gpr88\textsuperscript{+/+}; see Fig. 2A). First-generation animals expressing Cre under the control of A2A-R promoter (Gpr88\textsuperscript{A2A-Cre/lox}) were crossed a second time to eliminate the wild-type Gpr88 gene (Gpr88\textsuperscript{A2A-Cre/+}; background: 1.08%, C57BL/6; 16.78%, C57BL/6J; 0.01%, FVB/N; 53.17%, 129/SvPas; 0.01%, SJL/J; 29.54%, C57BL/6N). All mice were bred at the Institut Clinique de la Souris (Ims-mice, 5' GAGAGTGA AACCACAGGTGTGTACAC-3' and 5'-GTT TGT TTC CTC ACT GGC TGA GAG TC-3', for GPR88

Tissue preparation and fluorescent in situ hybridization

Mice (n = 4 Gpr88\textsuperscript{lox/lox}; 4 Gpr88\textsuperscript{A2A-Cre}) were killed by cervical dislocation, and fresh brains were extracted and embedded in OCT (Optimal Cutting Temperature Medium, Thermo Scientific), frozen, and kept at −80°C. Frozen brains were coronally sliced into 20-μm serial sections by using a cryostat (model CM3050, Leica) and placed on SuperFrost slides (Thermo Scientific). In situ hybridizations were performed using the RNAscope Multiplex Fluorescent Assay. Gpr88 probes were coupled to FITC, while D1R and D2R probes were coupled with Tritec and Cy5, respectively.

Relative expression of Gpr88 mRNA in Drd1- and Drd2-positive cells

Image acquisition was performed with the slide scanner NanoZoomer 2 HT and fluorescence module L11600-21 (Hamamatsu Photonics). To verify the specific excision of Gpr88 in Drd1 R and Drd2 R MSNs neurons, in situ hybridization images were analyzed using NDP.viewer software. For each brain, four slices were selected, as follows: two slices for the caudate–putamen (CPu; rostral, +0.98 mm from bregma; caudal, −0.58 mm from bregma); one slice for the nucleus accumbens (Nac; +1.34 mm relative to bregma); and one slice for the central nucleus of the amygdala (CeA; −1.22 mm from bregma). For each structure, regions of interest (ROIs) were determined by drawing two-dimensional boxes with defined surfaces. Counting was performed on one ROI with a surface of 1 mm² for the Nac, 0.250 mm² for the CeA, and two ROIs of 0.5 mm² for the each CPu slice (to include both dorsomedian and dorsolateral striatum; Fig. 1). Counting was balanced between right and left hemispheres. To evaluate the expression of Gpr88 mRNA in Drd1- and Drd2-expressing cells, counting was performed manually using the NDP.view software counting add-up. First, cells expressing Drd1 mRNA, but not Drd2 mRNA, were marked and counted. For each Drd1-positive cell, the coexpression of Gpr88 was verified and counted separately. This process was repeated for Drd2 mRNA-positive cells. Relative Gpr88 expression is represented as a percentage of the total Drd1- or Drd2-positive cells counted ([number Drd1 or Drd2 expressing cells coexpressing Gpr88 × 100]/total number of Drd1- or Drd2-expressing cells). Given the lack of difference in Gpr88 expression between lateral and medial CPu, the relative percentage of each was pooled for graphical representation and statistical analysis.

[\textsuperscript{35}S]-GTP\textgamma;S binding assay

[\textsuperscript{35}S]-GTP\textgamma;S assays were performed on membrane preparations, as described in our previous report (Pradhan et al., 2009).

To evaluate the activation of GPR88 in PFC, CPu, Nac, CeA, and hippocampus, structures were punched in six animals of each genotype (three males, three females), as previously described (Meirsm et al., 2016), and were pooled for membrane preparation. To perform [\textsuperscript{35}S]-GTP\textgamma;S assays on whole striatum mice were killed by cervical dislocation, and both striata were rapidly manually removed, frozen in dry ice, and stored at −80°C until use. Three membrane preparations were used for each genotype, gathering tissue from three animals each (males and females). Results are expressed as mean measures from the three membrane preparations. All assays were performed on membrane preparations. Membranes were prepared by homogenizing the tissue in an ice-cold 0.25 μL sucrose solution (10 vol/ml/g wet weight of tissue). Samples were then centrifuged at 2500 × g for 10 min. Supernatants were collected and diluted 10 times in buffer containing 50 μM Tris-HCl, pH 7.4, 3 mM MgCl\textsubscript{2}, 100 mM NaCl, and 0.2 mM EGTA, following which they were centrifuged at 23,000 × g for 30 min. The pellets were homogenized in 800 μL of ice-cold sucrose solution (0.32 M) and kept at −80°C. For each [\textsuperscript{35}S]-GTP\textgamma;S binding assay, 2 μg of protein/well was used. Samples were incubated with and without ligands, for 1h at 25°C in assay buffer containing 30 μM GDP and 0.1 μM [\textsuperscript{35}S]-GTP\textgamma;S. Bound radioactivity was quantified using a liquid scintillation counter. B\textsubscript{max} and K\textsubscript{d} values were calculated. Non-specific binding was defined as binding in the presence of 10 μM GTP\textgamma;S, and binding in the absence of agonist was defined as the basal binding.

Drugs

The GPR88 agonist compound 19 (Meirsm et al., 2016) was synthesized by Prestwick Chemicals and dissolved in water.
Figure 1. Molecular characterization of conditional A2AR-Gpr88 KO mice. A. Triple-fluorescent in situ hybridization probing Gpr88 (Aa, Ab, Ae, Af, Ai, and Aj; probe labeled in green), Drd2 (Ac–Af; probe labeled in orange), and Drd1 (Ag–Aj; probe labeled in red). Representative images are shown. In Gpr88flx/flx control animals, Gpr88 mRNA colocalizes with both Drd2 (Ae: merge GPR88/Drd2, white arrows) and Drd1 mRNA (Ai: merge GPR88/Drd1, yellow arrows). In contrast, Gpr88A2A-Cre conditional mice show almost no colocalization with Drd2 (Af: merge GPR88/D2R), while colocalization with Drd1 remains (Aj: merge GPR88/Drd1, yellow arrows). DAPI staining (blue) was used to label all cell nuclei. Scale bar, 25 μm. B. Quantification shows a strong decrease of Gpr88/Drd2 double-positive neurons (red), but not GPR88/Drd1 double-positive (blue) neurons, in the CPu, Nac, and CeA of Gpr88A2A-Cre conditional mice. Percentage of Gpr88 expression was calculated based on the total number of Drd1- or Drd2-positive cells counted [(number Drd1- or Drd2-expressing cells coexpressing Gpr88 × 100)/total number of Drd1- or Drd2-expressing cells]. Data are presented as the mean ± SEM. n = 4, Gpr88A2A-Cre; n = 4, Gpr88flx/flx; solid asterisks). *p < 0.05; **p < 0.01; ***p < 0.001 (Student’s t test).
Behavioral analysis

Open field locomotion
To assess basal locomotor activity in a novel environment, mice were placed in a dimly lit (15 lux) open field arena placed over a white Plexiglas infrared-lit platform. Locomotor activity was recorded during 30 min via an automated tracking system (Videotrack, ViewPoint). Only movements with speed exceeding 6 cm/s were taken into account for this measure.

Elevated plus maze
Anxiety-like behavior was first evaluated using the ethological (also known as unconditioned) anxiety test elevated plus maze (EPM). The EPM was a plus-shaped maze elevated 52 cm from the base, with a black Plexiglas floor consisting of two open and two closed arms (37 × 6 cm each) connected by a central platform (6 × 6 cm). The experiments were conducted under low-intensity light (15 lux). The movement and location of the mice were analyzed by an automated tracking system (Videotrack; ViewPoint, Lyon, France). Each mouse was placed on the central platform facing a closed arm and were observed for 5 min. Anxiety-like behavior was assessed by measures of the time spent and the number of entries in closed and open arms of the maze, and related time and activity ratios (time spent or distance traveled in open arms/total time spent or distance in arms). Risk-taking behavior was evaluated by analyzing the time spent in the distal part of the open arms (time spent in the last one-third of the open arm) and the number of head dips (total number of head dips and head dips from the distal part of the open arms). Finally, the distance traveled in the maze was used as a measure of locomotor activity.

Light/dark test
Anxiety-like behavior was next evaluated using the light/dark apparatus, which was composed of two rectangular compartments (20 × 20 × 14 cm) separated by a tunnel (5 × 7 × 10 cm; Imetronic). One compartment is constituted of a black floor and walls dimly lit (5 lux), whereas the other is constituted of a white floor and walls intensely lit (1000 lux). The apparatus is equipped with infrared beams and sensors. Mice were placed in the dark compartment, and behavior was automatically recorded for 5 min.

Novelty-suppressed feeding test
Conflict-based anxiety was measured using the novelty-suppressed feeding test. All mice were subjected to fasting 24 h before the beginning of the test, but water was provided ad libitum. Mice were isolated in a single cage 30 min before the beginning of the test. During the test, three food pellets (regular chow) were placed on a square piece of white filter paper positioned in the center of a brightly illuminated (60 lux) open field (50 × 50 cm) filled with ~2 cm of sawdust bedding. Each mouse was placed in a corner of the open field facing the open field wall. The latency to the first bite of the food pellet was recorded. The cutoff time was defined as 15 min. After the test was over, the animal was placed in the home cage and left alone for 5 min. The food intake during this period was scored.

Social interaction test
Social interaction was assessed on an open field (50 × 50 cm) dimly lit (<10 lux) using naive wild-type mice of the same age and weight as interactors. On the first day, all mice were individually placed in the open field arena and left for a 10 min period of habituation. The next day, mice were placed in the open field arena with a wild-type naive interactor, and a 10 min session was recorded. Nose and paw contacts as well as following and grooming were measured. If an interactor failed to engage in any interaction, data from the respective mice were excluded from analysis (one mouse was excluded).

Marble burying
Defensive burying was measured using the marble-burying test performed with 20 small glass marbles (15 mm) evenly spaced in a transparent single cage (21 × 11 × 17 cm) over 4 cm sawdust bedding. The cage was covered by a plastic lid in a room illuminated at 40 lux. The mice were left in the cage for 10 min, and the number of marbles buried more than halfway in the sawdust was counted.

Novelty preference
Novelty preference was assessed in unbiased computerized boxes that have been previously described (Imetronic; Le Merrer et al., 2012). Briefly, the apparatus was composed of two chambers separated by a central alley. Two sliding doors separated the compartments from the central alley. Chambers differed in global shape (but same total surface) and floor texture. Mice were confined to one of the chambers (the familiar chamber) for 15 min before being placed in the central corridor for 5 min. Then, both sliding doors were opened, and mice were allowed to freely explore the apparatus. Time spent in each chamber was recorded, and the novelty preference was calculated as the percentage of time spent in the unfamiliar compartment.

Fear conditioning
Context- and cue-related conditioned fear responses were evaluated using a fear-conditioning paradigm. Experiments were conducted in four dimly lit operant chambers (28 × 21 × 22 cm; Coulbourn Instruments), with a Plexiglas door and a metal bar floor connected to a shocker (Coulbourn Instruments). Chambers had a permanent house light and were equipped with a speaker for tone delivery. An infrared activity monitor, which was used to assess animal motion, was placed on the ceiling of each chamber. The activity/inactivity behavior was monitored continuously during a 100 ms period. Data are expressed in the duration of inactivity per second, and the total time of inactivity displayed by each subject during training and testing sessions was counted. The procedure was similar to one previously described (Goeldner et al., 2009). Briefly, on the first day, animals underwent one conditioning session; on the second day, contextual and cued fear conditioning were tested. The conditioning session was initiated with a 4 min habituation period followed by a 20-s-long tone of 20 KHz/75 dB [conditional stimulus (CS)] coupled with a 0.4 mA footshock [unconditional stimulus (US)] during the last second. Two minutes later,
as a similar CS–US pairing was presented, and the mice were removed from the apparatus 2 min after the footshock. On the following day, mice were exposed again to the conditioning chamber, and immobility was measured over 4 min to assess contextual fear conditioning. The same day, 5 h after context fear was measured, cued fear conditioning was assessed in modified chambers.

**Statistical analysis**
All data are expressed as the mean group value ± SEM and were analyzed using Student’s test or two-way ANOVA, whenever it was appropriate. When relevant, data were submitted to Sidak’s or Tukey’s multiple-comparison *post hoc* analysis. The criterion for statistical significance was *p* < 0.05. All statistics were performed using Prism 6 (GraphPad Software).

**Results**

**Gpr88<sup>A2A-Cre</sup>** mice show decreased Gpr88 mRNA levels in D<sub>2</sub>R neurons of caudate–putamen, nucleus accumbens, and central amygdala

To conditionally delete Gpr88 exon 2 in D<sub>2</sub>R MSNs, we crossed mice carrying two LoXP sites flanking the second exon of the Gpr88 gene with mice expressing the Cre recombinase under the control of the D<sub>2</sub>R MSN-specific Adora2a gene promoter (Durieux et al., 2009; Meirsman et al., 2016). Then, we quantified Gpr88 mRNA in Drd1-positive and Drd2-positive neurons in Gpr88<sup>lox/lox</sup> (control) and Gpr88<sup>A2A-Cre</sup> mice using triple *in situ* hybridization. Quantitative analysis was performed in four brain regions, including rostral CPu, caudal CPu, Nac, and CeA (Fig. 1). In control animals (*n* = 4), Gpr88 mRNA was present in striatal Drd1 (96.76% ± 0.28) and Drd2 (96.44% ± 0.74) expressing cells, as well as in the few (n ~ 17/ROI) cells coexpressing the two dopaminergic receptor mRNAs. In the CeA of control animals, Gpr88 was expressed in fewer Drd1-positive (68.31% ± 13.24) and Drd2-positive (78.26% ± 11.77) cells compared to the striatum, and did not significantly differ across the two cell types (*t*<sub>0</sub> = 0.56; *p* = 0.59). Expression of the Cre in A<sub>2</sub>A R expressing neurons had no effect on Gpr88 mRNA in D<sub>2</sub>R expressing neurons in any of the structures analyzed (*n* = 4). In contrast, the number of Gpr88<sup>lox/lox</sup>-positive cells was strongly reduced in D2R expressing MSNs of rostral ((25.89% ± 7.86); *t*<sub>0</sub> = 9.004; *p* < 0.001) and caudal CPu (40.24 ± 2.68%; *t*<sub>0</sub> = 19.68; *p* < 0.0001) as well as in Nac (40.08 ± 11.66%; *t*<sub>0</sub> = 4.736; *p* = 0.0032) as previously demonstrated with this Adora2a cre mice (Durieux et al., 2009, 2012; Ena et al., 2013). Also, mice expressing the Cre in A<sub>2</sub>A R expressing neurons had significantly lower number of Gpr88<sup>lox/lox</sup>-positive cells in D<sub>2</sub>R-neurons of the CeA (34.33% ± 8.2) compared to control animals (*t*<sub>0</sub> = 3.06; *p* = 0.02). Together, the data indicate that conditional Gpr88<sup>A2A-Cre</sup> mice show a selective decrease of Gpr88 transcript levels in D<sub>2</sub>R-neurons of striatum and CeA.

**Gpr88<sup>A2A-Cre</sup>** mice show decreased GPR88 agonist-induced [<sup>35</sup>S]-GTP<sub>Y</sub>S binding

To measure the consequences of Gpr88 gene knockout at protein level, we performed Gpr88 agonist-induced [<sup>35</sup>S]-GTP<sub>Y</sub>S binding assays in Gpr88<sup>A2A-Cre</sup> mice and their controls, as well as in total Gpr88<sup>+/−</sup> (negative control; Fig. 2B–F). Structures were chosen based on A<sub>2</sub>A R expression (Schiffmann et al., 1991; Schiffmann and Vanderhaeghen, 1993). We found a significant genotype effect in the CPu (Gpr88<sup>A2A-Cre</sup>: 282.2% ± 15.46; Gpr88<sup>lox/lox</sup>: 427.2% ± 22.61; genotype effect F<sub>2</sub> = 61.56; *p* < 0.0001), Nac (Gpr88<sup>A2A-Cre</sup>: 205.1% ± 5.54; Gpr88<sup>lox/lox</sup>: 342.4% ± 9.13; F<sub>2</sub> = 152.92; *p* < 0.0001), and CeA (Gpr88<sup>A2A-Cre</sup>: 141.2% ± 6.18; Gpr88<sup>lox/lox</sup>: 170.3% ± 5.90; F<sub>2</sub> = 37.48; *p* < 0.0001). Post hoc analysis (Tukey multiple comparisons) revealed significant differences between Gpr88<sup>A2A-Cre</sup> and Gpr88<sup>lox/lox</sup> for the two highest agonist concentrations (10<sup>−5</sup> and 10<sup>−6</sup> M) in the CPu, Nac, and CeA. This result demonstrates that the selective Gpr88 gene KO in D<sub>2</sub>R-expressing cells, observed at mRNA levels, translates into a significant reduction of protein levels in regions of high Gpr88 expression. Although the approach does not discriminate GPR88 signaling in D<sub>1</sub>R and D<sub>2</sub>R-expressing cells, the approximately 40% reduction in CPu membranes reflects the specific Gpr88 gene KO in D<sub>2</sub>R cells, representing ~40% of the dorsal striatum population of dopamine receptor-expressing neurons (Valjent et al., 2009).

**Both total and A<sub>2</sub>A-R-Gpr88 gene deletion increase basal locomotor activity**

Previous studies have demonstrated increased basal locomotor activity in total Gpr88 KO mice (Quintaña et al., 2012; Meirsman et al., 2016), as was also observed in mice with disrupted D<sub>2</sub>R MSN activities (Durieux et al., 2009, 2012; Bateup et al., 2010). We compared general locomotor activity in total and A<sub>2</sub>A-R-Gpr88 KO mice. Animals were individually placed in a dimly lit open field and analysis of forward locomotion revealed a significant increased activity for Gpr88<sup>A2A-Cre</sup> (n = 10, Gpr88<sup>A2A-Cre</sup>; n = 10, Gpr88<sup>lox/lox</sup>; genotype effect: F<sub>1</sub> = 28.93, *p* < 0.0001; mean locomotion: t<sub>19</sub> = 16.50, *p* < 0.0001) as well as Gpr88<sup>+/−</sup> mice (n = 12, Gpr88<sup>+/−</sup>; n = 12, Gpr88<sup>A2A-Cre</sup>; n = 12, Gpr88<sup>lox/lox</sup>; genotype effect: F<sub>1</sub> = 52.72, *p* < 0.0001; mean locomotion: t<sub>19</sub> = 6.65, *p* < 0.0001; Fig. 3). The deletion of Gpr88 in A<sub>2</sub>A-R-neurons is therefore sufficient to recapitulate the hyperlocomotion phenotype of Gpr88 KO mice, suggesting that the locomotor effect of GPR88 is mediated through receptors expressed in D<sub>2</sub>R MSNs.

**Both total and A<sub>2</sub>A-R-Gpr88 deletion decrease anxiety-like behavior**

Complete deletion of Gpr88 in mice decreased anxiety levels in several models of anxiety-like behavior (Meirsman et al., 2016). To examine whether this behavior is dependent on Gpr88 in A<sub>2</sub>A-R neurons, we evaluated anxiety-like behaviors of Gpr88<sup>A2A-Cre</sup> and Gpr88<sup>+/−</sup> mice in the standard light/dark and elevated plus maze test. Results indicate that both A<sub>2</sub>A-R-Gpr88 (n = 11, Gpr88<sup>A2A-Cre</sup>; n = 9, Gpr88<sup>lox/lox</sup>) and total KO mice (n = 11, Gpr88<sup>A2A-Cre</sup>; n = 9, Gpr88<sup>lox/lox</sup>) entered more frequently (Gpr88<sup>A2A-Cre</sup>; t<sub>18</sub> = 3.01, *p* = 0.008; Gpr88<sup>+/−</sup>; t<sub>18</sub> = 2.46, *p* = 0.023) and spent more time (Gpr88<sup>A2A-Cre</sup>; t<sub>18</sub> = 3.11, *p* = 0.006; Gpr88<sup>+/−</sup>; t<sub>18</sub> = 2.84, *p* = 0.01) exploring the aversive illuminated compartment of the light/dark apparatus (Fig. 4A–D). In the elevated
Figure 2. Agonist-induced GPR88 activation in conditional A2AR-Gpr88 KO mice. Introduction of LoxP sites does not alter GPR88 activation. A, GPR88-mediated [35S]-GTP-S binding is totally and partially abolished in the striatum of Gpr88−/− and Gpr88A2A-Cre mice, respectively. Gpr88+/+ and Gpr88flx/flx mice present similar GPR88 agonist-induced receptor activation. Two membrane preparations were used per genotype, with each membrane preparation gathering tissue from three animals (1 male/2 females or 2 males/1 female). B–F, Decreased activation of GPR88 in several brain regions of Gpr88A2A-Cre mice: GPR88-mediated [35S]-GTP-S binding is decreased in the CPu, Nac, and central amygdala of mutant animals. Data are represented as the mean ± SEM, A, Text: ***p < 0.001 (post hoc Tukey’s multiple-comparisons test of Gpr88flx/flx or Gpr88−/− vs Gpr88A2ACre and Gpr88−/−). B–F; n = 6, Gpr88A2A-Cre; n = 6, Gpr88−/−; n = 6, Gpr88flx/flx; text: ***p < 0.001 (post hoc Tukey’s multiple comparisons test).

plus maze, A2AR-Gpr88 (n = 12, Gpr88A2A-Cre, n = 13, Gpr88flx/flx) and total Gpr88 deletion (n = 11, Gpr88−/−; n = 9, Gpr88+/+) increased the open arms (OA) exploration (percentage of time in the OA: Gpr88A2A-Cre, t(23) = 4.13, p = 0.0004; Gpr88−/−, t(18) = 2.71, p = 0.015; percentage of the distance traveled in the OA: Gpr88A2A-Cre, t(23) = 3.43, p = 0.0023; Gpr88−/−, t(18) = 3.05, p = 0.007; Fig. 4E, H). Also, both mutants spent more time in the distal part of the open arm (Gpr88A2A-Cre, t(23) = 2.76, p = 0.011; Gpr88−/−, t(18) = 2.17, p = 0.043; Fig. 4G, J) and displayed a higher number of total and distal head dips (total: Gpr88A2A-Cre, t(23) = 3.54, p = 0.0017; Gpr88−/−, t(18) = 3.05, p = 0.007; distal: Gpr88A2A-Cre, t(23) = 2.95, p = 0.0072; Gpr88−/−, t(18) = 2.81, p = 0.012; Fig. 4F, J). There was no difference in the total distance traveled (mutant mice, t(23) = 0.14, p = 0.886; and control mice, t(18) = 0.43, p = 0.675) or the total number of arms entries (mutant mice, t(23) = 0.09, p = 0.925; control mice, t(18) = 0.65, p = 0.52; data not shown). Together,

Figure 3. Locomotor activity is similarly increased in A2AR-Gpr88 KO and total KO mice. A, B, When placed individually in a dimly lit open field for 30 min, both A2AR-Gpr88 KO mice (A) and total KO mice (B) traveled a longer distance than their control littermates. Line graphs show the distance traveled (in centimeters) in 5 min bins over a 30 min session. Bar graphs show the average total distance traveled (in centimeters) over the 30 min sessions. Data are represented as the mean ± SEM. n = 10, Gpr88A2A-Cre, n = 10, Gpr88flx/flx (A); n = 12, Gpr88−/−; n = 12, Gpr88+/+ (B). Open asterisks: ***p < 0.001 (repeated-measures ANOVA); solid asterisks: ***p < 0.001 (Student’s t test).
Figure 4. Anxiety-related responses are similarly increased in A2AR-Gpr88 KO and total KO mice. A–D, A2AR-Gpr88 mice (A, B) and total KO mice (C, D) enter more frequently and spent more time in the light compartment of the light/dark test. E–J, In the elevated plus maze, Gpr88flx/flx and Gpr88KO mice present higher open arms exploration ratios (E, H, respectively), more frequent total and distal head dips (F, I), and increased time spent in the distal zone of the open arms when compared with their control littermates (G, J). K–R, Social interactions were evaluated in a dimly lit open field with wild-type naive mice of the same age and gender. Both mutant animals display increased number of nose and paw contacts, as well as increased following behaviors. Gpr88 mice, but not Gpr88flx/flx mice, engaged less frequently in grooming episodes than control animals. Data are presented as the mean ± SEM. A, B: n = 11, Gpr88flx/flx, Gpr88KO, n = 9, Gpr88flx/flx, Gpr88KO. C: n = 10, Gpr88KO; n = 11, Gpr88(flx/flx, KO). D: n = 10, Gpr88(flx/flx, KO). E–G: n = 12, Gpr88flx/flx, Gpr88KO. H–J: n = 11, Gpr88KO; n = 9, Gpr88(flx/flx, KO). K–N: n = 10, Gpr88flx/flx, Gpr88KO; n = 9, Gpr88flx/flx. O–R: n = 6, Gpr88KO; n = 6, Gpr88KO. Solid asterisks: ∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001 (Student’s t test).

these data show that the GPR88 deletion in A2AR neurons fully accounts for the high levels of anxiety observed in total GPR88 KO mice in this study and our previous study (Meišman et al., 2016).

Because anxiety can affect social behavior, we scored for social interaction. Both mutant lines show increased nose contact (n = 10, Gpr88flx/flx, Gpr88KO; n = 9, Gpr88flx/flx, Gpr88KO; t(17) = 3.39, p = 0.004; n = 6, Gpr88KO; t(9) = 6, Gpr88flx/flx, Gpr88KO; t(10) = 10.12, p < 0.0001) and paw contact (t(17) = 2.73, p = 0.014; and t(10) = 3.94, p = 0.0028) and followed their interactors more frequently than did control littermates (t(17) = 2.72, p = 0.014; and t(10) = 12.03, p < 0.0001; Fig. 4K–R). GPR88 in A2AR neurons, therefore, also recapitulate the phenotype of total GPR88 KO mice in this test.

A2AR-Gpr88 gene deletion increases avoidance but does not regulate approach behaviors

Altered anxiety can be explained by an imbalance between approach-related and avoidance-related drives (Aupperle and Paulus, 2010). To further investigate the role of GPR88 in approach–avoidance conflict-based anxiety tests, we measured avoidance and approach behaviors separately in both mouse lines. Marble burying constitutes an unconditioned defensive behavior aiming to avoid threats, and is reversed by anxiolytic drugs (De Boer and Koolhaas, 2003). In the presence of 20 marbles, A2AR-Gpr88 mice (n = 7, Gpr88flx/flx, Gpr88KO; n = 9 Gpr88flx/flx; t(14) = 3.28, p = 0.005) and total KO mice (n = 7, Gpr88KO; n = 7, Gpr88flx/flx; t(12) = 2.42, p = 0.032) buried significantly fewer marbles than their control littermates.
Discussion

In the present study, we show that GPR88 in A2AR-expressing neurons regulates anxiety-like behaviors without affecting fear conditioning and drive toward novelty or food. In situ hybridization and GTPγS binding data indicate that deletion of this gene in A2AR neurons does not affect food intake.

Figure 5. A2AR-Gpr88 gene deletion increases avoidance but does not regulate approach behaviors. A, B. In the marble-burying test (Gpr88<sup>flx/flx</sup>; A2AR<sup>-/-</sup> Cre<sup>+</sup>, n = 11, Gpr88<sup>flx/flx</sup>; A2AR<sup>-/-</sup> Cre<sup>-/+</sup>, n = 10), both KO mice buried fewer marbles than controls. C, F. When assessing novelty preference, total KO mice (F), but not A2AR-Gpr88 KO mice (C), spent more time in the novel compartment when compared with their littermates. D, E, G. In the novelty-suppressed feeding test, Gpr88<sup>flx/flx</sup> A2AR<sup>-/-</sup> Cre<sup>-/+</sup> mice exhibit similar latencies to start eating and home cage food intake (D) than Gpr88<sup>flx/flx</sup> mice. Gpr88<sup>-/-</sup> mice display shorter latencies to start eating in the center of the arena compared with Gpr88<sup>-/+</sup> mice (G), eating normally when placed back in their home cage (E). Data are presented as the mean ± SEM. n = 11, Gpr88<sup>flx/flx</sup>; 9, Gpr88<sup>flx/flx</sup> A2AR<sup>-/-</sup> Cre<sup>-/+</sup>; n = 11, Gpr88<sup>flx/flx</sup> A2AR<sup>-/-</sup> Cre<sup>-/-</sup>; n = 8, Gpr88<sup>flx/flx</sup> A2AR<sup>-/-</sup> Cre<sup>-/-</sup>; n = 11, Gpr88<sup>flx/flx</sup> A2AR<sup>-/-</sup> Cre<sup>-/-</sup>; n = 11, Gpr88<sup>-/-</sup> A2AR<sup>-/-</sup> Cre<sup>-/-</sup>; n = 10, Gpr88<sup>-/-</sup> A2AR<sup>-/+</sup> Cre<sup>-/-</sup>; n = 10, Gpr88<sup>-/-</sup> A2AR<sup>-/+</sup> Cre<sup>-/+</sup>; n = 10, Gpr88<sup>-/+</sup> A2AR<sup>-/+</sup> Cre<sup>-/-</sup>; n = 10, Gpr88<sup>-/-</sup> A2AR<sup>-/-</sup> Cre<sup>-/-</sup>; n = 10, Gpr88<sup>-/-</sup> A2AR<sup>-/-</sup> Cre<sup>-/-</sup>. Solid asterisk: *p < 0.05, **p < 0.01 (Student’s t test).}

Total but not A<sub>2a</sub>R-Gpr88 deletion impairs fear conditioning

Fear and anxiety are related processes, and fear circuitry has been shown to be altered in anxiety disorders (Stein et al., 2007). To evaluate whether Gpr88 deletion affects fear learning and expression, we tested mice in a fear-conditioning paradigm (Fig. 6). During conditioning, post-shock immobility increased regardless of the genotype, which is suggestive of successful US–CS conditioning (n = 11, Gpr88<sup>flx/flx</sup> A2AR<sup>-/-</sup> Cre<sup>-/-</sup>; n = 11, Gpr88<sup>flx/flx</sup> A2AR<sup>-/-</sup> Cre<sup>-/-</sup>; F<sub>(1,20)</sub> = 23.25, p = 0.0001; n = 10, Gpr88<sup>-/-</sup>; n = 10, Gpr88<sup>-/-</sup>; F<sub>(1,19)</sub> = 17.05, p = 0.0006; time-effect repeated-measures ANOVA). When tested for contextual fear, Gpr88<sup>-/-</sup> mice displayed significantly less immobility than control animals (F<sub>(1,18)</sub> = 2.36, p = 0.039), but not for Gpr88<sup>flx/flx</sup> A2AR<sup>-/-</sup> Cre<sup>-/-</sup> mice (n = 7, Gpr88<sup>-/-</sup>; n = 7, Gpr88<sup>-/-</sup> Cre<sup>-/-</sup>; n = 7, Gpr88<sup>-/-</sup>; n = 7, Gpr88<sup>-/-</sup> Cre<sup>-/-</sup>; n = 7, Gpr88<sup>-/-</sup>; n = 7, Gpr88<sup>-/-</sup> Cre<sup>-/-</sup>). As in context fear measurement, cue presentation led to similar immobility scores between Gpr88<sup>flx/flx</sup> A2AR<sup>-/-</sup> Cre<sup>-/-</sup> mice and their controls (F<sub>(1,19)</sub> = 1.14, p = 0.27). As in context fear measurement, cue presentation led to similar immobility scores between Gpr88<sup>flx/flx</sup> A2AR<sup>-/-</sup> Cre<sup>-/-</sup> mice and their controls (F<sub>(1,20)</sub> = 1.34, p = 0.17). Moreover, in the novelty-suppressed feeding test (Fig. 5D,G), Gpr88<sup>-/-</sup> mice, but not Gpr88<sup>flx/flx</sup> A2AR<sup>-/-</sup> Cre<sup>-/-</sup> mice, show a decreased latency to feed. Thus, approach behaviors in both novelty preference and exploration of novel instead of familiar environments (Fig. 5A,B). In this test, therefore, conditional D<sub>2</sub>R mice recapitulate the behavioral deficit of total KO mice. However, when evaluating the motivation of mice for the exploration of novel instead of familiar environments (Fig. 5C,F), we observed a significant increase in novel environment exploration for Gpr88<sup>-/-</sup> mice (n = 7, Gpr88<sup>-/-</sup>; n = 7, Gpr88<sup>-/-</sup>; n = 7, Gpr88<sup>-/-</sup>; n = 7, Gpr88<sup>-/-</sup>; t<sub>(12)</sub> = 2.31, p = 0.039), but not for Gpr88<sup>flx/flx</sup> A2AR<sup>-/-</sup> Cre<sup>-/-</sup> mice (n = 7, Gpr88<sup>-/-</sup>; n = 7, Gpr88<sup>-/-</sup> Cre<sup>-/-</sup>; n = 9, Gpr88<sup>flx/flx</sup>; t<sub>(12)</sub> = 1.43, p = 0.17). Moreover, in the novelty-suppressed feeding test (Fig. 5D,G), Gpr88<sup>-/-</sup> mice, but not Gpr88<sup>flx/flx</sup> A2AR<sup>-/-</sup> Cre<sup>-/-</sup> mice, show a decreased latency to feed.

Thus, approach behaviors in both novelty preference and novelty-suppressed feeding tests are enhanced in total but not in A<sub>2a</sub>R Gpr88 KO mice. These data together suggest that GPR88 in A<sub>2a</sub>R neurons regulates threat avoidance, but not novelty approach behaviors.

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Gpr88<sup>-/-</sup><br>Gpr88<sup>flx/flx</sup><br>Gpr88<sup>A2A-Cre</sup>
cate that Gpr88 was efficiently deleted in neurons expressing the D_{2}R of Gpr88^{A2A-Cre} animals in dorsal as well as ventral striatum, and, to a smaller extent, in the central nucleus of the amygdala. Importantly, Gpr88 mRNA level in D_{1}R-MSNs was intact in Gpr88^{A2A-Cre} mice when compared with control mice not expressing the Cre recombinase. The selective deletion of Gpr88 in D_{2}R neurons in the striatum of Gpr88^{A2A-Cre} mice is consistent with the previous characterization of Adora2a-Cre mice showing specific expression of Cre recombinase in these neurons (Durieux et al., 2009, 2012; Ena et al., 2013). Furthermore, we confirm previous data indicating that Gpr88 is present in the vast majority of D_{1}R- and D_{2}R-expressing MSNs (Quintana et al., 2012).

Converging evidence supports the inhibitory function of striatal D_{2}R MSNs in motor output systems. Optogenetic bilateral excitation of these neurons was shown to decrease the initiation of locomotor activity (Kravitz et al., 2010), while ablation or disruption of these neurons increased motor activity (Durieux et al., 2009, 2012; Bateup et al., 2010). Several reports (Logue et al., 2009; Quintana et al., 2012), including our own work (Meirsman et al., 2016), indicate that Gpr88 gene deletion leads to general hyperactive behavior. In the present report, we show that the deletion of Gpr88 from A_{2A}R neurons is sufficient to increase locomotor activity. Considering the predominant expression of A_{2A}Rs in D_{2}R MSNs, this observation suggests that GPR88 normally promotes the demonstrated inhibitory function of D_{2}R MSNs on locomotor activity. The mechanisms underlying the positive modulatory role of GPR88 on D_{2}R MSNs remain to be clarified. Importantly, both total and A_{2A}R-Gpr88 KO mice show a similar hyperactivity when tested under the same experimental conditions, strongly suggesting that the presence of GPR88 in D_{2}R MSN fully accounts for this phenotype.

In a previous report, we showed that Gpr88^{+/−} mice present decreased anxiety-like behaviors (Meirsman et al., 2016). Also, recent reports suggest that D_{1}R and D_{2}R MSNs regulate emotional processing and goal-directed behavior (Hranilovic et al., 2008; Kravitz et al., 2012; Peciña et al., 2013; Brandão et al., 2015; Francis et al., 2015). In the light/dark and elevated plus maze tests, both Gpr88^{A2A-Cre} and Gpr88^{+/−} mice displayed similar decreased anxiety-like behaviors with increased exploration of the light compartment/open arm of the apparatus. This strongly suggests that GPR88 in D_{2}R neurons, but not in D_{1}R neurons, regulates anxiety-like behaviors; however, we cannot exclude that GPR88 also regulates emotional behavior in A2AR-expressing neurons at extrastriatal sites (Wei et al., 2014). Note that this anxiety phenotype cannot be explained by their overall hyperactive behavior since the total distance traveled or the number of entries did not differ from that of control animals.

In these ethological anxiety tests, the tendency to avoid threatening stimuli (bright light/exposed arms) is confronted with the inner drive toward exploration, and this conflict is thought to inhibit exploration (Crawley, 2000; Sousa et al., 2006; Bailey and Crawley, 2009; Aupperle and Paulus, 2010). As such, the low-anxiety phenotype of mice lacking GPR88 could result from increased drive toward novelty exploration, decreased avoidance of a threatening environment, or both factors. We therefore evaluated avoidance behavior in the marble-burying test that measures ethological defensive burying (Borsini et al., 2002; De Boer and Koelhaas, 2003). Both Gpr88^{+/−} and Gpr88^{A2A-Cre} mice buried fewer marbles than control littermates, showing decreased defensive burying that is consistent with reduced threat avoidance in these mice. To tackle approach behavior, we assessed novelty preference in both KO lines. Total but not A_{2A}R-Gpr88 KO mice showed enhanced preference for the novel compartment when presented with a choice for novel or familiar environment. Similarly, in the novelty-suppressed feeding test, total but not A_{2A}R-Gpr88 deletion decreased the latency to start eating. In this conflict test, both approach and avoidance components are enhanced by starving and neophagia, respectively. The absence of phenotype of Gpr88^{A2A-Cre} mice in these two tests could therefore be
explained by unaltered motivation toward new environment or food reinforcement.

Recent reports suggest that D1R MSNs encode predictive reward and mediate approach behavior, while D2R MSNs mediate aversive and defensive behavior (Hranić et al., 2008; Durieux et al., 2009; Hikida et al., 2010, 2013; Kravitz and Kreitzer, 2012; Kravitz et al., 2012, 2013; Kravitz and Bonci, 2013; Calabresi et al., 2014). For instance, D2R MSN neurotransmission blocking in the nucleus accumbens was found to disrupt aversive but not reward learning (Hikida et al., 2010). Moreover, the same authors showed that the impaired aversive behavior was dependent on D2R activation (Hikida et al., 2013). Interestingly, in the present report, we show decreased threat avoidance in mice lacking GPR88 in A2AR neurons. Together with the increased locomotion observed in A2AR-Gpr88 KO mice, we may therefore hypothesize that the lack of GPR88 in D2R MSNs disrupted the activity of this pathway. Further studies using electrophysiological approaches would help to confirm this hypothesis. Also, results from Gpr88<sup>−/−</sup> mice showing both decreased avoidance behavior and increased novelty and food approach suggest that GPR88 in D1R MSNs normally regulates approach behavior.

Finally, we tested the fear responses of total and A2AR-Gpr88 KO mice for the first time. Total deletion of GPR88 impaired both context and cued fear responses. Reduced fear responses in total KO mice is in agreement with altered cue-based learning previously reported in Gpr88 KO animals. The lack of phenotype in Gpr88<sup>A2A-Cre</sup> mice may be due to the D1R neuron-mediated mechanisms contributing to these behaviors. Alternatively, and because central amygdala functioning is essential in the acquisition and expression of fear conditioning (Wilensky et al., 2006), the partial Gpr88 deletion at the level of the amygdala may be insufficient to alter fear responses. Further studies using viral approaches will define the precise role of GPR88 function in amygdala-mediated fear responses.

In sum, our analysis of Gpr88<sup>A2A-Cre</sup> mice shows that GPR88 in A2AR MSNs regulates locomotor and anxiety behaviors. These results represent a first step toward understanding the circuit mechanisms underlying GPR88 function in the brain. Future studies will evaluate the role of GPR88 in D1R MSNs, and how this receptor regulates the D1R/D2R MSN balance. Finally, further demonstration of GPR88 implication in anxiety-related behaviors and threat evaluation definitely posit GPR88 blockade as a new target for the treatment of anxiety-related disorders (Aupperle and Paulus, 2010).

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