

Appendix 1 to Galet B, Ingallinesi M, Pegon J, et al. G-protein coupled receptor 88 knock-down in the associative striatum reduces the psychiatric symptoms in a translational male rat model of Parkinson's disease. *J Psychiatry Neurosci* 2020.

DOI: 10.1503/jpn.190171

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SUPPLEMENTAL DATA

Study design

Two hypotheses were emitted at the beginning of the project, namely that (i) the partial DA lesion of the rat dorsolateral striatum (DLS) would reproduce some of the psychiatric symptoms of Parkinson's disease (PD), and that (ii) inactivating *Gpr88* in different striatal areas of the lesioned rats would affect specific behavioral parameters. In order to test each hypothesis, several experimental groups were designed. (i) To assess the effect of the loss of DA, two groups of animals were compared: 6-OHDA-injected vs SHAM-injected. The rats from both groups were also transduced with an inactive control lentiviral vector, LV miR-neg sequence, in the different striatal compartments. (ii) To determine the effects of *Gpr88* inactivation in 6-OHDA-lesioned rats, stereotaxic injections of lentiviruses were performed either in DLS, dorsomedial (DMS) or ventral striatum (VS; *Nucleus Accumbens* Core). However, as experiments were underway, no major effects of *Gpr88* inactivation in the ventral striatum were observed, while intriguing interactions were emerging between the DLS and DMS tiers of the striatum. For the sake of clarity, we thus decided to limit the scope of this article to the DLS-DMS interactions in both the effects of the lesion and of *Gpr88*-KD.

The data were accumulated over 17 replication batches. Each batch consisted of 6-10 animals that were randomly distributed across the different experimental conditions, making sure that no 2 rats of the same conditions were housed together. In order to comply with the 3Rs guidelines, the appropriate sample size was calculated based on behavioral data from preliminary experiments, using the G*Power3 software.¹ To reach a statistical power of 0.9 with the alpha level set at 0.05, the recommended sample size was of 9-10 animals per condition, depending on the behavioral task. Replication batches were thus stopped once the recommendation was met.

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Finally, we observed an important level of cumulative variability in the quality and extent of the lesions and LV transductions. In order to limit the effect of this variability and enhance the reproducibility of our findings, strict inclusion criteria were applied. First of all, the DA loss had to affect at least 20% of the DLS, but no more than 15% of the neighboring DMS and VS. Next, sufficient EmGFP fluorescence had to be present in the area targeted with LV miR-*Gpr88*. Furthermore, the timeframe during which we performed our behavioral and histological analyses is posterior to the period of neuroinflammatory reaction with microglial activation that follows the 6-OHDA-induced neurodegeneration.² Rats were excluded from the statistical analyses in the rare occurrences of adverse events such as important weight loss or inflammatory reactions. Specifically, we visually checked for increases in cellular proliferation around the stereotaxic injection sites with a nuclear DAPI stain, which could be due to a neuroinflammatory reaction. In such cases (n=2), the animals were excluded from the study.

Stereotaxic injections of 6-OHDA and LV vectors

Before the beginning of the surgeries, the animals were first anaesthetized with 4% isoflurane (IsoVet, Osalia) in an induction chamber (Minerve Veterinary Equipment, Esternay, France) for 5 minutes before being placed into a Kopf stereotaxic surgery apparatus (Phymep, Paris, France). Anesthesia was maintained throughout the surgery with an isoflurane pump (Univentor, Zejtun, Malta). General and localized analgesia were induced with subcutaneous injections of Buprecare (buprenorphine; 0,05mg/kg) (Axience) and Xylovet (lidocaine; 17,5mg/kg) (Ceva) before beginning the surgery. 4µl of a solution of 6-OHDA (3µg/µl, 12µg total per hemisphere) in saline + 0.02% ascorbic acid (all chemicals from Sigma) or of a control solution (saline + 0.02% ascorbic acid only) were then injected bilaterally in the DLS using a 10µL Hamilton syringe (Phymep, Paris, France). The coordinates were as follow: anteroposterior (AP) + 0,7mm; mediolateral (ML) ± 3,8mm; dorsoventral (DV) -5,5mm (2µl) and -4,5mm (remaining 2µl) from

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bregma.³ The injections were performed at a rate of 0.5µl/min, and the syringe was left in place for 3 minutes after the end of each injection to allow for proper diffusion of the toxin. Following the surgeries, the wellbeing of the animals was checked daily. While some rats transiently lost weight, they typically recovered within 3 to 5 days.

Lentivirus production

The LV vectors were generated at the in-house iVector platform, using the BLOCK-iT Pol II miR RNAi expression vector kit (Invitrogen). The vector construct co-expressed an engineered miR sequence to drive *Gpr88* knock-down and EmGFP as a transduction marker. The modified miR consisted of a shRNA inserted within the miRNA 155 flanking sequences. One of the shRNA complementary sequences was designed to target either *Gpr88* mRNA (“miR-*Gpr88*”), or a control sequence that is not expressed in the genome (“miR-neg”). The use of a shRNA sequence allowed for high specificity of RNA interference under control of the PGK promoter, which is best suited for *in vivo* studies. The lentiviruses were stored in PBS at -80°C, at an average of $1,6 \times 10^5$ transducing units (TU) /µL.

6µL of the lentivirus solution were bilaterally injected in the DLS or DMS two weeks after the 6-OHDA injections, following the same general surgical procedure. The coordinates were however different, as the lentiviruses were injected at 4 different sub-sites per hemisphere (8 total) to insure sufficient knock-down of *Gpr88* expression. When targeting the DLS, the coordinates were the following: (1) AP +1,2 mm; ML ±3,6 mm; DV -5,5mm and -4,5mm (2) AP +0,2 mm, ML ±4mm, DV -5,5mm and -4,5mm from bregma. Regarding the DMS, the coordinates were: (1) AP +1,2 mm; ML ±2 mm; DV -5,5mm and -4,5mm (2) AP +0,2 mm, ML ±2,2mm, DV -5,5mm and -4,5mm from bregma.

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Behavioral tests

Each test was set up by the same experimenter and around the same time of day for each batch of animals, in between 9 AM and 4 PM. The experimenter was unaware of the status of the rats, which were brought in the testing rooms one hour before the beginning of the task, in order for them to acclimate to the environment. The luminosity was controlled for every experiment (35 lux), and the testing apparatus were cleaned before each test and between rats with a disinfectant solution (Aniospray, Dutscher).

Actimeter - General motor and exploratory behavior was assessed for 15 minutes using the Panlab Infrared Actimeter (Harvard Apparatus, Holliston, MA, USA). Horizontal, stereotyped and vertical movements were automatically quantified and cumulated into 5-minute segments.

Sucrose preference - Rats were isolated in enriched individual cages for 72h, during which time they were given access to two bottles containing either tap water, or tap water supplemented with 0,5% sucrose (Sigma). The first 24h were considered as an acclimation phase, and were not included in the analysis. After 48h, the position of the bottles was inverted to avoid side preference effects. Bottles were weighed daily in order to calculate the amount of consumed liquids. Sucrose preference was calculated as the percentage of sucrose intake / total intake. However, as the level of sucrose preference exhibited by control rats was lower than what we had observed during pilot experiments (at 0,25% sucrose), the concentration had to be re-evaluated after several batches of animals (0,5%). For this reason, the number of data points for this experiment is relatively lower. General consummatory behavior was also tracked by weighing the food dispenser at the beginning and end of the isolation phase.

Social Novelty Discrimination (SND) - Social interaction and selective attention were then evaluated using the social novelty discrimination task (SND) as previously described.^{4, 5} As this behavioral paradigm requires a preliminary isolation phase, we performed it at the end of the sucrose preference test. Briefly, a

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first juvenile was placed into the home cage of the tested rat for a presentation period (P1) of 30 min. The time spent by the tested rat investigating the juvenile (anogenital sniffing, pursuing, allogrooming) was timed manually for the first 5 min. At the end of P1, a second juvenile was introduced in the cage, and the time spent investigating the novel vs the familiar juvenile was timed by the experimenter (presentation period P2). The “discrimination ratio” was calculated as the time spent by the tested rat interacting with the novel juvenile over the familiar juvenile during P2.

Prepulse Inhibition (PPI) - Sensorimotor gating was assessed using an auditory prepulse inhibition apparatus (IMETRONIC, Pessac, France). The adapted protocol⁶ contained three phases: a first acclimation period of 10 minutes, followed by a phase of habituation to the startle stimulus, and ending with the testing phase. During the acclimation phase, a background white noise of 60dB was played, which persisted throughout the whole testing session. The habituation phase consisted of 10 startle-inducing auditory “pulses” played at 110dB (7Khz, 100ms), and at random intervals between 15-30 seconds. During the testing phase, four different types of stimuli were presented: a pulse alone (110dB), a prepulse-pulse pairing, a prepulse alone, or no sound (to assess background movement). The prepulses (20ms, 70-80dB) preceded the startle pulses by 100ms. Each condition was presented 10 times in a pseudo-randomized order, at random intervals between 15 and 30 seconds. Prepulse inhibition (%PPI) was measured as the reduction in startle response during prepulse–pulse conditions compared to pulse-alone trials.

Forced Swim Test (FST) - The rats were placed in a transparent cylinder filled up to 35 cm with 24°C (±1) water for 5 minutes, and recorded using a digital camera. A trained experimenter blinded to the conditions then analyzed the behavior of the animals using a previously described sampling method, calculating immobility, swimming, climbing and diving counts.⁷

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Immunolabelling and *in situ* hybridization (ISH)

Immunolabelling procedures were adapted from a previously published protocol⁸ using primary antibodies directed against Tyrosine Hydroxylase (TH) (Millipore MAB318, 1:400) and the truncated splice variant of the FosB **transcription factor** (Δ FosB) (Abcam AB11959, 1:500) TH immunolabelling was completed using a fluorophore-coupled secondary antibody (Alexa Fluor 647, Invitrogen A21235, 1:1000), while Δ FosB required DAB revelation for best results (BA-2000 secondary antibody, 1:250, and PK6100 kit from Vector Laboratories) (**Fig. S1A,C**).

ISH was performed with antisense digoxigenin-labeled complementary RNA probes designed to recognize *Gpr88*, 67-kDa glutamate decarboxylase (*Gad67*), proenkephaline (*Penk*), prodynorphine (*Pdyn*) mRNAs as previously described⁵ (**Fig. S1B, D**). A new probe was also designed following the same procedures to target regulator of G-protein signaling 4 (*Rgs4*) mRNA (GenBank accession number: NM017214, targeted nucleotides: 325 to 604).

Digitization and semi-quantitative analysis - Slides were then digitized using the Axio Scan.Z1 and ZEN software (Zeiss, Oberkochen, Germany). The resulting images were exported for processing in Fiji (NIH, Bethesda, MD, USA).⁹ As indirect fluorescent and colorimetric signals are not stoichiometrically related to biological content, the signal intensity was not quantified. Instead, a threshold was determined using control slides (secondary antibody alone/sense probe) or control areas within a slice (corpus callosum), and applied to all of the images from a same experiment. To evaluate the loss of dopaminergic terminals, the TH-positive signal was then quantified in each striatal area (DLS, DMS, VS) of every lesioned rat according to previously published methods.¹⁰ Regarding the nuclear markers (Δ FosB and all of the *ISH* targets), a fixed-size region of interest was drawn in the DLS and DMS, and the total signal-positive area was quantified (**see Fig.S2E**). For each striatum, the signal was measured over at least 3 anteroposterior

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locations between AP +0,2 mm and +1,2 mm, and averaged. The values were then normalized to those obtained in control rats (SHAM + miR-neg). A Fiji macro script was written to automatize the process. As the lesion and transduction extent varied, each striatum (2/brain) was considered as a biological replicate for statistical analyses.

Statistical Analyses

Data from the experiments were analyzed using the Prism 6.0 software (GraphPad Software Inc, La Jolla, CA, USA). Different tests were performed depending on the nature of the data and the driving hypotheses that were exposed in the “study design” section. For instance, regarding behavioral data, to assess the effects of the lesion (hypothesis 1), the two groups (SHAM-neg vs 6-OHDA-neg) were compared using two-tailed unpaired **Welch's t-tests**. Then, to evaluate the effects of *Gpr88*-KD in 6-OHDA-lesioned animals (hypothesis 2), the 6-OHDA + miR-*Gpr88* groups (DLS and DMS) were compared to the 6-OHDA + miR-neg animals using one-way ANOVAs followed by Dunnett multiple comparison tests, as the data distribution passed normality tests. However, some of the actimeter, PPI and SND data required the use of 2-way ANOVAs followed by Sidak or Dunnett corrections where appropriate to assess interactions with independent variables (time/prepulse intensity/novelty status). Finally, as each striatal area was differentially affected by the 6-OHDA lesion, the data from the immunolabelling and *ISH* experiments were analyzed using multiple t-tests followed by Holm-Sidak corrections.

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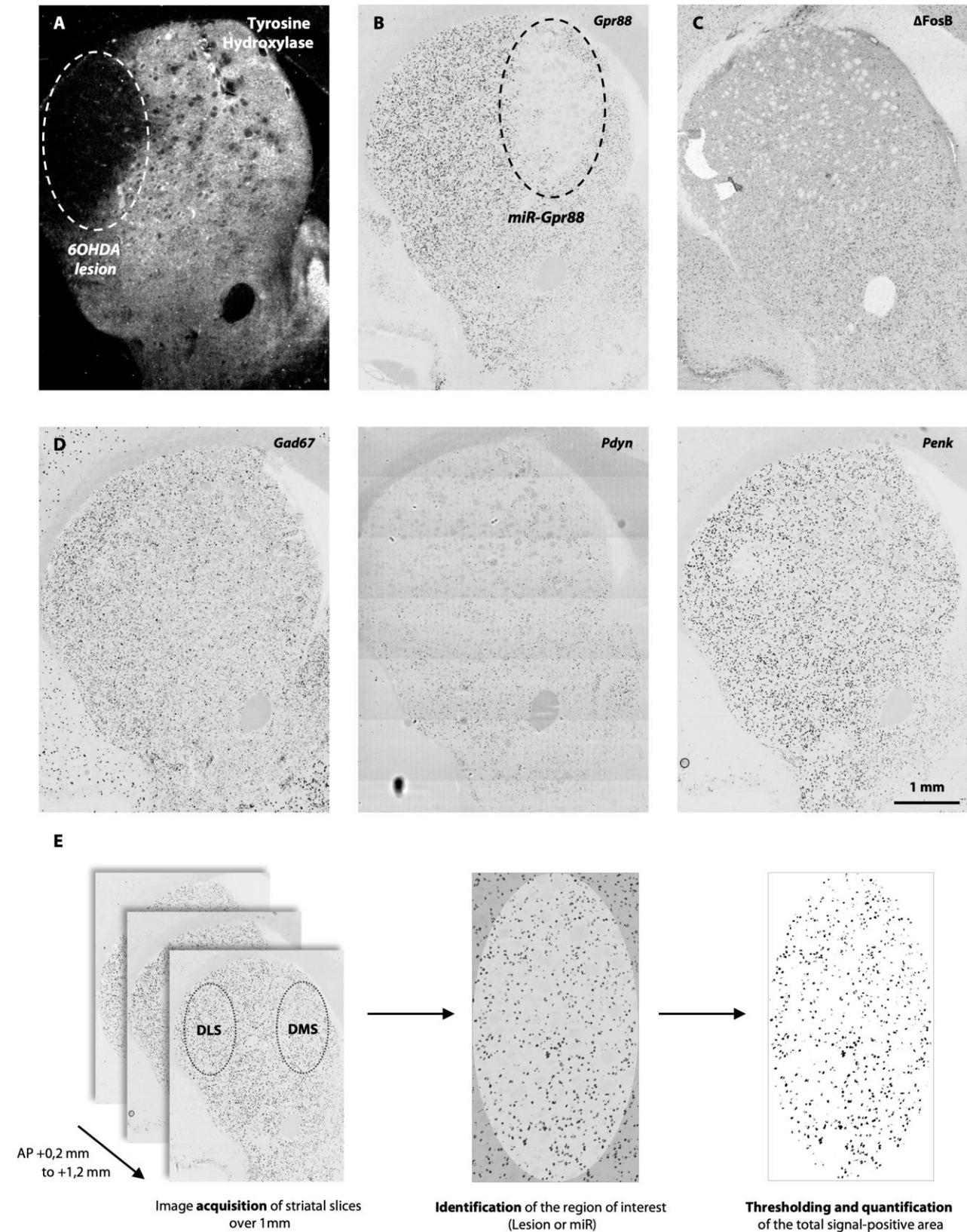
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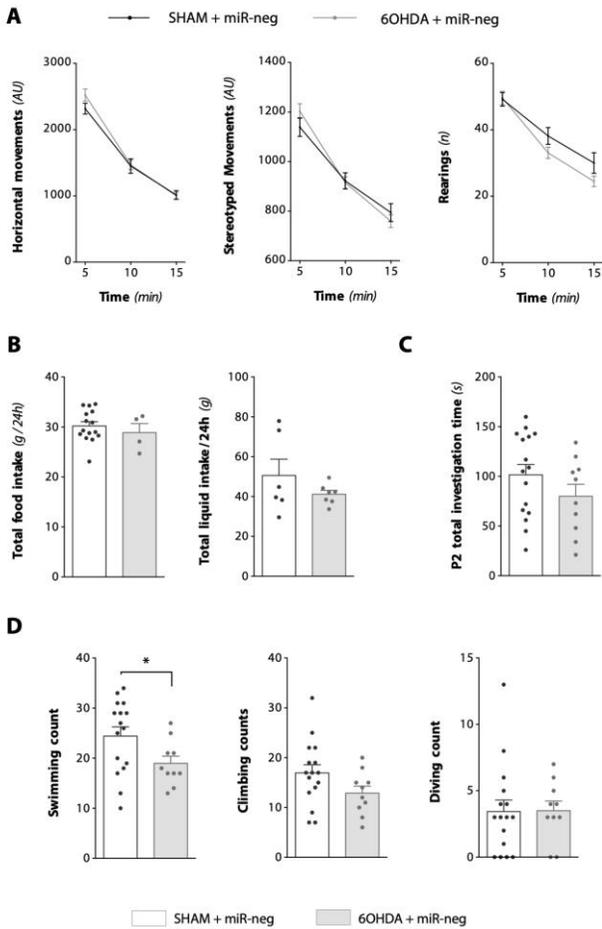
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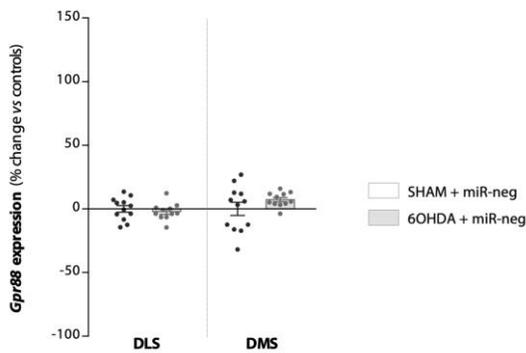
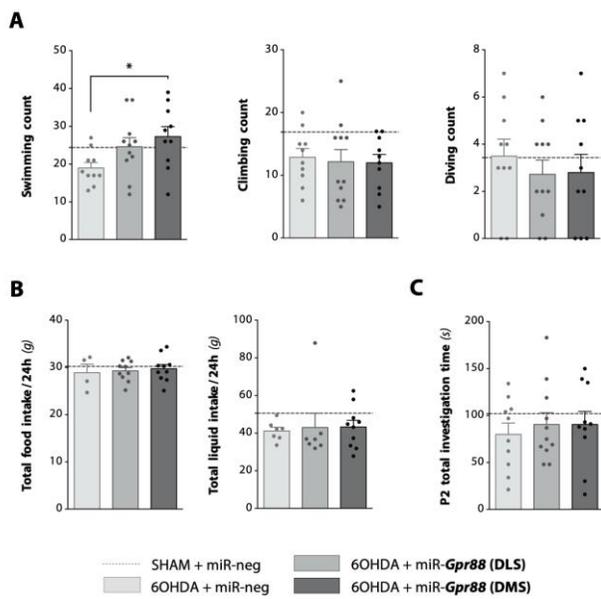


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Supplementary Figure 1 – Histological characterization.

(A-D) High resolution scans of 12 μ m coronal slices from the same animal (6-OHDA + miR-*Gpr88* in the DMS), stained for (A) TH, (B) *Gpr88*, (C) Δ FosB, (D) *Gad67*, *Pdyn* and *Penk*. (E) Summary of the image processing and quantification workflow, detailed in the Supplemental Data.

Supplementary Figure 2 – Behavioral effects of the 6-OHDA lesion: additional data.

(A) Effect of the 6-OHDA lesion 2 weeks after stereotaxic injection on motor parameters during the actimeter test [$n_{\text{SHAM + miR-neg}} = 15$; $n_{\text{6OHDA + miR-neg}} = 27$]. Effect of the 6-OHDA lesion after 4 weeks on: (B) consummatory behavior [$n_{\text{SHAM + miR-neg}} = 6-15$; $n_{\text{6OHDA + miR-neg}} = 4-7$], (C) total social investigation time during the discrimination task (P2) [$n_{\text{SHAM + miR-neg}} = 16$; $n_{\text{6OHDA + miR-neg}} = 10$], (D) swimming, climbing and diving behaviors during the FST [$n_{\text{SHAM + miR-neg}} = 16$; $n_{\text{6OHDA + miR-neg}} = 10$]. The increase in immobility count induced by the lesion (Fig. 3D) was mediated specifically by a decrease in swimming. Data are presented as mean \pm SEM. When two groups were compared, two-tailed, Welch's t-tests were performed (B and C). In the case of interactions with additional factors, two-way ANOVAs, followed by Sidak's multiple comparison test were used. * $p < 0.05$.

Supplementary Figure 3 – Behavioral effects of the Gpr88-KD: additional data.

(A) Effect of the *Gpr88*-KD on swimming, climbing and diving behaviors during the FST [$n_{\text{6OHDA + miR-neg}} = 10$; $n_{\text{6OHDA + miR-Gpr88-DLS}} = 11$; $n_{\text{6OHDA + miR-Gpr88-DMS}} = 10$]. KD in the DMS reduced the immobility count (Fig. 4D) by specifically increasing swimming behavior. (B) Effects of *Gpr88*-KD on consummatory behavior [$n_{\text{6OHDA + miR-neg}} = 4-7$; $n_{\text{6OHDA + miR-Gpr88-DLS}} = 7-10$; $n_{\text{6OHDA + miR-Gpr88-DMS}} = 10$], and (C) total social interaction duration in the SND task [$n_{\text{6OHDA + miR-neg}} = 10$; $n_{\text{6OHDA + miR-Gpr88-DLS}} = 11$; $n_{\text{6OHDA + miR-Gpr88-DMS}} = 10$]. Data are presented as mean \pm SEM, and were compared using one-way ANOVAs followed by Dunnett multiple comparisons tests. For reference, a dashed horizontal line indicates the values from the control group (SHAM miR-neg), that were presented in Fig. 3. * $p < 0.05$.

Supplementary Figure 4 – The 6-OHDA lesion does not affect Gpr88 expression.

The values were normalized to those of the control group (SHAM + miR-neg) [$n = 12$ in each condition]. Data are presented as mean \pm SEM, and were compared using multiple t-tests with Holm-Sidak corrections.