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# Mice lacking GPR88 show motor deficit, improved spatial learning and low anxiety reversed by delta opioid antagonist

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## Abstract

**Background—**GPR88 is an orphan G protein coupled receptor (GPCR) highly enriched in the striatum, and previous studies have focused on GPR88 function in striatal physiology. The receptor is also expressed in other brain areas and here we examined whether GPR88 function extends beyond striatal-mediated responses.

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A.C.M., J.L.M., B.L.K. and J.A.J.B. designed the experiments. A.C.M. and J.A.J.B. performed and analyzed behavioral and pharmacological experiments. A.C.M. performed and analyzed [$^{35}$S]-GTPγS binding experiments. L.P. Pellissier performed in vitro pharmacological experiments. J.A.J.B. performed and analyzed qRT-PCR. D.C. performed and analyzed HPLC dosage of monoamine levels in brain tissue. J.D. performed and analyzed DiI staining and quantification of dendritic spines. A.C.M., J.L.M., B.L.K. and J.A.J.B. interpreted the results. A.C.M., J.L.M., B.L.K. and J.A.J.B. wrote the article. B.L.K. and J.A.J.B. contributed equally to this work. All authors discussed the results and commented on the manuscript.

**Disclosure/conflict of interest**

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

**Methods—**We created Gpr88 knockout mice and examined both striatal and extra-striatal regions at molecular and cellular levels. We also tested striatum, hippocampus- and amygdala-dependent behaviors in Gpr88$^{-/-}$ mice using extensive behavioral testing.

**Results—**We found increased G protein coupling for delta (DOR) and mu opioid, but not other Gi/o coupled receptors, in the striatum of Gpr88 knockout mice. We also found modifications in gene transcription, dopamine and serotonin contents, and dendritic morphology inside and outside the striatum. Behavioral testing confirmed striatal deficits (hyperactivity, stereotypies, motor impairment in rotarod). In addition, mutant mice performed better in spatial tasks dependent on hippocampus (Y-maze, novel object recognition, dual solution cross-maze) and also showed markedly reduced levels of anxiety (elevated plus maze, marble burying, novelty suppressed feeding). Strikingly, chronic blockade of DOR using naltrindole partially improved motor coordination, and normalized spatial navigation and anxiety of Gpr88$^{-/-}$ mice.

**Conclusion—**We demonstrate that GPR88 is implicated in a large repertoire of behavioral responses that engage motor activity, spatial learning and emotional processing. Our data also reveal functional antagonism between GPR88 and DOR activities in vivo. The therapeutic potential of GPR88 therefore extends to cognitive and anxiety disorders, possibly in interaction with other receptor systems.

### Keywords

GPR88 agonist-induced GTPγS binding; medium spiny neurons; ethological scoring of anxiety; gene clustering; spatial learning; psychiatric disorders

## INTRODUCTION

The orphan G protein coupled receptor (GPCR) GPR88 is a striatal-enriched gene (1-5) whose expression is modified by neuropharmacological interventions (6-9). Within the striatum, GPR88 is homogenously distributed throughout dorsal (caudate putamen or CPu) and ventral (nucleus accumbens or NAc) areas. Gpr88 gene is detected in projection medium spiny neurons (MSNs) of both striatonigral and striatopallidal pathways, under the control of corticostriatal inputs (3). At present, only one synthetic agonist has been reported (10, 11) and functional studies of GPR88 have used genetic approaches (2, 12, 13). The analysis of mice lacking the Gpr88 gene demonstrates an essential role for GPR88 receptors in dopamine neurotransmission and striatal physiology. Mutant mice show altered basal dopamine and higher phosphoDARPP-32 levels in the striatum (2), as well as increased MSN excitability and firing rates (13). In addition, behavioral deficits evocative of striatal dysfunction were reported, including increased apomorphine and amphetamine effects on locomotor activity (2), or reduced motor coordination and altered cue-based learning (13). Finally, amphetamine locomotor effects were inhibited by local silencing of Gpr88 in the NAc (12).

Previous studies have focused on GPR88 function in the striatum, however Gpr88 expression is not confined to this brain structure. Extrastriatal Gpr88-expressing brain regions are discrete but widely distributed from cortical areas (layer IV) to inferior olive (Allen Brain Atlas; (2, 14)). GPR88 mRNA is absent in hippocampus but present in

prefrontal cortex (PFC), septum and parasubiculum, which receive hippocampal inputs (15). Finally, GPR88 is abundant in the amygdala, prominently in the central nucleus (CeA) (14) and to a lesser extent in lateral, cortical and intercalated nuclei, as well as in the anterior part of the bed nucleus of the stria terminalis (BNST) (14). We therefore hypothesized that, beyond striatal-related responses, GPR88 may modulate a wide variety of behaviors, notably hippocampus- and amygdala-dependent behaviors.

We created a Gpr88 knockout mouse line and investigated the impact of Gpr88 gene deletion on several molecular and cellular endpoints in both striatal and extra-striatal regions. We also examined striatum, hippocampus- and amygdala-dependent behaviors in Gpr88$^{-/-}$ mice using an extensive set of behavioral tasks. Our data demonstrate that GPR88 activity regulates monoamine neurotransmission, influences neural connectivity inside and outside the striatum including hippocampus and amygdala, and is implicated in a vast repertoire of behavioral responses that engage cognitive and emotional processing. Intriguingly, most behavioral deficits in Gpr88 mutant mice were reversed by pharmacological blockade of delta opioid receptors (DOR), whose activity seems to oppose GPR88 function.

## METHODS AND MATERIALS

### Subjects

Male and female Gpr88$^{+/+}$ and Gpr88$^{-/-}$ mice aged 8-10 weeks were bred in-house. Animals were group-housed (except during nest building test) and maintained on a 12hr light/dark cycle (lights on at 7:00 AM) at controlled temperature (22±1°C). Food and water were available ad libitum throughout all experiments, unless otherwise stated. All experiments were analyzed blind to genotypes. All experimental procedures were reviewed and approved by the local ethic comity (CREMEAS, 2003-10-08-[1]-58).

### Generation of Gpr88$^{-/-}$ mice

Gpr88 floxed mice (Gpr88$^{fl/fl}$) were generated at the Institut Clinique de la Souris using Cre-LoxP technology. Briefly, exon 2 was flanked by loxP sites and a Lox-FRT neomycin-resistance cassette was inserted downstream exon 2 using homologous recombination (Figure 1A and Supplement 1). F1 heterozygous Gpr88$^{fl/+}$ mice were bred with CMV-Flip mice in order to remove the neomycin cassette, and produce a conditional Gpr88 floxed line. For this study, we further created constitutive knockout animals by breeding conditional animals with a general CMV-Cre driver line (16, 17). This led to germ-line deletion of Gpr88 exon 2 on a hybrid 50% C57BL/6J–50% 129Sv genetic background. Gpr88$^{fl/fl}$ × CMV-Cre$^{Tg/+}$ and Gpr88$^{+/+}$ × CMV-Cre$^{0/+}$ were used as experimental (Gpr88$^{-/-}$ mice) and control (Gpr88$^{+/+}$) animals, respectively.

### Drugs

Chemical compounds used for [$^{35}$S]-GTPγS binding and in vivo pharmacology experiments, their doses and route of administration are described in Supplement 1.

### [$^{35}$S]-GTPγS binding

The assay was performed on membrane preparations from striatum and other brain regions and performed as described previously (21) (see dissection in Figure S1 and Methods in Supplement 1).

### Real-time quantitative PCR analysis

Real-time quantitative PCR analysis was performed on brain samples as described previously (9, 19, 22) (see dissection in Figure S1 and Methods in Supplement 1).

### Neurochemical assay for biogenic amine dosage

Monoamine levels across brain regions were measured by HPLC as described previously (22, 23) (see dissection in Figure S1 and Methods in Supplement 1).

### DiI staining and quantification of dendritic spines

DiI labelling was performed on fixed slides, dendrites were then imaged using a Leica SP5 confocal laser scanning microscope and dendritic spine density was evaluated on 3D stacks using the plug-in AutoSpine of Neurolucida software package (Version 7.51) (full Methods in Supplement 1). Typical DiI fluorescent striatal MSN is displayed in Figure S2C (Supplement 1).

### Behavioral experiments

Striatal-dependent behavioral responses were evaluated by evaluating locomotor activity in a novel environment, ability to build a nest, motor stereotypies, time to grasp a string and skill motor learning on the accelerating rotarod. Hippocampus-dependent behaviors were assessed using a continuous alternation paradigm in the Y-maze, a novel object recognition task and a dual solution cross-maze task followed by reversal. Anxiety-like behavior was evaluated by ethological scoring in the elevated plus-maze (EPM) and assessed in the marble burying and novelty-suppressed feeding (NSF) tests.

To assess the effects of chronic naltrindole administration, a first cohort of animals underwent successively EPM (day 8) and NSF (day 16) tests. A second cohort was trained on the accelerating rotarod (starting day 8), and then tested for Y-maze exploration (day 24) (detailed protocols in Supplement 1).

### Statistical analyses

Statistical analyses were performed using Statistica 9.0 software (StatSoft, Maisons-Alfort, France). For all comparisons, values of $p<0.05$ were considered as significant. Statistical significance in behavioral experiments and monoamine dosage was assessed using two to five-way analysis of variance (genotype, gender, treatment, trial and session effects) followed by Newman-Keuls post-hoc test, except for nesting score, which significance was tested using the non-parametric Kruskal-Wallis' analysis of variance. For [$^{35}$S]-GTPγS binding, statistical significance was calculated using two-way analysis of variance (genotype and dose effects). Statistical and unsupervised clustering analysis of qPCR data were performed as previously described (9).

# RESULTS

## Molecular and cellular alterations are detected throughout Gpr88$^{-/-}$ mouse brains

We first verified the absence of receptor expression in Gpr88$^{-/-}$ mice. The Gpr88 transcript was undetectable in mutant brains by in situ hybridization (not shown). Further, the GPR88 agonist Compound 19 (24) elicited strong [$^{35}$S]-GTPγS binding in striatal membranes from control mice ($EC_{50}$=940.6±7.12 nM and $B_{max}$=361.3±18.0) but was ineffective in Gpr88$^{-/-}$ samples, demonstrating lack of GPR88 receptor signaling in mutant animals (Figure 1B).

Using the [$^{35}$S]-GTPγS assay, we also examined whether the absence of GPR88 in mutant mice modifies signaling efficacy of other Go/Gi coupled receptors expressed in the striatum. We selected best-studied GPCRs, including dopamine D2, glutamate metabotropic, muscarinic acetylcholine (m2/m4), mu opioid (MOR) and delta opioid (DOR) receptors (Figure 1B). Quinpirole, glutamate and carbachol dose-dependently increased [$^{35}$S]-GTPγS binding in striatal membrane preparations, with efficacies and potencies similar in GPR88 mutants and controls. Intriguingly, carbachol and the two opioid agonists DAMGO (MOR agonist) and SNC-80 (DOR agonist) induced a slightly but significantly stronger [$^{35}$S]-GTPγS response in mutant samples (statistics in Table S2 in Supplement 2). Gpr88 ablation therefore leads to enhanced signaling for muscarinic and opioid receptors.

We then quantified the expression of 89 genes in PFC, CPu, NAc, CeA and CA1 using qRT-PCR (Table S3 in Supplement 2). Genes were selected as key actors of GABA, glutamate and monoamine signaling pathways or were striatal gene markers. Cluster analysis of qRT-PCR data identified groups of genes sharing similar expression profiles (Figures 1C and S2A in Supplement 1), organized qRT-PCR data in five main clusters. Genes with increased expression gathered in clusters (a) and (d); most genes with down-regulated expression in PFC clustered in (b), whereas cluster (c) grouped genes with decreased expression in CPu and NAc. Limited regulation of gene expression was observed in cluster (e). This analysis reveals that Gpr88 deletion modifies gene transcription in regions where the receptor is abundant (CPu, NAc, CPF and CeA), and also in areas with no detectable expression (CA1). Gene regulation appeared region-specific, with most down-regulated expression observed in striatal regions and up-regulation detected in PFC and CeA (see examples of individual gene expression profiles in Figure S2B in Supplement 1).

We examined the impact of Gpr88 deletion on monoamines in areas of high receptor density (CPu, NAc and CeA), as well as in other brain regions where the receptor is weakly expressed (PFC) or undetectable (dorsal and ventral hippocampus or HPCd and HPCv, dorsal raphe or DRN, ventral tegmental area/substantia nigra pars compacta or VTA/SNc). Levels of DA and 5HT, and their metabolites are shown in Figure 1D and Table S4 in Supplement 2. In agreement with previous findings (2) (but see (13)), we found decreased DA levels in CPu, but not NAc of mutant mice. DA content was also lower in CeA, and higher in SNc/VTA. DA was otherwise unchanged in other brain regions (PFC, HPCd and HPCv), and the DA metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) was not modified in any sample (Table S4 in Supplement 2). 5HT and its metabolite 5-hydroxyindoleacetic acid (5HIAA) were higher in the DRN of Gpr88$^{-/-}$ mice. No significant modification was

detected in other brain structures. Monoamine levels are increased at their site of synthesis (DA in SNc/VTA and 5HT in DRN).

We compared dendritic spine morphology in the CPu, NAc, CeA and CA1 of Gpr88$^{-/-}$ mice (Figure 1E, F). Spine density was significantly reduced in CPu, NAc and CeA, as well as in hippocampus CA1.

## Striatal-dependent behaviors are impaired in Gpr88$^{-/-}$ mice

Similar to previous investigators (3, 5), we tested behaviors involving striatal circuits. We first assessed locomotor activity in a novel environment (Figure 2A; Table S5 in Supplement 2). Gpr88$^{-/-}$ animals and controls were exposed to an open-field for 5 daily consecutive 30-min sessions. As observed earlier (5), mutant animals displayed increased forward locomotion and this hyperactivity failed to habituate over days of testing.

Altered striatal function often results in the development of spontaneous motor stereotypies. Gpr88$^{-/-}$ mice displayed decreased number of rearing, grooming, and burying episodes (Figure 2B; Table S5 in Supplement 2) and also spent more time burying due to markedly longer episodes than controls, as well as increased frequency of circling, indicating altogether that motor stereotypies develop as a consequence of Gpr88 deletion. We also used the nest-building test to further evaluate striatal function (25). Male, but not female, Gpr88$^{-/-}$ mice showed impaired ability to build a nest (Figure 2C; Table S5 in Supplement 2).

We evaluated procedural abilities of mutant using a motor skill-learning task on the accelerating rotarod (26, 27). Mice were trained to run for 3 trials a day during 4 consecutive daily sessions. In both mutant and control groups, females performed better than males. Consistent with previous report (13), Gpr88$^{-/-}$ mice performed poorly in this test. Moreover, mutants strikingly failed to ameliorate over trials and sessions, suggesting a marked impairment of motor skill learning (Figure 2D; Table S5 in Supplement 2). Mutant mice achieved similar performance as controls in the string task, suggesting that motor coordination was not affected in this test (Figure 2E). Muscular strength was not altered in Gpr88$^{-/-}$ animals, as shown in the grip test (Figure 2F).

Together, these data indicate that striatal-dependent behaviors are impaired in Gpr88$^{-/-}$ mice.

## Hippocampus-dependent behaviors are facilitated in Gpr88$^{-/-}$ mice

Hippocampal and striatal activities compete to drive behavior during learning and memory tasks (28). Altered striatal function in Gpr88$^{-/-}$ mice may thus influence hippocampal-dependent behaviors. We performed three behavioral tests to tackle hippocampal function. First, hippocampal-dependent navigation was evaluated by scoring spontaneous alternation in a Y-maze (Figure 3A; Table S5 in Supplement 2). Consistent with locomotor hyperactivity, Gpr88$^{-/-}$ mice entered more often into arms of the maze during a 5-min exploration session, males being more active. Mutant mice showed a trend towards higher spontaneous alternation and returned significantly less into the same arm, indicative of less perseverative errors, while alternate arms returns were unchanged.

Second, we used a novel object recognition task to assess the ability to discriminate either novel objects or their spatial location (19). In trial 1, mice were presented two copies of an unfamiliar object in an open field (familiarization phase); in trial 2, one of the two objects was displaced to a novel location (place phase); in trial 3, the unmoved object was replaced by a novel object (object phase). Mutant animals explored the objects more often, but not longer, than controls across phases, further demonstrating hyperactivity (Figure 3B; Table S5 in Supplement 2). During place phase (Trial 2), knockout animals spent more time exploring the displaced object compared to controls, suggesting facilitated hippocampal-mediated recognition of object location (Figures 3B and S3 in Supplement 1).

Lastly, we tested mutants in a dual-solution cross-maze task, which specifically addresses hippocampal/striatal balance in learning. In this task, performance at early stages of acquisition and reversal rely preferentially on hippocampus-mediated allocentric strategy (place), and then switches to striatal-dependent egocentric strategy (response) at later stages as habitual behavior develops (see Supplement 1). Mutant animals acquired the task more rapidly and reached higher levels of performance than controls (Figure 3C; Figure S3 in Supplement 1; Table S5 in Supplement 2), although they initially displayed longer choice latencies. A probe trial performed after 8 training sessions revealed that $Gpr88^{-/-}$ mice had already shifted towards an egocentric strategy to solve the task, whereas $Gpr88^{+/+}$ animals still used an allocentric strategy at this stage ($\chi^2=23.27$, $p<0.0001$). Reversal confirmed higher levels of performance in mutant mice, which acquired the novel rule more rapidly than controls. A probe trial performed after 2 reversal sessions indicated that mutant mice had consistently re-shifted to an allocentric strategy ($\chi^2=111.00$, $p<0.0001$).

Altogether, our results concur to demonstrate that hippocampus-dependent behaviors are facilitated in mice lacking GPR88.

## Levels of anxiety are diminished in $Gpr88^{-/-}$ mice

GPR88 is densely expressed in the CeA and, further, Gpr88 mutant mice showed significant modifications of transcriptional activity, dendritic spines morphology and dopamine levels in this region, suggesting potential effects on anxiety-like behavior. We used three tests to address different aspects of anxiety.

In the EPM, $Gpr88^{-/-}$ mice travelled a longer distance in the open arms, and spent more time in their distal parts (Figure 4A; Table S5 in Supplement 2). Moreover, ethological measures (29) revealed increased exploration in mutant mice, as shown by more frequent flat back approaches, stretched attend postures and head-dips than controls, specifically in the distal part of the open arms. Also, individual head-dips lasted longer in $Gpr88^{-/-}$ mice, suggesting altogether that GPR88 receptor deletion reduces anxiety levels in this test. In the anxiety-induced marble-burying test, $Gpr88^{-/-}$ mice buried less marbles than $Gpr88^{+/+}$ animals (Figure 4B), consistent with lower anxiety. Finally, we exposed $Gpr88^{-/-}$ to the novelty-suppressed feeding (NSF) test, a conflict task challenging approach/avoidance behavior (30). Mutant mice needed less time to start feeding in the center of the arena, and ate more chow when returned to their home cage compared to controls (Figure 4C), suggestive of decreased conflict anxiety.

Together, these data indicate that disruption of the Gpr88 gene results in decreased anxiety-like behaviors in mice.

### Chronic blockade of delta opioid receptors partly normalizes behavioral phenotypes of Gpr88$^{-/-}$ mice

We noticed that behavioral features of Gpr88$^{-/-}$ animals remarkably oppose several aspects of behavioral phenotypes that we previously identified in mice lacking DORs. In contrast to GPR88 knockout mice, DOR knockout mutants show facilitated striatal-dependent behaviors and impaired hippocampal-dependent responses in the dual-solution cross-maze task (19), as well as higher levels of anxiety in the elevated plus maze (31). In addition, [$^{35}$S]-GTPγSdata indicate excessive DOR signaling in GPR88 mutant mice (Figure 1B). We therefore tested the hypothesis that GPR88 and DOR activities compete in vivo. To this aim, we examined whether chronic inhibition of DORs (to mimic gene invalidation as closely as possible) using the antagonist naltrindole (NTI, 0.3 mg/kg s.c.) would normalize behavioral phenotypes of Gpr88$^{-/-}$ animals in the rotarod, Y-maze, EPM and NSF tests.

Chronic NTI administration transiently reduced motor skill deficits in Gpr88 mutant mice. Overall, control mice performed better than mutants in this test, and this effect varied across sessions (Figure 5A; Table S6 in Supplement 2). When analyzing rotarod data for each session separately, we found that NTI significantly improved motor performance of Gpr88$^{-/-}$ animals in session 2, suggesting a transient effect during early skill learning. During Y-maze exploration, chronic NTI normalized spontaneous alternation in Gpr88$^{-/-}$ mice, by increasing significantly the number of same arm returns, without effect on arm entries (Figure 5B; Figure S4A in Supplement 1; Table S6 in Supplement 2).

The chronic NTI treatment also normalized anxiety levels of Gpr88$^{-/-}$ animals in two behavioral tests. In the EPM (Figure 5C; Figure S4B in Supplement 1; Table S6 in Supplement 2), NTI failed to modify exploration ratios, but normalized ethological exploration parameters in mutant mice. The total number of flat back approaches (FBA) and stretched attend postures (SAP), as well as the number of distal FBA, SAP and head-dips, which are remarkably higher in mutant mice, were reduced to control levels upon NTI treatment. In the NSF test, chronic NTI administration increased latency to feed of mutant mice to control level, without effect on food intake (Figure 5D; Table S6 in Supplement 2).

## DISCUSSION

The present study reports that Gpr88 gene knockout in mice broadly modifies brain physiology and behavior. We show transcriptional, anatomical and neurochemical modifications in several brain areas. We confirm striatal dysfunction and further demonstrate that Gpr88 knockout facilitates hippocampus-dependent learning and decreases levels of anxiety. We finally provide evidence that excessive DOR signaling contributes to this behavioral profile. Whether molecular and behavioral phenotypes observed in Gpr88 knockout mice arise from developmental compensations or lack of tonic GPR88 receptor activity in adult mutant mice, or both, deserves further investigation. In a previous report, virally mediated inactivation of Gpr88 in the NAc of adult rats reduced amphetamine-induced hyperlocomotion (12), demonstrating a tonic role of GPR88 activity for this

particular behavior. Future studies will involve time-controlled genetic inactivation, as well as pharmacology with existing (10) or novel GPR88 drugs to tease out developmental from adult GPR88 activities.

Consistent with previous reports (2, 13), we found impaired striatal function in mutant mice. Knockout animals show non-habituating hyperactivity in a novel environment, a primary landmark of striatal dysfunction (32), and increased activity in several other tests (Y-maze, novel object recognition). Increased circling and duration of burying episodes suggest stereotypic behavior and difficulty ending a behavioral sequence, both major features of striatal deficit (33). Male Gpr88$^{-/-}$ mice failed in the building nests, a behavior normally developing in males for thermoregulation purposes (34) and sensitive to striatal deficits (25, 35). Note that this phenotype may be linked to hyperactivity (36-38). Finally, severe deficit in procedural learning on the rotarod points towards dorsal striatal (CPu) dysfunction (39, 40). Notably also, mutant mice show transcriptional, cellular and neurochemical modifications in the striatum, mostly in the CPu, including down-regulated D2R-MSN and glutamate synapse gene markers, low dendritic spine density and DA contents (as previously shown using microdialysis (2)). Meanwhile, DA levels were higher in the midbrain, suggesting defective release. Similar findings were reported after dopamine-depleting lesions mimicking Parkinson's disease (41, 42) with modifications occurring preferably in D2R-MSNs at least in early stages (41, 43). Whether hyperactivity, motor skill learning deficit and increased exploration in Gpr88 mutants arise from alterations of either D2R-MSN or D1R-MSN activities, or both (44), in the CPu, remains to be clarified using cell-specific Gpr88 gene knockout.

An interesting finding is that, in contrast to striatal-mediated behaviors, hippocampus-dependent responses are enhanced in Gpr88 knockout mice. We found less perseverative arm reentries (Y-maze), indicative of enhanced hippocampus-dependent working memory (45), and higher preference for the displaced object in a task (novel object recognition) that requires hippocampal integrity (46-48). In the cross-maze, mutant mice acquired the dual solution task, requiring distal extra-maze cues, earlier than controls, and shifted sooner after spatial reversal, two behavioral landmarks of improved hippocampal function (49-52). Gpr88$^{-/-}$ mice display modified spine density and gene expression in the hippocampus, whereas Gpr88 expression is undetectable in this region (Allen Brain Atlas; (2, 14)). In mutant mice therefore, lack of GPR88 receptor activity in hippocampal inputs and outputs where the receptor is normally expressed (striatum, PFC, septum, amygdala, parasubiculum) likely contributes to anatomical changes in the hippocampus and, possibly, to improved spatial navigation. Alternatively, functional competition between striatum and hippocampus may account for this behavioral phenotype. Indeed, hippocampal lesion/inactivation facilitates dorsal striatal function (51, 53, 54), although striatal lesion/inactivation fails to conversely facilitate hippocampal-dependent spatial learning (55-57), possibly due to differential implication of MSN subpopulations. Previous studies showed that striatal deletion of the adenosine A2a receptor (Adora2a) gene decreases excitability of D2R-MSNs and facilitates spatial learning (58-60). Reduced A2a receptor expression in the CPu of Gpr88 null mice (Figure S2 in Supplement 1), or decreased D2R-MSN excitability by other mechanisms, may thus have facilitated hippocampal-dependent behaviors in Gpr88$^{-/-}$ animals.

We report for the first time decreased anxiety levels in Gpr88$^{-/-}$ mice. Both classical parameters and ethological measures suggest that levels of anxiety are lower in the EPM. Decreased marble burying in mutant mice can also be interpreted as a sign of lower anxiety (61) or may result from hyperactivity (38, 62, 63). Mutant animals also showed reduced conflict anxiety (NSF) and together the data demonstrate that Gpr88 knockout leads to hyperactive exploratory behavior when mice are exposed to a novel and potentially hostile environment. Altered gene transcription, neuronal morphology and DA levels in the CeA, where Gpr88 is highly expressed, likely contributed to this phenotype (64-67). Gpr88 expression, however, is also detectable in several brain regions involved in regulating anxiety and approach (30), including ventral striatum (NAc and olfactory tubercles), anterior BNST and lateral septum. In the future, site-specific Gpr88 gene knockout should clarify brain sites for anxiolytic activity of GPR88 receptors.

Because of the remarkable opposing phenotypes of Gpr88 and DOR knockout mice in several tests, we probed the GPR88/DOR interaction behaviorally and the partial normalization of Gpr88$^{-/-}$ mice phenotype by chronic DOR blockade was striking. Chronic NTI alleviated motor skill learning deficit in Gpr88$^{-/-}$ mice at an early stage, when motor learning engages dorsal striatal D2R-MSNs (44), suggesting that excessive DOR activity in Gpr88$^{-/-}$ mice could contribute to this process, possibly at the level of D2R-MSNs. In the Y-maze, DOR blockade decreased spontaneous alternation of Gpr88$^{-/-}$ mice back to control levels, consistent with impaired hippocampal-dependent navigation in DOR knockout mice (19). Finally, NTI normalized low anxiety levels in Gpr88$^{-/-}$ mice in both EPM and NSF, in accordance with previously reported anxiogenic effects of DOR gene knockout and blockade (31, 68), notably in the CeA (69). We therefore suggest that GPR88 activity normally acts as a brake on DOR activity to regulate these behaviors. Restoration of normal behaviors in double GPR88/DOR null mutants may confirm this remarkable GPR88/DOR balance in the future. Functional interactions between GPR88 and DOR revealed in this study could operate at systems levels, through functional competition at the level of downstream effectors within neurons, or via physical interactions between the two receptors, and all these possibilities will need to be tested in future studies. Our observation that DOR, muscarinic and MOR coupling to Gi/Go proteins is enhanced in striatal membranes from Gpr88$^{-/-}$ mice (GTPγS assay) supports the notion that GPR88 receptors inhibit DOR, MOR and muscarinic receptor function, and possibly signaling of other GPCRs, at cellular level. The analysis of potential cellular interactions in the striatum, including down-regulated expression of Rgs4 (present study, (13)), that was shown to repress opioid receptor signaling (70, 71), will deserve further investigations.

In conclusion, this study confirms that GPR88 receptors are a promising target in pathologies involving deficient striatal function, such as Parkinson Disease or Attention Deficit/Hyperactivity Disorder, as facilitating GPR88 activity is expected to alleviate striatal dysfunction. Furthermore, our work extends implications of receptor function to areas of cognitive and anxiety disorders where, on the contrary, inhibiting GPR88 signaling could exert beneficial effects on spatial leaning and working memory, or improve some aspects of anxiety-related conditions. The therapeutic potential of GPR88 as a target, therefore, is broad, likely complex, and mechanistically highly novel.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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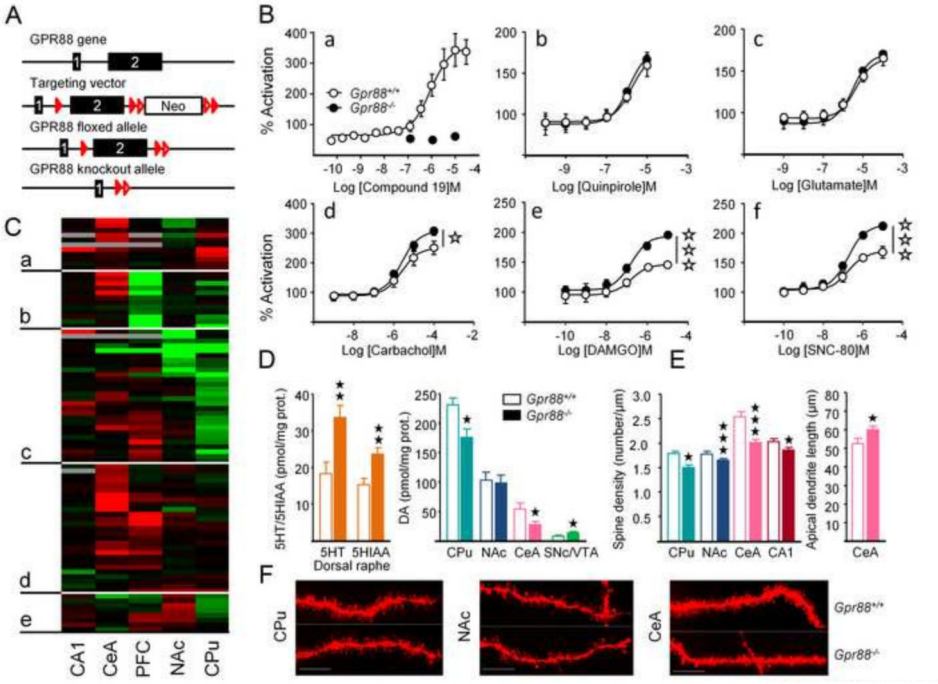


**Figure 1. Deletion of the Gpr88 gene display modifies GPCR signaling in the striatum and monoamine neurotransmission at striatal and extrastriatal sites**

(A) Gene targeting strategy to generate a Gpr88$^{-/-}$ mouse line. (B) a: GPR88-mediated [$^{35}$S]-GTPγS binding is abolished in the striatum of mutant animals; b-f: [$^{35}$S]-GTPγS binding assay shows facilitated delta (SNC80) and mu (DAMGO) opioid receptor function in striatal membranes of Gpr88$^{-/-}$ mice, whereas signaling properties of Go/Gi coupled D2 dopamine (quinpirole), metabotropic glutamate (glutamate), muscarinic (carbachol) receptors are unchanged. (C) Clustering analysis of gene expression data across PFC, CPu, NAc, CeA and CA1 classifies genes in five clusters (a-e). Most genes with increased expression in the CeA and PFC of mutant mice gather in clusters (a) and (d); most genes with down-regulated expression in the PFC are found in (b), whereas cluster (c) groups genes with decreased expression in the CPu and NAc. Little regulation of gene expression is observed in cluster (e). In the CPu of Gpr88$^{-/-}$ mice, 19 genes are down-regulated, 7 being highly or specifically expressed D2R MSNs (Drd2, Adora2, Cnr1, Foxp1, Rgs4, Gpr6, Nr4a1, Penk) and 6 playing a role in glutamate transmission (gene names in Figure S2 (Supplement 1) and Table S3 (Supplement 2)). In the CeA, 24 over 27 genes with regulated expression show increased transcription, 11 of which coding for subunits of GABA-A receptors or main actors of glutamate signaling (most significant up-regulation for Grm5 and Homer3) and 8 coding for MSN-enriched proteins (Arpp21, Bcl11b, Cartpt, Gpr6, Hpca, Nr4a1, Oprk1, Oprm1, Pde10a, Wfs1). Examples of individual gene expression profiles are displayed in Figure S2B in Supplement 1. (D) Contents in serotonin (5HT) and its metabolite 5-hydroxyindoleacetic acid (5HIAA) are increased in the dorsal raphe of Gpr88$^{-/-}$ animals, while dopamine contents are increased in the midbrain of these animals (SNc/VTA) but decreased in the CPu and CeA (Table S4 in Supplement 2). (E) Spine density is reduced in the CPu, NAc, CeA and CA1 of Gpr88$^{-/-}$ mice. (F) Dil fluorescent terminal dendritic segments from MSNs in the CPu, NAc and CeA of Gpr88$^{+/+}$ and

Gpr88$^{-/-}$ mice. Scale bar: 10 μm. Data are presented as mean ± SEM. Open stars: *p < 0.05; **p< 0.01; *** p< 0.001 (Two-way ANOVA). Solid Stars: *p < 0.05, **p < 0.01, ***p < 0.001 (post-hoc: Newman–Keuls test). CA1: CA1 of the dorsal hippocampus; CeA: central nucleus of the amygdala; PFC: prefrontal cortex; CPu: caudate putamen; NAc: nucleus accumbens, SNc: substantia nigra, pars compacta; VTA: ventral tegmental area.

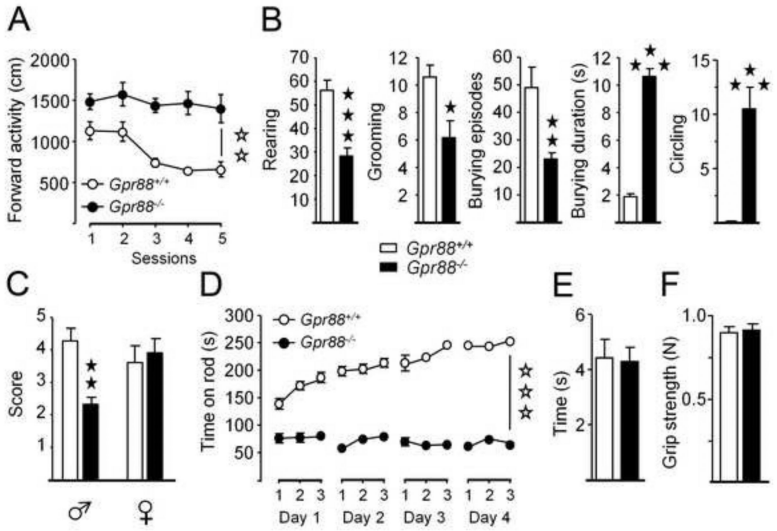


**Figure 2. Gpr88$^{-/-}$ mice display impaired striatal dependent behaviors**

(A) Gpr88$^{-/-}$ mice travel a longer distance in the open field as compared to controls and this hyperactivity fails to habituate over sessions. (B) Mutant animals display less rearing, grooming and burying episodes but spend longer time burying and make more circles than Gpr88$^{+/+}$ mice. (C) Male, and not female, mice lacking Gpr88 fail to build a nest. (D) Gpr88 deletion impairs motor coordination and skill learning in the accelerating rotarod test, while forelimb motor coordination (E) and strength (F) are preserved. Data are presented as mean ± SEM. Open stars: **p < 0.01, *** p< 0.001 (genotype effect). Solid Stars: *p < 0.05, **p < 0.01, ***p < 0.001 (Newman–Keuls post-hoc test or Kruskal Wallis' analysis of variance).

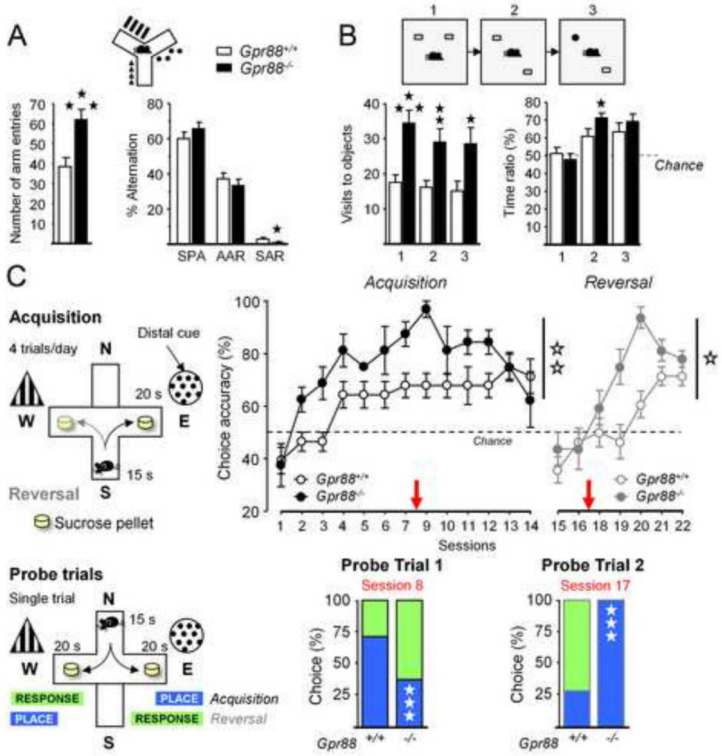


**Figure 3. Gpr88$^{-/-}$ animals show facilitated hippocampus-dependent behaviors**

(A) When exploring a Y-maze, mutant mice displayed more arm entries, evoking hyperactivity, and made less perseverative arm reentries. (B) In the novel object recognition test, Gpr88 null mice visited the objects more often and spent more time exploring the moved object during phase 2 (place phase). (C) Mutant animals acquired earlier and better a dual solution cross-maze task using distal extra-maze cues, shifted sooner to a response strategy to solve the task (probe trial 1), and reacquired more rapidly this task after spatial reversal than Gpr88$^{+/+}$ controls, by shifting sooner to an allocentric strategy (probe trial 2). More parameters are displayed in Figure S3 in Supplement 1. Data are presented as mean ± SEM. Solid stars: *p < 0.05, **p < 0.01, ***p < 0.001 (post-hoc: Newman–Keuls test). Open stars: *p < 0.05; **p< 0.01 (genotype effect). See Figure S2 in Supplement 1 for more parameters.

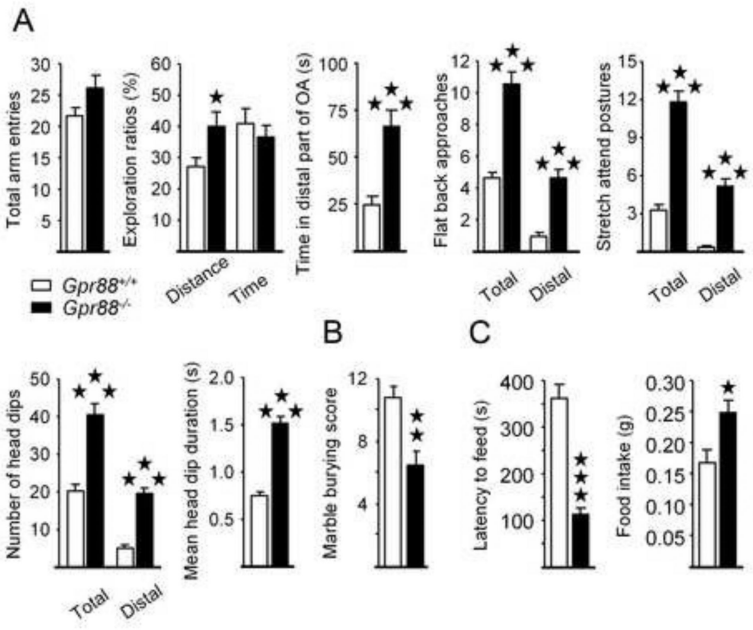


**Figure 4. Gpr88 null mice display low levels of anxiety**

(A) During the elevated-plus maze test, if Gpr88$^{-/-}$ mice display similar number of arm entries as Gpr88$^{+/+}$ controls, they travel a longer distance in the open arms, and spend more time in the distal part of these arms than Gpr88$^{+/+}$ animals; moreover knockout mice display more frequent flat back approaches, stretched attend postures and head-dips than controls, specifically in the distal part of the open arms, with individual head-dips lasting longer than in Gpr88$^{+/+}$ counterparts.(B) Mutant animals bury less marbles than controls in the marble burying test. (C) In the novelty-suppressed feeding test, Gpr88 null mice display shorter latencies to start eating in the center of the arena compared to Gpr88$^{+/+}$ animals, and eat more when placed back in their home cage. Data are presented as mean ± SEM. Solid stars: *p < 0.05, **p < 0.01, ***p < 0.001 (post-hoc: Newman–Keuls test).

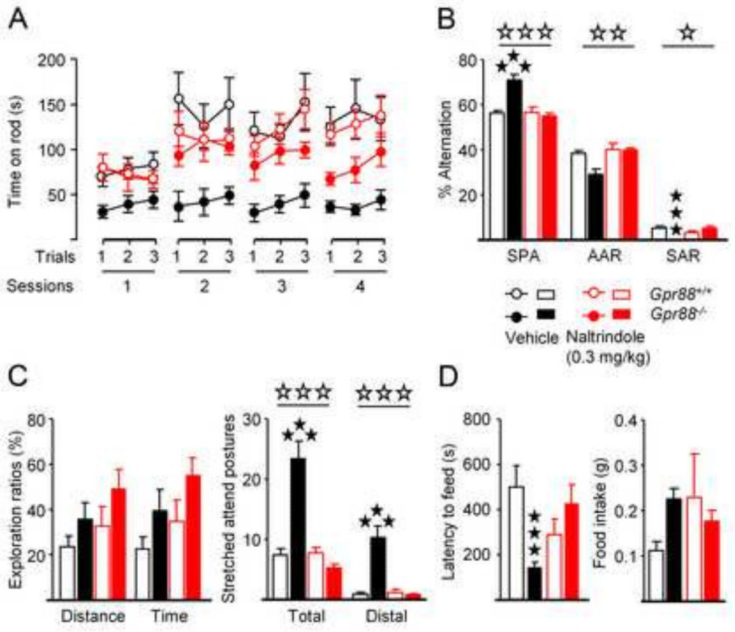


**Figure 5. Chronic blockade of delta opioid receptor activity normalizes behavior in mice lacking GPR88 receptors**

(B) Chronic naltrindole administration (0,3mg/kg) transiently (session 2) restores motor skill learning deficit in mice lacking GPR88 receptors. (C) In the Y-maze, this treatment increases significantly their number of same arm returns. (D) Naltrindole had no effect on exploration ratios during the elevated plus-maze test in mutants, but normalized their number of stretched attend postures, (E) as well as their latency to feed in the center of the open field in the novelty-suppressed feeding test. More parameters are displayed in Figure S4 in Supplement 1. Data are presented as mean ± SEM. Open stars: *p < 0.05; **p< 0.01; ***p < 0.001 (upper panel: genotype effect; lower panel: treatment effect). Solid stars: *p < 0.05, **p < 0.01, ***p < 0.001 (post-hoc: Newman– Keuls test).