Disorders of the Nervous System

GPR88 in A_{2A}R Neurons Enhances Anxiety-Like Behaviors

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Abstract

GPR88 is an orphan G-protein-coupled receptor highly expressed in striatal dopamine D_1 (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 A_{2A}R (A_{2A}R)-Cre-driven recombination, and compared anxiety-related responses in both total and *A_{2A}R-Gpr88* KO mice. *A_{2A}R-Gpr88* KO mice showed a selective reduction of *Gpr88* mRNA in D2R-expressing, but not D_1R-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 A_{2A}R-expressing neurons. Further, *A_{2A}R-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, *A_{2A}R-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 A_{2A}R neurons. We conclude that *Gpr88* expressed in A_{2A}R 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 D_1 and D_2 receptors (Rs). To evaluate the contribution of GPR88 in D_2R-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 D_1R 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 (Levoye et al., 2006; Rask-Andersen et al., 2011; Ghanemi, 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 can all be related to the strong enrichment of GPR88 in the striatum (Swerdlow 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 (Meirsman 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 D_1 receptors (Rs; D_1R-MSNs coexpressing substance P) and dopamine D_2R MSNs coexpressing adenosine A_{2A}R (A_{2A}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 D_2R 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 D_2R modulates reward and emotional processing (Hranilovic et al., 2008; Peciña et al., 2013; Brandão et al., 2015), while the activation of D_2R neurons in mice induced depressive-like behavior (Francis et al., 2015).

To gain a better understanding of how GPR88 in D_2R MSNs regulates emotional processing, we generated a conditional knockout (KO) of *Gpr88* in neurons expressing A_{2A}R (*A_{2A}R-Gpr88* KO) known to be selectively expressed in D_2R MSNs (Schiffmann et al., 1991; Schiffmann and Vanderhaeghen, 1993). To evaluate the contribution of GPR88 in D_2R MSNs, we compared behavioral responses of *A_{2A}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 and fear responses.

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, $Gpr88^{A2A\text{-}Cre}$ mice were compared with their littermates ($Gpr88^{flx/flx}$), and $Gpr88^{-/-}$ mice were compared with $Gpr88^{+/+}$ mice. An independent cohort of naive 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 $Gpr88^{A2A\text{-}Cre}$ mice

Gpr88-floxed mice ($Gpr88^{flx/flx}$), total *Gpr88* KO mice ($Gpr88^{-/-}$; Meirsman et al., 2016), and *Adora2a-Cre* mice (Durieux et al., 2009) were produced as previously described. Briefly, to generate a total KO, $Gpr88^{flx/flx}$ 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-

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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 A_{2A}R-expressing neurons ($Gpr88^{A2A\text{-}Cre}$), *Adora2a-Cre* mice were crossed with $Gpr88^{flx/flx}$ 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 ($Gpr88^{flx/flx}$) compared with wild-type animals ($Gpr88^{+/+}$; see Fig. 2*A*). First-generation animals expressing Cre under the control of A_{2A}R promotor ($Gpr88^{A2A\text{-}Cre/+}$) were crossed a second time to eliminate the wild-type *Gpr88* gene ($Gpr88^{A2A\text{-}Cre}$; 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-Institut de Génétique et Biologie Moléculaire et Cellulaire.

Mice were genotyped using PCR-based genotyping with the following primers: $Gpr88^{-/-}$ mice, 5'-GAAGAGTGA AACCACAGGTGTGTACAC-3' and 5'-GTT TGT TTC CTC ACT GGC TGA GAG TC-3' for $GPR88^{+/+}$; and $Gpr88^{A2A\text{-}Cre}$ mice, 5'-GTC CTA GGT GTG GAT ATG ACC TTA G-3' and 5'-GTT TGT TTC CTC ACT GGC TGA GAG TC 3'. To verify the presence of Cre and myosine (with the latter as a positive control) the following primers were used: 5'-GAT CGC TGC CAG GAT ATA CG-3', 5'-CAT CGC CAT CTT CCA GCA G-3', 5'-TTA CGT CCA TCG TGG ACA GC-3', and 5'-TGG GCT GGG TGT TAG CCT TA-3'.

Tissue preparation and fluorescent *in situ* hybridization

Mice ($n = 4$ $Gpr88^{flx/flx}$; 4 $Gpr88^{A2A\text{-}Cre}$) 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 D_1R and D_2R probes were coupled with Tritc 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 D_2R 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 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 mm^2 for the Nac, 0.250 mm^2 for the CeA, and two ROIs of 0.5 mm^2 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 D_1R- and D_2R-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.

[^{35}S]-GTPγS binding assay

[^{35}S]-GTPγS 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 (Meirsman et al., 2016), and were pooled for membrane preparation. To perform [^{35}S]-GTPγS 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 M 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 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 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 [3^{5S}]GTPγS binding assay, 2 μg of protein/well was used. Samples were incubated with and without ligands, for 1 h at 25°C in assay buffer containing 30 mM GDP and 0.1 nM [^{35}S]GTPγS. Bound radioactivity was quantified using a liquid scintillation counter. B_{max} and K_d values were calculated. Nonspecific binding was defined as binding in the presence of 10 μM GTPγS, and binding in the absence of agonist was defined as the basal binding.

Drugs

The GPR88 agonist compound 19 (Meirsman et al., 2016) was synthesized by Prestwick Chemicals and dissolved in water.

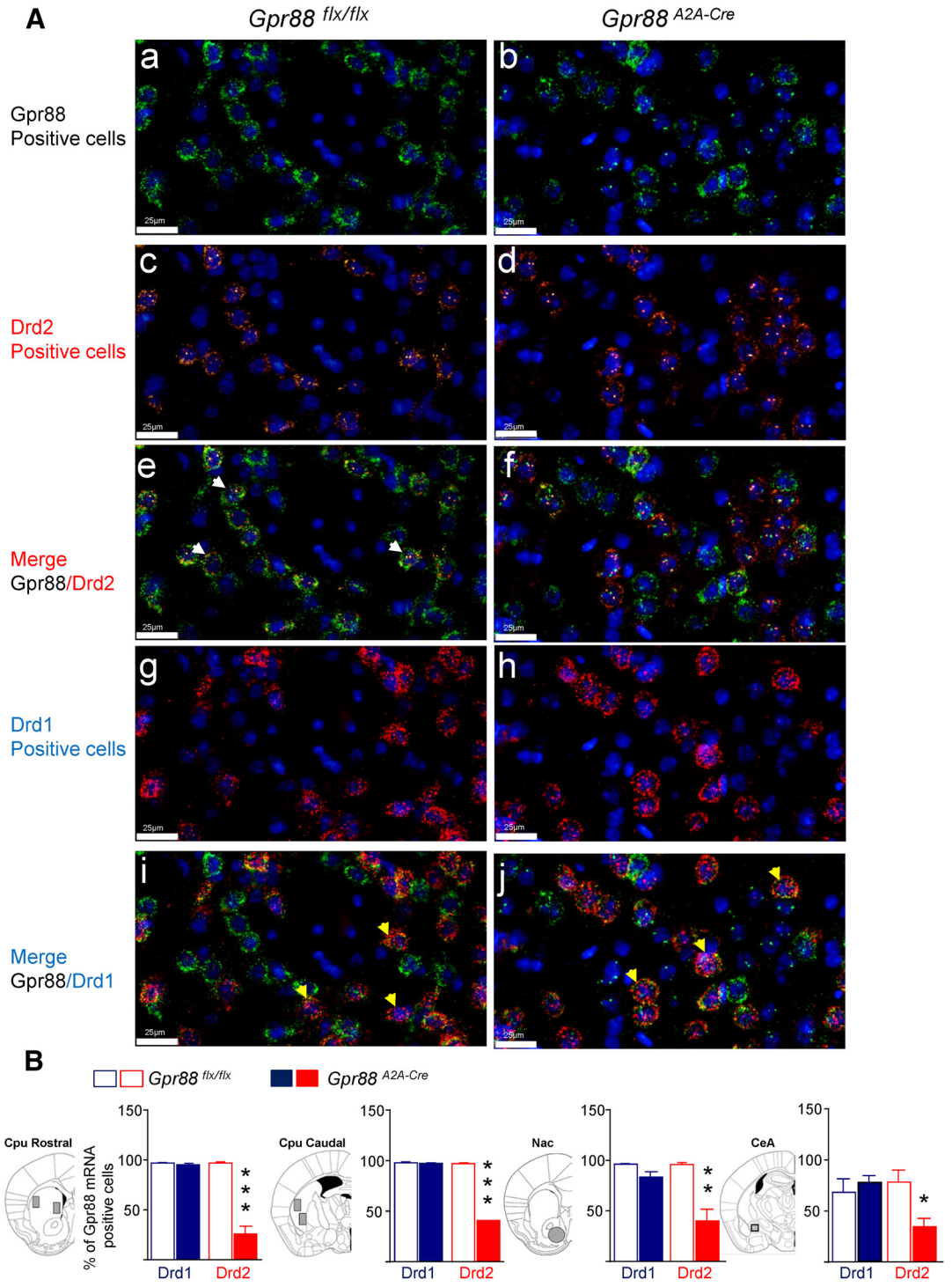


Figure 1. Molecular characterization of conditional $A_{2A}R$-*Gpr88* KO mice. ***A***, Triple-fluorescent *in situ* hybridization probing Gpr88 (***Aa***, ***Ab***, ***Ae***, ***Af***, ***Ai***, and ***Aj***; probe labeled in green), Drd2 (***Ac–Af***; probe labeled in orange), and Drd1 (***Ag–Aj***; probe labeled in red). Representative images are shown. In *Gpr88*$^{flx/flx}$ 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/D_2R), 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 $\times$ 100)/total number of Drd1- or Drd2-expressing cells]. Data are presented as the mean $\pm$ SEM. $n = 4$, Gpr88A2A-Cre; $n = 4$, Gpr88$^{flx/flx}$; 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; View Point, 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,

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$^{A2A\text{-}Cre}$ mice show decreased *Gpr88* mRNA levels in D_2R neurons of caudate–putamen, nucleus accumbens, and central amygdala

To conditionally delete *Gpr88* exon 2 in D_2R 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_2R 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*$^{flx/flx}$ (control) and *Gpr88*$^{A2A\text{-}Cre}$ 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 ($n \sim 17$/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 ($t_{(6)}=0.56$; $p = 0.59$). Expression of the Cre in A_{2A}R expressing neurons had no effect on *Gpr88* mRNA in D_1R expressing neurons in any of the structures analyzed ($n = 4$). In contrast, the number of *Gpr88*-positive cells was strongly reduced in D2R expressing MSNs of rostral ((25.89%± 7.86); $t_{(6)} = 9.004$; $p < 0.001$) and caudal CPu (40.24 ± 2.68%; $t_{(6)} = 19.68$; $p < 0.0001$) as well as in Nac (40.08 ± 11.66%; $t_{(6)}=4.736$; $p = 0.0032$) as previously demonstrated with this *Adora2a* cre mice (Durieux et al., 2009, 2012; Ena et al., 2013). Also, mice expressing the Cre in A_{2A}R expressing neurons had significantly lower number of *Gpr88*-positive cells in D_2R-neurons of the CeA (34.33%±8.2) compared to control animals ($t_{(6)} = 3.06$; $p = 0.02$). Together, the data indicate that conditional *Gpr88*$^{A2A\text{-}Cre}$ mice show a selective decrease of *Gpr88* transcript levels in D_2R-neurons of striatum and CeA.

Gpr88$^{A2A\text{-}Cre}$ mice show decreased GPR88 agonist-induced [S35]-GTPγS binding

To measure the consequences of *Gpr88* gene knockout at protein level, we performed GPR88 agonist-induced [^{35}S]-GTPγS binding assays in *Gpr88*$^{A2A\text{-}Cre}$ mice and their controls, as well as in total *Gpr88*$^{-/-}$ (negative control; Fig. 2*B–F*). Structures were chosen based on A_{2A}R expression (Schiffmann et al., 1991; Schiffmann and Vanderhaeghen, 1993). We found a significant genotype effect in the CPu (*Gpr88*$^{A2A\text{-}Cre}$: 282.2%±15.46; *Gpr88*$^{flx/flx}$: 427%±22.61; genotype effect $F_{(2,30)}=61.56$; $p < 0.0001$), Nac (*Gpr88*$^{A2A\text{-}Cre}$: 205.1%±5.54; *Gpr88*$^{flx/flx}$: 342.4%±9.13; $F_{(2,30)}=152.9$; $p < 0.0001$), and CeA (*Gpr88*$^{A2A\text{-}Cre}$: 141.2%±6.18; *Gpr88*$^{flx/flx}$: 170.3%±5.90; $F_{(2,30)}=37.48$; $p < 0.0001$). *Post hoc* analysis (Tukey multiple comparisons) revealed significant differences between *Gpr88*$^{A2A\text{-}Cre}$ and *Gpr88*$^{flx/flx}$ for the two highest agonist concentrations (10^{-5} and 10^{-6} M) in the CPu, Nac, and CeA. This result demonstrates that the selective *Gpr88* gene KO in D_2R-expressing cells, observed at mRNA level, translates into a significant reduction of protein levels in regions of high *Gpr88* expression. Although the approach does not discriminate GPR88 signaling in D_1R and D_2R-expressing cells, the approximately 40% reduction in CPu membranes reflects the specific *Gpr88* gene KO in D_2R cells, representing ~40% of the dorsal striatum population of dopamine receptor-expressing neurons (Valjent et al., 2009)

Both total and A_{2A}R-*Gpr88* gene deletion increase basal locomotor activity

Previous studies have demonstrated increased basal locomotor activity in total *Gpr88* KO mice (Quintana et al., 2012; Meirsman et al., 2016), as was also observed in mice with disrupted D_2R MSN activities (Durieux et al., 2009, 2012; Bateup et al., 2010). We compared general locomotor activity in total and *A$_{2A}$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*$^{A2A\text{-}Cre}$ ($n = 10$, Gpr88$^{A2A\text{-}Cre}$; $n = 10$, *Gpr88*$^{flx/flx}$; genotype effect: $F_{(1,108)} = 28.93$, $p < 0.0001$; mean locomotion: $t_{(10)} = 15.60$, $p < 0.0001$) as well as *Gpr88*$^{-/-}$ mice ($n = 12$, Gpr88$^{-/-}$; $n = 12$, *Gpr88*$^{+/+}$; genotype effect: $F_{(1,132)} = 52.72$, $p < 0.0001$; mean locomotion: $t_{(10)}=6.65$; $p < 0.0001$; Fig. 3). The deletion of *Gpr88* in A_{2A}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_2R MSNs.

Both total and *A$_{2A}$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_{2A}R neurons, we evaluated anxiety-like behaviors of *Gpr88*$^{A2A\text{-}Cre}$ and *Gpr88*$^{-/-}$ mice in the standard light/dark and elevated plus maze test. Results indicate that both *A$_{2A}$R-Gpr88* ($n = 11$, *Gpr88*$^{A2A\text{-}Cre}$; $n = 9$, *Gpr88*$^{flx/flx}$) and total KO mice ($n = 11$, *Gpr88*$^{A2A\text{-}Cre}$; $n = 9$, *Gpr88*$^{flx/flx}$) entered more frequently (*Gpr88*$^{A2A\text{-}Cre}$: $t_{(18)}= 3.01$, $p = 0.008$; *Gpr88*$^{-/-}$: $t_{(19)}= 2.46$, $p = 0.023$) and spent more time (*Gpr88*$^{A2A\text{-}Cre}$: $t_{(18)}= 3.11$, $p = 0.006$; *Gpr88*$^{-/-}$: $t_{(19)}= 2.84$, $p = 0.01$) exploring the aversive illuminated compartment of the light/dark apparatus (Fig. 4*A–D*). In the elevated

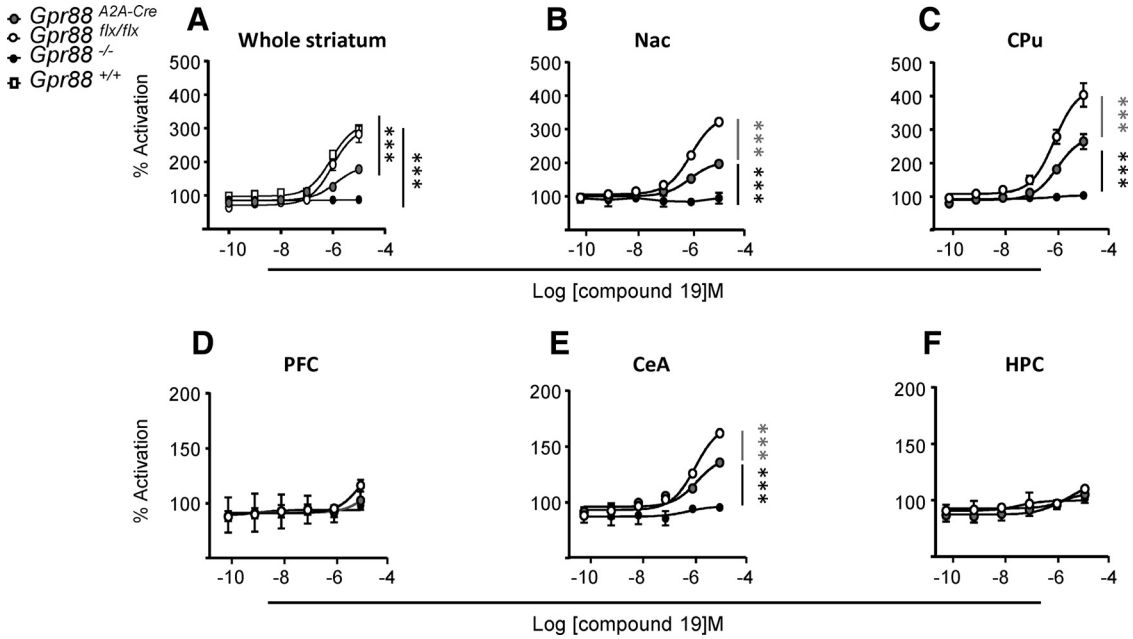


Figure 2. Agonist-induced GPR88 activation in conditional $A_{2A}R$-*Gpr88* KO mice. Introduction of LoxP sites does not alter GPR88 activation. ***A***, GPR88-mediated [^{35}S]-GTPγS was totally and partially abolished in the striatum of *Gpr88*$^{-/-}$ and *Gpr88*$^{A2A\text{-}Cre}$ mice, respectively. *Gpr88*$^{+/+}$ and *Gpr88*$^{flx/flx}$ 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 *Gpr88*$^{A2A\text{-}Cre}$ mice: GPR88-mediated [^{35}S]-GTPγS 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 *Gpr88*$^{flx/flx}$ or Gpr88$^{+/+}$ vs *Gpr88*$^{A2A\text{-}Cre}$ and *Gpr88*$^{-/-}$; ***B–F***); $n = 6$, *Gpr88*$^{A2A\text{-}Cre}$; $n = 6$, *Gpr88*$^{-/-}$; $n = 6$, *Gpr88*$^{flx/flx}$; text: ***$p < 0.001$ (*post hoc* Tukey's multiple comparisons test).

plus maze, $A_{2A}R$-*Gpr88* ($n = 12$, *Gpr88*$^{A2A\text{-}Cre}$; $n = 13$, *Gpr88*$^{flx/flx}$) and total *Gpr88* deletion ($n = 11$, *Gpr88*$^{-/-}$; $n = 9$, *Gpr88*$^{+/+}$) increased the open arms (OA) exploration (percentage of time in the OA: *Gpr88*$^{A2A\text{-}Cre}$, $t_{(23)} = 4.13$, $p = 0.0004$; *Gpr88*$^{-/-}$, $t_{(18)} = 2.71$, $p = 0.015$; percentage of the distance traveled in the OA: *Gpr88*$^{A2A\text{-}Cre}$, $t_{(23)} = 3.43$, $p = 0.0023$; *Gpr88*$^{-/-}$, $t_{(18)} = 3.05$, $p = 0.007$; Fig. 4*E*,*H*). Also, both mutants spent more time in the distal part of the open arm (*Gpr88*$^{A2A\text{-}Cre}$, $t_{(23)} = 2.76$, $p = 0.011$; *Gpr88*$^{-/-}$, $t_{(18)} = 2.17$, $p = 0.043$; Fig. 4*G*,*J*) and displayed a higher number of total and distal head dips (total: *Gpr88*$^{A2A\text{-}Cre}$, $t_{(23)} = 3.54$, $p = 0.0017$; *Gpr88*$^{-/-}$, $t_{(18)} = 3.05$, $p = 0.007$; distal: *Gpr88*$^{A2A\text{-}Cre}$, $t_{(23)} = 2.95$, $p = 0.0072$; *Gpr88*$^{-/-}$, $t_{(18)} = 2.81$, $p = 0.012$; Fig. 4*F*,*I*). 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,

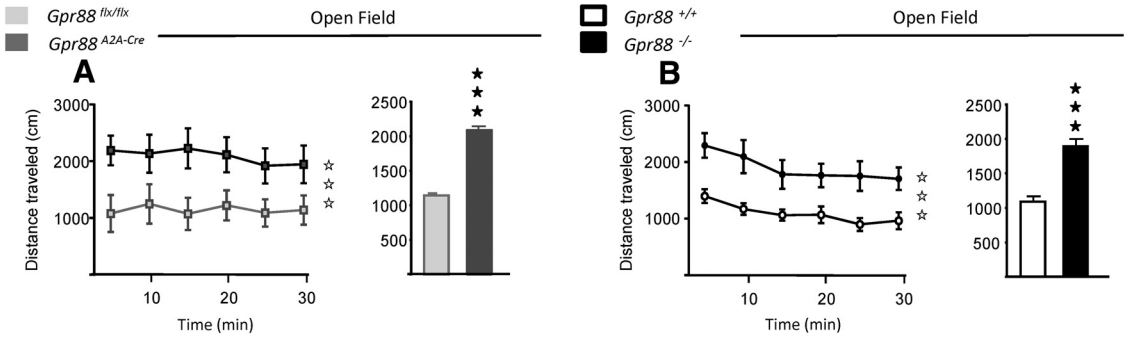


Figure 3. Locomotor activity is similarly increased in *A2AR-Gpr88* KO and total KO mice. ***A***, ***B***, When placed individually in a dimly lit open field for 30 min, both *A2AR-Gpr88* KO mice (***A***) and total KO mice (***B***) traveled a longer distance than their control littermates. Line graphs 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. $n = 10$, *Gpr88*$^{A2A\text{-}Cre}$; $n = 10$, *Gpr88*$^{flx/flx}$ (***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).

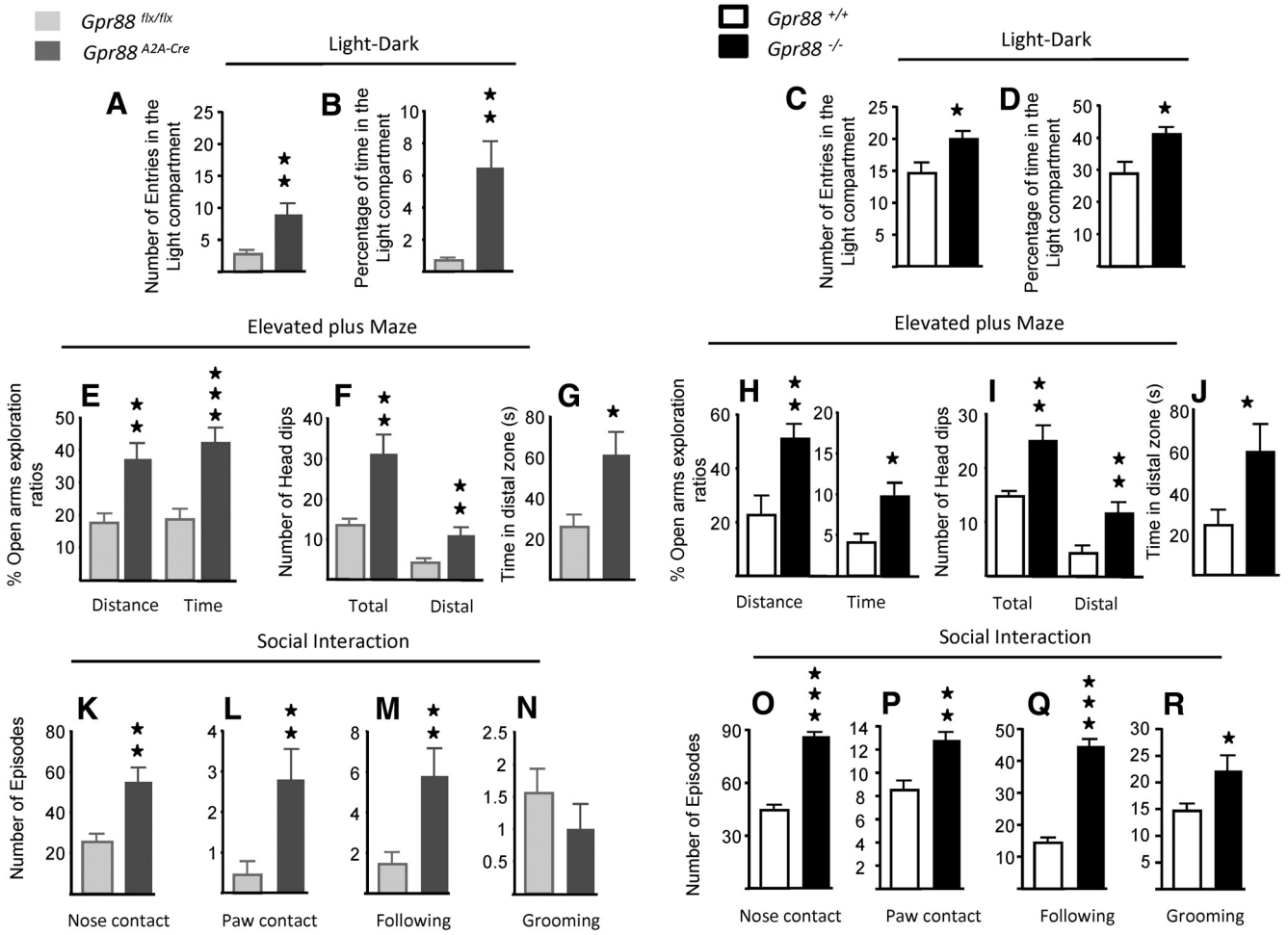


Figure 4. Anxiety-related responses are similarly increased in *A2AR-Gpr88* KO and total KO mice. ***A–D***, *A2AR-Gpr88* mice (***A***, ***B***) and total KO mice (***C***, ***D***) enter more frequently and spent more time in the light compartment of the light/dark test. ***E–J***, In the elevated plus maze, $Gpr88^{A2A\text{-}Cre}$ and $Gpr88^{-/-}$ 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 $Gpr88^{A2A\text{-}Cre}$ mice, engaged less frequently in grooming episodes than control animals Data are presented as the mean ± SEM. ***A***, ***B***: $n = 11$, $Gpr88^{A2A\text{-}Cre}$; $n = 9$ $Gpr88^{flx/flx}$; ***C***, ***D***: $n = 10$, $Gpr88^{-/-}$; $n = 11$, $Gpr88^{+/+}$; ***E–G***: $n = 12$, $Gpr88^{A2A\text{-}Cre}$; $n = 13$, $Gpr88^{flx/flx}$; ***H–J***: $n = 11$, $Gpr88^{-/-}$; $n = 9$, $Gpr88^{+/+}$; ***K–N***: $n = 10$, $Gpr88^{A2A\text{-}Cre}$; $n = 9$, $Gpr88^{flx/flx}$; ***O–R***: $n = 6$, $Gpr88^{-/-}$; $n = 6$, $Gpr88^{+/+}$. Solid asterisks: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (Student's *t* test).

these data show that the GPR88 deletion in $A_{2A}R$ neurons fully accounts for the high levels of anxiety observed in total GPR88 KO mice in this study and our previous study (Meirsman et al., 2016).

Because anxiety can affect social behavior, we scored for social interaction. Both mutant lines show increased nose contact ($n = 10$, $Gpr88^{A2A\text{-}Cre}$; $n = 9$, $Gpr88^{flx/flx}$: $t_{(17)} = 3.39$, $p = 0.004$; $n = 6$, $Gpr88^{-/-}$; $n = 6$, $Gpr88^{+/+}$: $t_{(10)} = 10.12$, $p < 0.0001$) and paw contact ($t_{(17)} = 2.73$, $p = 0.014$; and $t_{(10)} = 3.94$, $p = 0.0028$) and followed their interactor more frequently than did control littermates ($t_{(17)} = 2.72$, $p = 0.014$; and $t_{(10)} = 12.03$, $p < 0.0001$; Fig. 4*K–R*). GPR88 in $A_{2A}R$ neurons, therefore, also recapitulate the phenotype of total GPR88 KO mice in this test.

$A_{2A}R$-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, *$A_{2A}R$-Gpr88* mice ($n = 7$, $Gpr88^{A2A\text{-}Cre}$; $n = 9$ $Gpr88^{flx/flx}$: $t_{(14)} = 3.28$, $p = 0.005$) and total KO mice ($n = 7$, $Gpr88^{-/-}$; $n = 7$, $Gpr88^{+/+}$: $t_{(12)} = 2.42$, $p = 0.032$) buried significantly fewer marbles then their control littermates

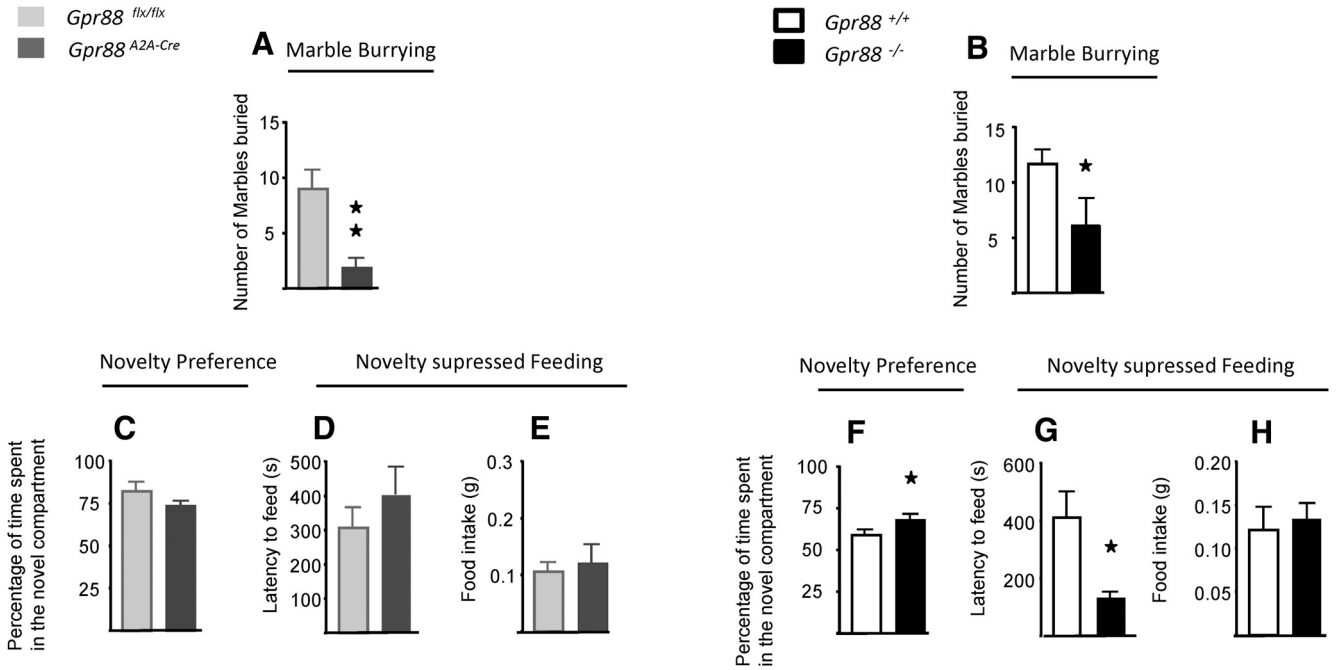


Figure 5. *A2AR-Gpr88* gene deletion increases avoidance but does not regulate approach behaviors. ***A***, ***B***, In the marble-burying test ($Gpr88^{A2A\text{-}Cre}$, ***A***; for $Gpr8^{-/-}$, ***B***), both KO mice buried fewer marbles than controls. ***C***, ***F***, When assessing novelty preference, 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, $Gpr88^{A2A\text{-}Cre}$ mice exhibit similar latencies to start eating and home cage food intake (***D***) than $Gpr88^{flx/flx}$ mice. $Gpr88^{-/-}$ mice display shorter latencies to start eating in the center of the arena compared with $Gpr88^{+/+}$ mice (***G***), eating normally when placed back in their home cage (***E***). Data are presented as the mean ± SEM. $n = 11$, $Gpr88^{A2A\text{-}Cre}$; $n = 9$, $Gpr88^{flx/flx}$ (***A***); $n = 7$, $Gpr88^{-/-}$; $n = 7$, $Gpr88^{+/+}$ (***B***); $n = 8$, $Gpr88^{A2A\text{-}Cre}$; $n = 6$, $Gpr88^{flx/flx}$ (***C***); $n = 11$, $Gpr88^{A2A\text{-}Cre}$; $n = 11$, $Gpr88^{flx/flx}$ (***D***, ***E***); $n = 7$, $Gpr88^{-/-}$; $n = 7$, $Gpr88^{+/+}$ (***F***); $n = 7$, $Gpr88^{-/-}$; $n = 7$, $Gpr88^{+/+}$ (***G***, ***H***). Solid asterisk: $*p < 0.05$, $**p < 0.01$ (Student's *t* test).

(Fig. 5*A*,*B*). In this test, therefore, conditional D_2R 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. 5*C*,*F*), we observed a significant increase in novel environment exploration for $Gpr88^{-/-}$ mice ($n = 7$, $Gpr88^{-/-}$; $n = 7$ $Gpr88^{+/+}$: $t_{(12)} = 2.31$, $p = 0.039$), but not for $Gpr88^{A2A\text{-}Cre}$ mice ($n = 7$, $Gpr88^{A2A\text{-}Cre}$; $n = 9$, $Gpr88^{flx/flx}$: $t_{(12)} = 1.43$, $p = 0.17$). Moreover, in the novelty-suppressed feeding test (Fig. 5*D*,*G*) $Gpr88^{-/-}$ mice, but not $Gpr88^{A2A\text{-}Cre}$ 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 $A_{2A}R$ *GPR88* KO mice. These data together suggest that GPR88 in $A_{2A}R$ neurons regulates threat avoidance, but not novelty approach behaviors.

Total but not $A_{2A}R$-*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 ($n = 11$, $Gpr88^{A2A\text{-}Cre}$; $n = 11$, $Gpr88^{flx/flx}$: $F_{(1,20)} = 23.25$, $p = 0.0001$; $n = 10$, $Gpr88^{-/-}$; $n = 10$, $Gpr88^{+/+}$: $F_{(1,18)} = 17.05$, $p = 0.0006$; time-effect repeated-measures ANOVA). When tested for contextual fear, $Gpr88^{-/-}$ mice displayed significantly less immobility than control animals ($t_{(18)} = 2.36$, $p = 0.029$), which is in agreement with the altered hippocampal functioning previously reported in *Gpr88* KO mice (Meirsman et al., 2016). A similar profile was found when mice were presented with a US-paired tone. In fact, ANOVA indicates a significant genotype effect for $Gpr88^{-/-}$ cue testing ($F_{(1,18)} = 8.89$, $p = 0.008$), and *post hoc* analysis (Sidak's multiple-comparisons test) indicates a significant immobility decrease for both cues presented. In $Gpr88^{A2A\text{-}Cre}$ mice, context-related immobility scores were similar to $Gpr88^{flx/flx}$ control mice ($t_{(20)} = 1.14$, $p = 0.27$). As in context fear measurement, cue presentation led to similar immobility scores between $Gpr88^{A2A\text{-}Cre}$ mice and their controls ($F_{(1,20)} = 1.06$, $p = 0.31$). Overall, these data show that complete deletion of *Gpr88* impairs fear expression in mice; however, deletion of this gene in $A_{2A}R$ neurons does not affect fear expression.

Discussion

In the present study, we show that GPR88 in $A_{2A}R$-expressing neurons regulates anxiety-like behaviors without affecting fear conditioning and drive toward novelty or food. *In situ* hybridization and GTPγS binding data indi-

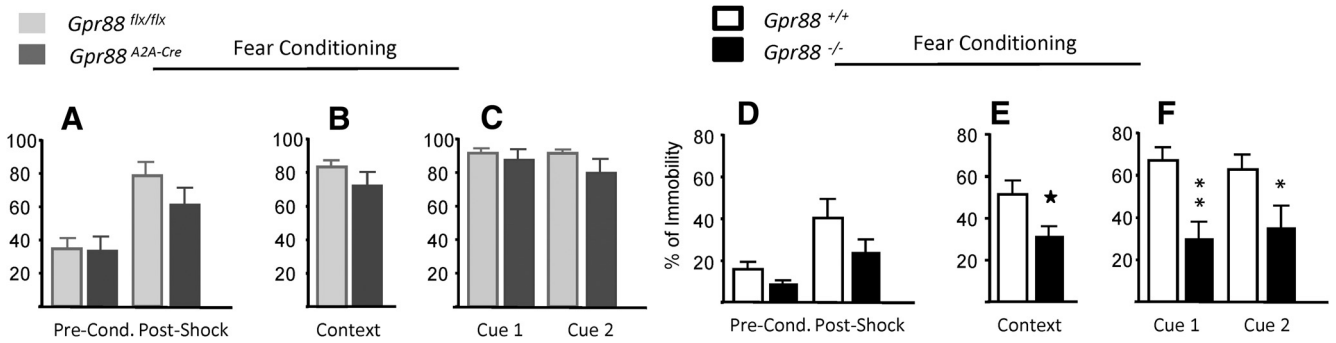


Figure 6. Total but not *A2AR-Gpr88* gene deletion impairs fear conditioning. To assess whether *Gpr88* deletion affects fear responses, we tested mice in a fear-conditioning test. ***A***, ***D***, During the conditioning session, mutant and control animals displayed similar levels of immobility before and after tone–shock pairing when compared with control mice. ***E***, Twenty-four hours later, $Gpr88^{-/-}$ mice displayed significantly lower context fear than $Gpr88^{+/+}$ mice. ***F***, The percentage of immobility of $Gpr88^{-/-}$ was also decreased when tested for cued fear memory. Deletion of *Gpr88* in A_{2A}R-expressing neurons did not affect context (***B***) or cued (***C***) fear memories. $n = 11$, $Gpr88^{A2A\text{-}Cre}$; $n = 11$, $Gpr88^{flx/flx}$ (***A–C***); $n = 10$, $Gpr88^{-/-}$; $n = 10$, $Gpr88^{+/+}$ (***D–F***). Solid asterisks: $*p < 0.05$ (Student's *t* test); text asterisks: $*p < 0.05$, $**p < 0.01$ (*post hoc* Sidak's multiple comparisons test).

cate that *Gpr88* was efficiently deleted in neurons expressing the D_2R of $Gpr88^{A2A\text{-}Cre}$ 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_1R-MSNs was intact in $Gpr88^{A2A\text{-}Cre}$ mice when compared with control mice not expressing the Cre recombinase. The selective deletion of *Gpr88* in D_2R neurons in the striatum of $Gpr88^{A2A\text{-}Cre}$ 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_1R- and D_2R-expressing MSNs (Quintana et al., 2012).

Converging evidence supports the inhibitory function of striatal D_2R 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_2R MSNs, this observation suggests that GPR88 normally promotes the demonstrated inhibitory function of D_2R MSNs on locomotor activity. The mechanisms underlying the positive modulatory role of GPR88 on D_2R 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_2R 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_2Rs and D_2R 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 $Gpr88^{A2A\text{-}Cre}$ 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_2R neurons, but not in D_1R neurons, regulate 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 Koolhaas, 2003). Both $Gpr88^{-/-}$ and $Gpr88^{A2A\text{-}Cre}$ 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 $Gpr88^{A2A\text{-}Cre}$ mice in these two tests could therefore be

explained by unaltered motivation toward new environment or food reinforcement.

Recent reports suggest that D_1R MSNs encode predictive reward and mediate approach behavior, while D_2R MSNs mediate aversive and defensive behavior (Hranilovic 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, D_2R 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 D_2R activation (Hikida et al., 2013). Interestingly, in the present report, we show decreased threat avoidance in mice lacking GPR88 in A_{2A}R neurons. Together with the increased locomotion observed in A_{2A}R-*Gpr88* KO mice, we may therefore hypothesize that the lack of GPR88 in D_2R MSNs disrupted the activity of this pathway. Further studies using electrophysiological approaches would help to confirm this hypothesis. Also, results from *Gpr88*$^{-/-}$ mice showing both decreased avoidance behavior and increased novelty and food approach suggest that GPR88 in D_1R MSNs normally regulates approach behavior.

Finally, we tested the fear responses of total and *A_{2A}R-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*$^{A2A\text{-}Cre}$ mice may be due to the D_1R 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*$^{A2A\text{-}Cre}$ mice shows that GPR88 in A_{2A}R 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 D_1R MSNs, and how this receptor regulates the D_1R/D_2R 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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