Research Article: New Research | Disorders of the Nervous System

GPR88 in A2AR-neurons enhances anxiety-like behaviors

A2AR-Gpr88 knockout and emotional responses

Aura Carole Meirsm1, Anne Robé1, Alban de Kerchove d’Exaerde2 and Brigitte Lina Kieffer1,2,3

1Département de Médecine Translationnelle et Neurogénétique, Institut de Génétique et de Biologie Moléculaire et Cellulaire, INSERM U-964, CNRS UMR-7104, Université de Strasbourg, 67400 Illkirch-Graffenstaden, France
2Laboratory of Neurophysiology, Université libre de Bruxelles, ULB Neuroscience Institute, C.P. 601, Route de Lennik, 808, Brussels 1070, Belgium
3Department of Psychiatry, Faculty of Medicine, Douglas Research Center, McGill University, Montréal H4H 1R3, Canada


Received: 12 July 2016
Accepted: 14 July 2016
Published: 21 July 2016

Author contributions: A.C.M. and B.L.K. designed research; A.C.M. and A.R. performed research; A.C.M. and A.R. analyzed data; A.C.M. and B.L.K. wrote the paper; A.d.K.d. contributed unpublished reagents/analytic tools.

Funding: National Institute of Health
NIH-NIAAA #16658
Funding: National Institute of Health
NIH-NIDA #005010

Conflict of Interest: The authors report no biomedical financial interests or potential conflicts of interest.

National Institute of Health [NIH-NIAAA #16658].

Corresponding author: Brigitte L. Kieffer, Douglas Hospital Research Center, Perry Pavilion Room E-3317.1, 6875 boulevard LaSalle, Montreal (Quebec) H4H 1R3, Canada. Phone: 514 761-6131 ext: 3175 Fax: 514 762-3033. E-mail: brigitte.kieffer@douglas.mcgill.ca

Cite as: eNeuro 2016; 10.1523/ENEURO.0202-16.2016

Alerts: Sign up at eneuro.org/alerts to receive customized email alerts when the fully formatted version of this article is published.
GPR88 in A2AR-neurons enhances anxiety-like behaviors

Running title: A2AR-Gpr88 knockout and emotional responses.

Aura Carole Meirsman¹, Anne Robé¹; Alban de Kerchove d’Exaerde²; Brigitte Lina Kieffer¹-³

¹ Département de Médecine Translationnelle et Neurogénétique, Institut de Génétique et de Biologie Moléculaire et Cellulaire, INSERM U-964, CNRS UMR-7104, Université de Strasbourg, 67400 Illkirch-Graffenstaden, France
² Laboratory of Neurophysiology, Université libre de Bruxelles, ULB Neuroscience Institute, C.P. 601, Route de Lennik, 808, Brussels 1070, Belgium,
³ Douglas Research Center, Department of Psychiatry, Faculty of Medicine, McGill University, Montréal, H4H 1R3, Canada

Corresponding author: Brigitte L. Kieffer, PhD
Douglas Hospital Research Center, Perry Pavilion Room E-3317.1, 6875 boulevard LaSalle, Montreal (Quebec) H4H 1R3, Canada.
Phone: 514 761-6131 ext: 3175 Fax: 514 762-3033.
E-mail address: brigitte.kieffer@douglas.mcgill.ca

Number of pages: 32
Number of figures: 6
Number of words in Abstract: 224
Number of words in Introduction: 520
Number of words in Discussion: 1040

Conflict of interest:
The authors report no biomedical financial interests or potential conflicts of interest.

Acknowledgments:
We thank J. A. J. Becker for his valuable contribution in social interaction testing and data analysis and M. Osikowicz for all her help in preparing the manuscript. We also thank the Mouse Clinic Institute (Illkirch, France) for the generation of mice lines. We thank A. Matifas, G. Duval and D. Memetov for animal care. This work was supported by the Centre National de la Recherche Scientifique (CNRS), Institut National de la Santé et de la Recherche Médicale (INSERM) and Université de Strasbourg. We also thank the ATHOS Consortium, including the Fonds Unigue Interministériel (FUI), the Région Alsace and our partners, Domain Therapeutics (Illkirch, France) and Prestwick Chemicals (Illkirch, France) for critical support in this project. We finally thank the National Institutes of Health (NIH-NIAAA #16658 and NIH-NIDA #005010) for financial support and FRS–FNRS (Belgium) (AKE), and Action de Recherche Concertée (FWB) (AKE). A.C.M. acknowledges doctoral fellowship from Fondation Française pour la Recherche Médicale (FRM: FDT20140930830). AKE is a Senior Research Associate of the FRS–FNRS (Belgium) and a WELBIO investigator.
ABSTRACT

GPR88 is an orphan G protein-coupled receptor highly expressed in striatal D1R 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 \textit{Gpr88} knockout mice using A2AR-Cre-driven recombination, and compared anxiety-related responses in both total and A2AR-\textit{Gpr88} KO mice. A2AR-\textit{Gpr88} KO mice showed a selective reduction of \textit{Gpr88} mRNA in D2R- 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 to those observed in total \textit{Gpr88} KO mice, demonstrating that the previously reported anxiogenic activity of GPR88 operates at the level of A2AR-expressing neurons. Further, A2AR-\textit{Gpr88} KO mice showed no change in novelty preference and novelty-suppressed feeding, while these responses were increased and decreased, respectively, in the total \textit{Gpr88} KO mice. Also, A2AR-\textit{Gpr88} KO mice showed intact fear conditioning, while the fear responses were decreased in total \textit{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 \textit{Gpr88} expressed in A2AR neurons enhances ethological anxiety-like behaviors without affecting conflict anxiety and fear responses.

KEYWORDS: D2R-medium spiny neurons/ G-protein coupled receptors/ striatum/ amygdala/ anxiety-like behavior
SIGNIFICANCE STATEMENT

GPR88, a striatal enriched orphan G protein-coupled receptor has been implicated in regulation of anxiety-like behaviors. In the striatum, GPR88 is most abundant in both medium spiny neurons expressing dopamine D1 receptors and dopamine D2 receptors. 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 knockout 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 towards understanding 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 about 40% marketed drugs, and are major players in biomedicine (Rask-Andersen et al., 2011). Orphans 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 PPI, 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., 2015), implicating this receptor in emotional processing and in 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; Bohm 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 (D1R-MSNs co-expressing substance P) and dopamine D2 receptors (D2R-MSNs co-expressing adenosine A2A receptor - A2AR) and regulates 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 MSNs subpopulations (Logue et al., 2009). However, the precise mechanism by which GPR88 regulates MSNs transmission to alter behavior remains unknown. In recent years, research on MSNs subtypes 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-MSNs transmission may disrupt inhibitory controls and avoidance in a decision conflict task (Hikida et al., 2010; Hikida et al., 2013). Moreover, studies in humans and rodents suggest that the dopamine D2R modulates reward and emotional processing (Hranilovic et al., 2008; Pecina et al., 2013; Brandao et al., 2015) while activation of D2R-neurons in mice induced depressive-like behavior (Francis et al., 2015).

To gain better understanding of how GPR88 in D2R-MSNs regulates emotional processing, we generated a conditional knockout of Gpr88 in neurons expressing A2A (A2AR-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 A2AR-Gpr88 KO mice with those of total Gpr88 knockout (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 do not regulate conflict anxiety and fear responses.

METHODS AND MATERIALS
Subjects. Mice (male and female) aged 9-15 weeks where bred in house and grouped-house 3-5 animals per cage. Animals where maintained on a 12hr light/dark cycle at controlled temperature (22±1°C). Food and water were available ad libitum throughout all experiments except for novelty suppressed feeding test. All experiments where approved by the local ethic comity (COMETH 2014-029). For all experiments Gpr88A2A-Cre mice were compared to their littermates (Gpr88flx/flx) and Gpr88/− mice were compared to 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 48h after the latter. All behavioral testing were performed and analyzed blind to genotypes.

Generation of Gpr88/− and Gpr88A2A-Cre mice. Gpr88-floxed mice (Gpr88flx/flx), total Gpr88 KO (Gpr88/−) (Meirsman et al., 2015) 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 CMV-Cre mice expressing Cre recombinase under the cytomegalovirus promoter. This led to germ-line deletion of Gpr88 exon 2 under a mixed background (13.96% C57B1/6; 60.94% C57B1/6J; 0.05% FVB/N; 25% 129/SvPas; 0.05% SJL/J).

To generate a conditional KO of Gpr88 in A2aR-expressing neurons (Gpr88A2A-Cre) Adora2a-Cre mice were crossed with Gpr88flx/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 (Gpr88flx/flx) compared to wild type animals (Gpr88+/+) (Figure 2A). First generation animals expressing the Cre under the control of A2aR promoter (Gpr88 A2A-Cre+/+) were...
crossed a second time to eliminate the wild-type Gpr88 gene (Gpr88\textsuperscript{A2A-Cre}) (background: 1.08% C57B1/6; 16.78% C57B1/6J; 0.01% FVB/N; 53.17% 129/SvPas; 0.01% SJL/J; 29.54% C57B1/6N). All mice were bred at Institut Clinique de la Souris-Institut de Génétique et Biologie Moléculaire et Cellulaire.

Mice were genotyped using PCR-based genotyping with the following primers:

- 5’GAAGAGTGAAACCACAGGTGTACAC 3’, 5’ GTT TGT TTC CTC ACT GGC TGA GAG TC 3’ for GPR88 +/+ and 5’ GTC CTA GGT GTG GAT ATG ACC TTA G 3’, 5’ GTT TGT TTC CTC ACT GGC TGA GAG TC 3’ for Gpr88 −/− and Gpr88\textsuperscript{A2A-Cre}.

To verify the presence of Cre and Myosine (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’ and 5’ TTA CGT CCA TCG TGG ACA GC 3’, 5’ TGG GCT GGG TGT TAG CCT TA 3’.

**Tissue preparation and fluorescent in situ hybridization.** Mice (n = 4 Gpr88\textsuperscript{flx/flx}; 4 Gpr88\textsuperscript{A2A-Cre}) were sacrificed by cervical dislocation and fresh brains were extracted and embedded in OCT (Optimal Cutting Temperature medium, Thermo scientific, Waltham, MA, USA) frozen and kept at -80°C. Frozen brains were coronally sliced into 20µm serial sections by using cryostat (CM3050 Leica, Wetzar, Germany) and placed in superfrost slides (Thermo scientific, Waltham, MA, USA). 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 Tritc and Cy5 respectively.
Relative expression of \textit{Gpr88} mRNA in \textit{Drd1} and \textit{Drd2} positive cells. Image acquisition was performed with the slide scanner NanoZoomer 2 HT and fluorescence module L11600-21 (Hamamatsu Photonics, Japan). To verify the specific excision of \textit{Gpr88} in D2R-MSNs neurons \textit{In situ} hybridization images were analyzed using NDP viewer software. For each brain, 4 slices were selected: two slices for the caudate-putamen (CPu) (Rostral: +0.98mm from Bregma; Caudal: -0.58mm from Bregma) one slice for the nucleus accumbens (Nac) (+1.34mm 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 1mm$^2$ for the Nac, 0.250 mm$^2$ for the CeA and two ROI of 0.5mm$^2$ for the each CPu slice (to include both dorsomedian and dorsolateral striatum, see Figure 1). Counting was balanced between right and left hemispheres. To evaluate expression of \textit{Gpr88} mRNA in D1R and D2R expressing cells, counting was performed manually using the NDP view counting add-up. First, cells expressing \textit{Drd1} mRNA but not \textit{Drd2} mRNA were marked and counted. For each \textit{Drd1} positive cell, co-expression of \textit{Gpr88} was verified and counted separately. This process was repeated for \textit{Drd2} mRNA positive cells. Relative \textit{Gpr88} expression is represented as a percentage of total \textit{Drd1} or \textit{Drd2} positive cells counted [(number \textit{Drd1} or \textit{Drd2} expressing cells co-expressing \textit{Gpr88} x 100)/ total number of \textit{Drd1} or \textit{Drd2} expressing cells]. Given the lack of difference in \textit{Gpr88} expression between lateral and medial CPu, relative percentage of each was pooled for graphical representation and statistical analysis.
[35S]-GTPγS binding assay: [35S]-GTPγS assays were performed on membrane preparations as described in our previous report (Pradhan et al., 2009).

To evaluate the activation of GPR88 in prefrontal cortex (PFC), caudate-putamen (CPu), nucleus accumbens (Nac), central nucleus of the amygdala (CeA) and hippocampus (HPC), structures were punched in 6 animals of each genotype (3 males 3 females) as previously described (Meirsman et al., 2015) and pooled for membrane preparation. To perform [35S]-GTPγS assays on whole striatum mice were sacrificed by cervical dislocation and both striatum were rapidly manually removed, frozen in dry ice and stored at -80°C until use. Three membrane preparations were used per genotype, gathering tissue from three animals each (males and females). Results are expressed by meaning measures from the three membrane preparation.

All assays were performed on membrane preparations. Membranes were prepared by homogenizing the tissue in 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 TrisHCl (pH 7.4), 3 mM MgCl2, 100 mM NaCl, 0.2 mM EGTA, following which they were centrifuged at 23 000 g for 30 min. The pellets were homogenized in 800μL ice-cold sucrose solution (0.32 M) and kept at -80°C. For each [35S]GTPγS binding assay 2μg of protein per well was used. Samples were incubated with and without ligands, for 1 hour at 25°C in assay buffer containing 30 mM GDP and 0.1 nM [35S]GTPγS. Bound radioactivity was quantified using a liquid scintillation counter. Bmax and Kd values were calculated. Non-specific 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., 2015) was kindly synthesized by Prestwick Chemicals (Illkirch, France) and dissolved in water.

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 minutes via an automated tracking system (videotrack; View Point, Lyon, France). Only movements which speed exceed 6 cm/s were taken into account for this measure.

Elevated plus-maze (EPM): Anxiety-like behavior was first evaluated using the ethological (also known as unconditioned tests) anxiety tests elevated plus maze (EPM). The EPM was a plus-shaped maze elevated 52 cm from base, with 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). 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 observed for 5 min. Anxiety-like behavior was assessed by measures of the time spent and 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 1/3 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 measures of locomotor activity.

**Light-Dark Test:** Anxiety-like behavior was next evaluated using the light/dark apparatus composed of two rectangular compartments (20 x 20 x 14 cm) separated by a tunnel (5 x 7 x 10 cm) (Imetronic, Pessac, France). One compartment is constituted of black floor and walls dimly lit (5 lux), whereas the other is made 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, 3 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 x 50 cm) filled with approximately 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 cut off time was defined as 15 min. After the test was over the animal was placed in his homecage 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 x 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 recorder. 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 exclude from analysis (One mouse was excluded).

**Marble burying:** Defensive burying was measured using the marble burying test carried out using 20 small glass marbles (15 mm) evenly spaced in a transparent single cage (21 X 11 X 17 cm) over 4cm 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 minutes and the number of marbles buried more than half in sawdust was counted.

**Novelty preference:** Novelty preference was assessed in unbiased computerized boxes previously described (Imetronic, Pessac, France (Le Merrer et al., 2012). Briefly, 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 (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 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 lighted operant chambers (28 x 21 x 22 cm, Coulbourn Instruments, Allentown, US), 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, used to assess animal motion, was placed on the ceiling of each chamber. The activity/inactivity
behavior was monitored continuously during 100 ms period. Data are expressed in duration of inactivity per sec and the total time of inactivity displayed by each subject during training and testing sessions was counted. The procedure was similar as previously described (Goeldner et al., 2009). Briefly, the first day animals underwent one conditioning session and, 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. The following day, mice were exposed again to the conditioning chamber and immobility was measured during 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 mean group value ± standard error of the mean (SEM) and 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 GraphPad Prism 6 (GraphPad Software, Inc, USA).

RESULTS

*Gpr88* A2A-Cre mice show decreased *Gpr88* mRNA levels in D2R neurons of caudate-putamen, nucleus accumbens and central amygdala.
To conditionally delete *Gpr88* exon 2 in D2R-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 D2R-MSNs-specific *Adora2a* gene promoter (Durieux et al., 2009; Meirsman et al., 2015). Then, we quantified *Gpr88* mRNA in Drd1-positive and Drd2-positive neurons in *Gpr88*^flx/flx^ (control) and *Gpr88*^A2A-Cre^ mice using triple *in situ* hybridization. Quantitative analysis was performed in four brain regions, including rostral Caudate-Putamen (CPu), caudal CPu, nucleus accumbens (Nac) and central nucleus of the amygdala (CeA) (Figure 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 co-expressing 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=0.56; p=0.59). Expression of the Cre in A2A expressing neurons had no effect on *Gpr88* mRNA in D1R 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=9.004; p<0.001) and caudal (40.24%±2.68; t=19.68; p<0.0001) CPu as previously demonstrated with this *Adora2a cre* mice (Durieux et al., 2009; Durieux et al., 2012; Ena et al., 2013). Also, mice expressing the Cre in A2A expressing neurons had significantly lower number of *Gpr88*-positive cells in D2R-neurons of the CeA (34.33%±8.2) compared to control animals (t=3.06; p=0.02). Together, the data indicate that conditional *Gpr88*^A2A-Cre^ mice show a selective decrease of *Gpr88* transcript levels in D2R-neurons of striatum and CeA.
Gpr88 A2A-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 [S35]-GTPγS binding assays in Gpr88A2A-Cre mice and their controls, as well as in total Gpr88−/− (negative control) (Figure 2B, C, D, E, F). Structures were chosen based on A2AR expression (Schiffmann et al., 1991; Schiffmann and Vanderhaeghen, 1993). We found a significant genotype effect in the CPu (Gpr88A2A-Cre: 282.2%±15.46; Gpr88flx/flx: 427%±22.61; genotype effect $F_{(2,30)}=61.56; p<0.0001$), Nac (Gpr88A2A-Cre: 205.1%±5.54; Gpr88flx/flx: 342.4%±9.13; $F_{(2,30)}=152.9; p<0.0001$) and CeA (Gpr88A2A-Cre: 141.2%±6.18; Gpr88flx/flx: 170.3%±5.90; $F_{(2,30)}=37.48; p<0.0001$). Post hoc analysis (Tukey multiple comparisons) revealed significant differences between Gpr88A2A-Cre and Gpr88flx/flx for the two highest agonist concentrations ($10^{-5}$ M and $10^{-6}$M) in the CPu, Nac and CeA. This result demonstrates that the selective Gpr88 gene KO in D2R-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 D1R and D2R-expressing cells, the approximately 40% reduction in CPu membranes reflects the specific Gpr88 gene KO in D2R cells, representing approximately 40% of the dorsal striatum population of dopamine receptor-expressing neurons (Valjent et al., 2009).

Both total and A2AR-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., 2015), as was also observed in mice with disrupted D2R-
MSN activities (Durieux et al., 2009; Bateup et al., 2010; Durieux et al., 2012). We compared general locomotor activity in total and A2A-R-\textit{Gpr88} KO mice. Animals were individually placed in a dimly lit open field and analysis of forward locomotion revealed a significant increased activity for \textit{Gpr88} \textit{A2A-Cre} (n=10 \textit{Gpr88}^{A2A-Cre}, n=10 \textit{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 \textit{Gpr88} \textit{A2A-Cre} (n=10 \textit{Gpr88}^{A2A-Cre}, n=10 \textit{Gpr88}^{Flx/Flx}; genotype effect: F\(_{(1, 132)}\) = 52.72, \(p<0.0001\); mean locomotion: t\(_{(10)}\) = 6.65, \(p<0.0001\)) (Figure 3). Deletion of \textit{Gpr88} in A2AR-neurons is therefore sufficient to recapitulate the hyperlocomotion phenotype of \textit{Gpr88} KO mice, suggesting that the locomotor effect of GPR88 is mediated through receptors expressed in D2R-MSNs.

Both total and A2A-R-\textit{Gpr88} deletion decrease anxiety-like behavior.

Complete deletion of \textit{Gpr88} in mice decreased anxiety levels in several models of anxiety-like behavior (Meirsman et al., 2015). To examine whether this behavior is dependent on GPR88 in A2AR-neurons we evaluated anxiety-like behaviors of \textit{Gpr88}^{A2A-Cre} and \textit{Gpr88}^{-/-} mice in the standard light-dark and elevated plus maze test. Results indicate that both A2A-R-\textit{Gpr88} (n=11 \textit{Gpr88}^{A2A-Cre}, n=9 \textit{Gpr88}^{Flx/Flx}) and total KO mice (n=11 \textit{Gpr88}^{A2A-Cre}, n=9 \textit{Gpr88}^{Flx/Flx}) entered more frequently (\textit{Gpr88}^{A2A-Cre}: t\(_{(18)}\) = 3.01, \(p=0.008\); \textit{Gpr88}^{-/-}: t\(_{(19)}\) = 2.46, \(p=0.023\)) and spent more time (t\(_{(18)}\) = 3.11, \(p=0.006\) and t\(_{(19)}\) = 2.84, \(p=0.01\) for \textit{Gpr88}^{A2A-Cre} and \textit{Gpr88}^{-/-} respectively) exploring the aversive illuminated compartment of the light-dark apparatus (Figure 4A-D). In the elevated plus maze, A2A-R-\textit{Gpr88} (n=12 \textit{Gpr88}^{A2A-Cre}, n=13 \textit{Gpr88}^{Flx/Flx}) and total \textit{Gpr88} deletion (n=11 \textit{Gpr88}^{-/-}, n=9 \textit{Gpr88}^{Flx/Flx}) increased the open arms exploration (% time in the OA: t\(_{(23)}\) = 4.13, \(p=0.0004\) and t\(_{(18)}\) = 2.71, \(p=0.015\); % of distance traveled in the OA: t\(_{(23)}\) = 3.43, \(p=0.0023\); t\(_{(18)}\) =
3.05, $p=0.007$ for $Gpr88^{A2A-Cre}$ and $Gpr88^{-/-}$, respectively) (Figure 4E and H). Also, both mutants spent more time in the distal part of the open arm ($t_{(23)} = 2.76$, $p=0.011$ and $t_{(18)} = 2.17$, $p=0.043$ for $Gpr88^{A2A-Cre}$ and $Gpr88^{-/-}$, respectively) (Figure 4G and J) and displayed higher number of total and distal head dips (total: $t_{(23)} = 3.54$, $p=0.0017$ and $t_{(18)} = 3.05$, $p=0.007$. Distal: $t_{(23)} =$ 2.95, $p=0.0072$ and $t_{(18)} = 2.81$, $p=0.012$ for $Gpr88^{A2A-Cre}$ and $Gpr88^{-/-}$, respectively) (Figure 4F and I). There was no difference in total distance traveled ($t_{(23)} = 0.14$, $p=0.886$ and $t_{(18)} = 0.43$, $p=0.675$) or total number of arms entries ($t_{(23)} = 0.09$, $p=0.925$ and $t_{(18)} = 0.65$, $p=0.52$) between mutants and control mice (data not shown). Together, 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 and our previous (Meirsman) study.

As anxiety can affect social behavior, we scored for social interaction. Both mutant lines show increased nose ($n=10$ $Gpr88^{A2A-Cre}$, $n=9$ $Gpr88^{Flx/Flx}$, $t_{(17)} = 3.39$, $p=0.004$; $n=6$ $Gpr88^{-/-}$, $n=6$ $Gpr88^{+/+}$: $t_{(10)} = 10.12$, $p<0.0001$) 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 control littermates ($t_{(17)} = 2.72$, $p=0.014$ and $t_{(10)} = 12.03$, $p<0.0001$) (Figure 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 mice lines. Marble burying constitutes an unconditioned defensive
behavior aiming to avoid threats, reversed by anxiolytic drugs (De Boer and Koolhaas, 2003). In presence of 20 marbles A2A-R-Gpr88 (n=7 Gpr88 A2A-Cre, n=9 Gpr88 Fk8/Fk8: 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 less marbles than their control littermates (Figure 5A and B). In this test, therefore, conditional D2R-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 (Figure 5C and 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 Gpr88 A2A-Cre mice (n=7 Gpr88 A2A-Cre, n=9 Gpr88 Fk8/Fk8: t (12) =1.43, p=0.17). Moreover, in the novelty suppressed feeding test (Figure 5D and G) Gpr88−/− but not Gpr88 A2A-Cre show decreased latency to feed. Thus, approach behaviors in both novelty preference and novelty suppressed feeding tests are enhanced in total but not A2A-R GPR88 KO mice. These data together suggest that GPR88 in A2A-R neurons regulate threat avoidance but not novelty approach behaviors.

Total but not A2A-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 (Figure 6). During conditioning, post-shock immobility increased regardless of the genotype suggestive of successful US-CS conditioning (n=11 Gpr88 A2A-Cre, n=11 Gpr88 Fk8/Fk8: F (1, 20) =23.25, p=0.0001 and n=10 Gpr88−/−, n=10 Gpr88−/−: F (1, 18) =17.05, p=0.0006; time effect RM ANOVA). When tested for contextual fear, Gpr88−/− displayed significantly less immobility than control animals (t (18) =2.36, p=0.029)
in agreement with altered hippocampal functioning previously reported in Gpr88 KO mice (Meirsman et al., 2015). 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$^{A2A-Cre}$ mice context-related immobility scores were similar to Gpr88$^{fx/fx}$ control mice ($t_{(20)} = 1.14, p = 0.27$). Like context fear measure, cue presentation led to similar immobility scores between Gpr88$^{A2A-Cre}$ 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 A2AR neurons does not affect fear expression.

**DISCUSSION**

In the present study we show that GPR88 in A2A-R-expressing neurons regulates anxiety-like behaviors without affecting fear conditioning and drive toward novelty or food. In situ hybridization and GTP$\gamma$S binding data indicate that Gpr88 was efficiently deleted in neurons expressing the D2R 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 D1R-MSNs was intact in Gpr88$^{A2A-Cre}$ mice when compared to control mice not expressing the Cre recombinase. The selective deletion of Gpr88 in D2R-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; Durieux et al., 2012; Ena et al., 2013).
Furthermore we confirm previous data indicating that GPR88 is present in the vast majority of D1R and D2R expressing MSNs (Quintana et al., 2012).

Converging evidence support the inhibitory function of striatal D2R-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; Bateup et al., 2010; Durieux et al., 2012).

Several reports (Logue et al., 2009; Quintana et al., 2012) including our own work (Meirsman et al., 2015) indicate that Gpr88 gene deletion leads to general hyperactive behavior. In the present report we show that deletion of A2AR-neurons Gpr88 is sufficient to increase locomotor activity. Considering the predominant expression of A2AR in D2R-MSNs, this observation suggests that GPR88 normally promotes the demonstrated inhibitory function of D2R-MSNs on locomotor activity. Mechanisms underlying the positive modulatory role of GPR88 on D2R-MSNs remain to be clarified. Importantly, both total and A2AR-Gpr88 KO mice show a similar hyperactivity when tested under the same experimental conditions, strongly suggesting that GPR88 in D2R-MSN fully accounts for this phenotype.

In a previous report we showed that Gpr88\(^{-/-}\) mice present decreased anxiety-like behaviors (Meirsman et al., 2015). Also, recent reports suggest that D2Rs and D2R-MSNs regulate emotional processing and goal directed behavior (Hranilovic et al., 2008; Kravitz et al., 2012; Pecina et al., 2013; Brandao et al., 2015; Francis et al., 2015). In the light-dark and elevated plus maze 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 D2R- but not D1R-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 number of entries did not differ from 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^{-/-} as well as Gpr88^{A2A-Cre} mice buried less marbles than control littermates, showing decreased defensive burying consistent with reduced threat avoidance in these mice.

To tackle approach behavior, we assessed novelty preference in both KO lines. Total but not A2AR-Gpr88 KO mice showed enhanced preference for the novel compartment, when presented a choice for novel or familiar environment. Similarly, in the novelty suppressed feeding test, total but not A2AR-Gpr88 deletion decreased the latency to start eating. In this conflict test, both approach and avoidance component 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 towards 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 (Hranilovic et al., 2008;
Durieux et al., 2009; Hikida et al., 2010; Kravitz and Kreitzer, 2012; Kravitz et al., 2012; Hikida et al., 2013; Kravitz and Bonci, 2013; Kravitz et al., 2013; Calabresi et al., 2014). For instance, D2R-MSNs 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 lack of GPR88 in D2R-MSNs disrupted this pathway’s activity. Further studies using electrophysiological approaches would help confirm this hypothesis. Also, results from Gpr88−/− mice showing both decreased avoidance behavior and increased novelty and food approach suggest that GPR88 in D1R-MSNs neurons normally regulates approach behavior.

Finally, we tested fear responses of total and A2AR-Gpr88 KO mice for the first time. Total deletion of Gpr88 impaired both context and cue 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 Gpr88A2AR-Cre mice may be due to D1R neurons-mediated mechanisms contributing to these behaviors. Alternatively, and because central amygdala functioning is essential in 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 Gpr88A2AR-Cre mice shows that GPR88 in A2AR-MSNs regulates locomotor and anxiety behaviors. These results represent a first step towards understanding
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-MSNs balance. Finally, further demonstration of GPR88 implication in anxiety-related behaviors and threat evaluation definitely posit GPR88 blockade as a new target for treatment of anxiety-related disorders (Aupperle and Paulus, 2010).

**AUTHOR CONTRIBUTION**

A.C.M. and B.L.K. designed the experiments. A.C.M. performed and analyzed behavioral experiments. A.C.M. and A.R performed and analyzed *in situ* hybridization and \[^{35}\text{S}]-\text{GTP}\gamma\text{S} binding experiments. A. K. E. provided the *Adora2a Cre* mice. A.C.M. and B.L.K. interpreted the results and wrote the article. All authors discussed the results, commented and edited on the manuscript.

**REFERENCES**


Del Zompo M et al. (2014) Association study in three different populations between the GPR88 gene and major psychoses. Molecular genetics & genomic medicine 2:152-159.


FIGURE LEGENDS

Figure 1. Molecular characterization of conditional A2A-R-Gpr88 KO mice. A, Triple fluorescent in situ hybridization probing Gpr88 (Aa and Ab, Ae and Af, Ai and Aj; probe labeled in green), Drd2 (Ac and Ad, Ae and Af; probe labeled in orange) and Drd1 (Ag and Ah, Ai and Aj; probe labeled in red). Representative images are shown. In Gpr88 Flx/Flx control animals Gpr88 mRNA co-localizes 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 co-localization with Drd2 (Af: merge GPR88/D2R) while co-localization with Drd1 remains (Aj: merge GPR88/Drd1, yellow arrows). Dapi staining (blue) was used to label all cells nuclei. (Scale bar: 25µm). 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 co-expressing Gpr88 x 100)/ total number of Drd1 or Drd2 expressing cells]. Data are presented as mean ± SEM. n=4 Gpr88A2A-Cre; n=4 Gpr88flx/flx; solid stars: one star p < 0.05, two stars p < 0.01, three stars p < 0.001 (Student t test).
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]-GTPγS was totally and partially abolished in the striatum of \textit{Gpr88}/\textit{-} and \textit{Gpr88} \textit{A2A-Cre} mice respectively. \textit{Gpr88} \textit{+/+} and \textit{Gpr88} \textit{flx/flx} mice present similar GPR88 agonist-induced receptor activation. Two membrane preparations were used per genotype, each membrane preparation gathering tissue from three animals (1 male/2 females or 2 males/1 female). (B, C, D, E, F) Decreased activation of GPR88 in several brain regions of \textit{Gpr88} \textit{A2A-Cre} mice: GPR88-mediated [35S]-GTPγS binding is decreased in the CPu, Nac and central amygdala of mutant animals. Data are represented as mean ± SEM. (A) Text stars (*): three stars $p < 0.001$ (post-hoc: Tukey’s multiple comparisons test of \textit{Gpr88} \textit{Flx/Flx} or \textit{Gpr88} \textit{+/+} versus \textit{Gpr88} \textit{A2A-Cre} and \textit{Gpr88} \textit{-/-}) (B, C, D, E, F) $n=6$ \textit{Gpr88} \textit{A2A-Cre}; $n=6$ \textit{Gpr88} \textit{-/-}; $n=6$ \textit{Gpr88} \textit{flx/flx}; Text stars: three stars $p < 0.001$ (post-hoc: Tukey’s multiple comparisons test).

Figure 3. Locomotor activity is similarly increased in A2AR-Gpr88 KO and total KO mice. When placed individually in a dimly lit open field for 30 min, both A2AR-Gpr88 KO (A) and total (B) traveled a longer distance then their control littermates. Line graphs show the distance traveled (cm) in 5 min bins over a 30 min session. Bar graphs show the average total distance traveled (cm) over the 30 min sessions period. Data are represented as mean ± SEM. (A) $n=10$ \textit{Gpr88} \textit{A2A-Cre}, $n=10$ \textit{Gpr88} \textit{flx/flx} (B) $n=12$ \textit{Gpr88} \textit{-/-}, $n=12$ \textit{Gpr88} \textit{+/+} Open stars: three stars $p <0.001$ (RM ANOVA). Solid stars: three stars $p < 0.001$ (Student t test).

Figure 4. Anxiety-related responses are similarly increased in A2AR-Gpr88 KO and total KO mice. A2AR-Gpr88 (A, B) and total KO mice (C, D) enter more frequently and spent more time in
In the elevated plus-maze, Gpr88\textsuperscript{A2A-Cre} and Gpr88\textsuperscript{-/-} mice present higher open arms exploration ratios (E and 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 to their control littermates (G, J). Social interactions were evaluated in a dimly lighted open field with wild type naïve mice of the same age and gender (K-R). Both mutant animals display increased number of nose, and paw contacts, as well as increased following behaviors. Gpr88\textsuperscript{-/-} but not Gpr88\textsuperscript{A2A-Cre} engaged less frequently in grooming episodes than control animals.

Data are presented as mean ± SEM. (A and B) n=11 Gpr88\textsuperscript{A2A-Cre}, n=9 Gpr88\textsuperscript{flx/flx}; (C and D) n=10 Gpr88\textsuperscript{-/-}, n=11 Gpr88\textsuperscript{+/+}; (E-G) n=12 Gpr88\textsuperscript{A2A-Cre}, n=13 Gpr88\textsuperscript{flx/flx} (H-J) n=11 Gpr88\textsuperscript{-/-}; n=9 Gpr88\textsuperscript{+/+}; (K-N) n=10 Gpr88\textsuperscript{A2A-Cre}, n=9 Gpr88\textsuperscript{flx/flx} (O-R) n=6 Gpr88\textsuperscript{-/-}; n=6 Gpr88\textsuperscript{+/+} Solid stars: one star p < 0.05, two stars p < 0.01, three stars p < 0.001 (Student t test).

**Figure 5. A2AR-Gpr88 gene deletion increases avoidance but does not regulate approach behaviors.** In the marble burying test (A for Gpr88\textsuperscript{A2A-Cre} and B for Gpr88\textsuperscript{-/-}) both KO mice buried less marbles than controls. When assessing novelty preference total (F) but not A2AR-Gpr88 KO mice (C) spent more time in the novel compartment when compared to their littermates. In the novelty-suppressed feeding test, Gpr88\textsuperscript{A2A-Cre} exhibit similar latencies to start eating and home cage food intake (D) than Gpr88\textsuperscript{flx/flx} mice. Gpr88\textsuperscript{-/-} mice display shorter latencies to start eating in the center of the arena compared to Gpr88+/+ animals (G), eating normally when placed back in their home cage (E). Data are presented as mean ± SEM. (A) n=11 Gpr88\textsuperscript{A2A-Cre}, n=9 Gpr88\textsuperscript{flx/flx}; (B) n=7 Gpr88\textsuperscript{-/-}; n=7 Gpr88\textsuperscript{+/+}; (C) n=8 Gpr88\textsuperscript{A2A-Cre}, n=6 Gpr88\textsuperscript{flx/flx}.
(D, E) n=11 Gpr88<sup>A2A-Cre</sup>; n=11 Gpr88<sup>flx/flx</sup>; (F) n=7 Gpr88<sup>−/−</sup>; n=7 Gpr88<sup>+/+</sup>; (G, H) n=7 Gpr88<sup>−/−</sup>; Solid stars: one star \( p < 0.05 \), two stars \( p < 0.01 \) (Student t test).

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. During the conditioning session, mutant and control animals displayed similar levels of immobility before and after tone-shock pairing when compared to control mice (A and D). 24h later, Gpr88<sup>−/−</sup> mice displayed significantly lower context fear than Gpr88<sup>+/+</sup> mice (E). The percentage of immobility of Gpr88<sup>−/−</sup> was also decreased when tested for cue fear memory (F). Deletion of Gpr88 in A2AR-expressing neurons didn’t affect context (B) or cue (C) fear memories; (A-C) n=11 Gpr88<sup>A2A-Cre</sup>; n=11 Gpr88<sup>flx/flx</sup>; (D-F) n=10 Gpr88<sup>−/−</sup>; n=10 Gpr88<sup>+/+</sup>; Solid stars: one star \( p < 0.05 \) (Student t test). Text stars (*): one star \( p < 0.05 \), two stars \( p < 0.01 \) (post-hoc: Sidak’s multiple comparisons test).
A

Gpr88<sup>Flox/Flox</sup> vs Gpr88<sup>A2A-Cre</sup>

Gpr88 Positive cells

Drd2 Positive cells

Merge Gpr88/Drd2

Drd1 Positive cells

Merge Gpr88/Drd1

B

Meirsman et al Figure 1
Meirisman et al Figure 2
Meirsman et al Figure 3
Meirisman et al Figure 4
Meisrman et al Figure 5
Meirson et al Figure 6