SUPPLEMENTARY INFORMATION

Gpr88 Deletion Impacts Motivational Control Without Overt Disruptions to Striatal Dopamine

Spark *et al.*

Supplementary Methods

Gpr88$^{-/-}$ CRISPR mice

Gpr88 CRISPR mice were generated on a C57BL/6J background using CRISPR/Cas9 gene editing using sgRNA1 5′ AAAGAACAGGAGAACGGGGG 3′ sgRNA2 5′ AGCCCCAGGGAAGATCCCAG 3′. CRISPR-Cas9 RNAs (crRNA) were ordered from Integrated DNA Technologies (IDT) and incubated with transactivating CRISPR RNA (tracrRNA) to form a functional guide RNA complex. Cas9 nuclease was purchased from IDT (IDT Alt-R® S.p. HiFi Cas9 Nuclease V3) and incubated with the guide RNAs to form a ribonucleoprotein (RNP) complex. The ssDNA repair template was generated using the Guide-it Long ssDNA Production System (Takara) according to the manufacturer's instructions. Cas9 nuclease (30ng/ml), gRNAs 1 & 2 (30ng/ml) & ssDNA repair template (30ng/ml) were microinjected into the pronucleus of C57BL/6J zygotes at the pronuclei stage. Injected zygotes were transferred into the uterus of pseudo pregnant F1 females.

Touchscreen apparatus

The touchscreen automated system (Campden Instruments Ltd.. Cambridge UK) was used as previously described (1,2). In brief, the system is composed of a touch-sensitive screen, a liquid reward-delivery magazine situated opposite to the touchscreen, infra-red (IR) beams and black

Perspex side walls. A black Perspex mask with five response windows was placed in front of the touchscreen to limit the area that the mice were allowed to touch. The operant chamber was placed inside a sound- and light- attenuated box with house light, tone generator, IR camera and ventilating fan. Strawberry flavoured milk (3D Devondale, Australia (Experiment 1) or Nippy's, NSW, Australia (Experiment 2)) was used as liquid reward. The system was controlled using Whiskers and Animal Behaviour Environment Test (ABET) software (Lafayette Instrument Company, Lafayette, IN, USA).

Behavioural procedures

For all behavioural experiments, mice were housed under a reversed light-dark cycle condition to allow testing during the active phase. Mice were acclimatised from a standard light-dark cycle to the reversed light-dark cycle for two weeks prior to any intervention. One week prior to touchscreen operant testing, mice (approx. 10 weeks of age) were weighed for three consecutive days to establish their free feeding body weight and then food restricted to reach 85% of their free feeding body weight by giving limited amounts of chow pellets every day. For two days immediately prior to the beginning of operant training, mice were exposed to a small amount of the liquid reward in their home cages to prevent neophobia. Touchscreen training and tasks protocols were adapted from (2).

Touchscreen operant training

Operant training was conducted as previously described (2). Briefly, to habituate mice to the testing apparatus, mice were placed in the touchscreen chamber free to explore for 20 minutes over two consecutive days. During initial touch training, a stimulus was presented (white square) for 30 seconds. Once the stimulus was turned off, a tone was issued, the reward magazine was lit and 20 μL of reward was dispensed. A 5 second inter-trial interval (ITI) followed and a new

trial began. If the white square was touched while illuminated, the stimulus was switched off, the tone issued, and the magazine was lit. Triple reward delivery was issued on these trials. The session ended after the mouse consumed 30 rewards or after 60 minutes, whichever came first. The criterion to move to the next training phase was to consume 30 rewards. During the Fixed Ratio Training, mice underwent fixed ratio (FR) 1 training which consisted of 20 µL reward delivery after a single touch response to the stimulus. The session ended after completion of 30 trials or after 60 minutes. Mice then underwent FR2 (two responses required for reward delivery), FR3 (three responses required for reward delivery) and FR5 (five responses required for reward delivery; 4 days) to ensure mice established robust responding Completion criteria for FR training was 30 trials within 60 minutes.

[^{18}F]DOPA PET

30-45 minutes prior to [^{18}F]DOPA treatment, mice were dosed with peripherally-restricted inhibitors of L-DOPA metabolism, benserazide (formulated in MilliQ H_2O at 10mg/kg in a 5 ml/kg dose volume, i.p.) and entacapone (formulated in 20% (2-hydroxypropyl)-β-cyclodextrin in dH_2O pH=4-5 at 40 mg/kg in 5 ml/kg dose volume, i.p). Prior to scanning, mice were anaesthetised with isoflurane in air and a tail-vein cannula was inserted. Animals were then placed in the µPET-CT scanner (Siemens, Germany) and a 60-minute scan commenced. After 1 minute of baseline reads, mice were dosed with [^{18}F]DOPA (4.31 ± 0.85 MBq). At the end of the PET scan, a 10-minute CT scan commenced for attenuation correction and to provide structural references. Following this, animals were transferred to the MRI scanner (Bruker, USA) to acquire a structural MRI using FLASH with TR/TE = 60/8 ms, resolution = 0.156 x 0.156 x 0.156 mm^3 (FOV = 14.976 x 14.976 x 7.8 mm^3, matrix size = 96 x 96 x 50), slice thickness = 7.8 mm, slice number = 1, average = 4.

PET data was histogrammed into 47 frames (4 x 20s, 10 x 3s, 14 x 5s, 6 x 30s, 4 x 60s, 7 x 300s, 2 x 600s), reconstructed using filtered back projection and corrected for CT attenuation, radiotracer decay and deadtime. Using Inveon Research Workspace software (Siemens, Germany), structural MRI was registered to PET and CT data and used to define 3D ROIs of the striatum and cerebellum, which was used as a reference region in lieu of arterial input.

Time activity curves (TACs) for the striatum and cerebellum were generated and modelled by Patlak graphical analysis. Striatal K_i^{Cer}, the influx rate constant, was calculated from a linear regression of data between 10-60 minutes relative to the cerebellum. K_i^{Cer} provides a composite measure of striatal dopamine synthesis, storage, release and metabolism.

Striatal GTPγ[^{35}S] binding

Striatal membranes were prepared from tissue dissected from adult *Gpr88*$^{-/-}$ mice (12-40 weeks of age, males and females). In brief, striatal tissue was kept on ice and homogenised using a hand held homogeniser (Polytron PT1200E, ThermoFisher Scientific, Waltham, Massachusetts, USA) in an iced-cold buffer containing 20mM HEPES and 10mM EDTA and pH 7.4. Homogenised tissue was then centrifuged at 500g for 5 min at 4 ℃ (Heraeus Multifuge 3SR+ Centrifuge, ThermoFisher Scientific, Waltham, Massachusetts, USA) the pellet was homogenised and centrifuged again as above. The resulting supernatant was then centrifuged at 40,000g for 1h at 4 ℃ (Sorvall Evolution RC, ThermoFisher Scientific, Waltham, Massachusetts, USA). The pellet was then resuspended in an iced cold buffer containing 20mM HEPES, 1mM EDTA, pH 7.4 and passed through a 30G needle. Protein concentration was determined using a BCA assay (Thermo Fisher Scientific, CA, USA) following manufacturer instructions. GTPγ[^{35}S] binding was assessed using 20 µg of protein per well. In brief, membrane preparations were incubated with and without ligands (Pramipexole Dihydrochloride (Sigma #1598), at concentrations from

30 µM to 300 pM, diluted in assay buffer, and Haloperidol (Sigma #1512), at a single concentration of 10 nM, diluted in 1% DMSO in assay buffer) for 1h at 37 °C in assay buffer (25 mM HEPES, 100 mM NaCl, 5 mM MgCl2, 2 mM CaCl2, 0.2 mM EGTA, 0.01% Pluronic F127 and 20 µg/mL saponin, pH 7.4) containing 100 µM GDP and 0.2 nM GTPγ[^{35}S]. The reaction was stopped by rapid filtration through Whatman GF/C filters, which were immediately washed with an ice-cold wash buffer (0.9% NaCl), dried and dissolved in Microscint^{TM}-0, and counted using a MicroBeta$^{2®}$ 2450 Microplate Counter (PerkinElmer, Waltham, Massachusetts, USA). Basal binding was assumed to be the specific GTPγ[^{35}S] binding in absence of agonist.

Quantitative real time-PCR (qRT-PCR)

Brain tissue was collected from ventral striatum (WT male n=4, female n=4; *Gpr88*$^{Cre/Cre}$ male n=4, female n=5), dorsal striatum (WT male n=5, female n=8; *Gpr88*$^{Cre/Cre}$ male n=6, female n=8), and hypothalamus (WT male n=6, female n=8; *Gpr88*$^{Cre/Cre}$ male n=6, female n=8) and quickly frozen in dry ice. RNA was extracted from each sample using the Bioline RNA ISOLATE II RNA mini kit (Bioline, London, UK) following the manufacturer instructions. cDNA was synthesised using the tetro-cDNA synthesis kit (Bioline, London, UK) following the manufacturer instructions. The qRT-PCR was performed using LightCycler 480 SYBR green (Roche, Basel, Switzerland) and the Bio-Rad CFX384 Touch Real-Time PCR Detection System (Bio-Rad, CA, USA). CFX Manager Software (Bio-Rad, CA, USA) was used to analyse RT-qPCR data and normalise gene expression to house-keeping genes by employing the DCt method where the average triplicate Ct value for each sample was subtracted. *Gpr88*$^{Cre/Cre}$ DCt values were then normalised to WT samples for each gene of interest to obtain the DDCt. β-actin and GAPDH were used as reference house-keeping genes.

Gene	Forward sequence	Reverse sequence
mComt	CACTGGAAAGACCGCTACCT	TTGTCAGCTAGGAGCACCGT
mDbh	CCGTTTCTCCGACTGGAAGT	TGTGTAGTGTAGGCGGATGC
mDdc	TCTTGGGTTGGTCTGCTTCC	CACACAGCAAAGCGTAGCAC
mDrd1	AGGTTGAGCAGGACATACGC	TTGCTTCTGGGCAATCCTGT
mDrd2	CCAGTGAACAGGCGGAGAAT	GGCTATACCGGGTCCTCTCT
mDrd3	TCACTCGACAGAACAGCCAG	AAAACTGCCGAATGGGATGC
mMaoa	GCCCTGTGGTTCTTGTGGTA	GGCCAGAGCCACCTACAAAT
mMaob	CCCTTGCTGAAGAGTGGGAC	AGAGCGTGGCAATCTGCTTT
mSlc6a3	GACCTGGCCAATGTCTGGAG	TCTGTTGAACTGCCCGAGAG
mTh	GCCGTCTCAGAGCAGGATAC	CATCCTCGATGAGACTCTGCC
mSlc18a1	GCAGAACCCACCAGCTAAGG	CTTCGTGGGCCTCTGGATTG
mSlc18a2	GCTGCTGTTTGCCTCCAAAG	TGCAGAATCCAGCAAACATGG
mCartpt	CGAGAAGAAGTACGGCCAAGT	GTCACACAGCTTCCCGATCC
mGpr88	CTGCTGCGTCTTCCTGCT	GGAACTCCTCGTTCCTCCAC
mPpp1r1b	CCACCCAAAGTCGAAGAGAC	GAGGCCTGGTTCTCACTCAA
mRgs4	GAGTGCAAAGGACATGAAACATC	TTTTCCAACGATTCAGCCCAT
mGrm8	GGAGAGAGTGGTGTTGAGGC	GTCTTGGTTCACGTGGGATT
mGAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
mActin	CATTGCTGACAGGATGCAGAAGG	TGCTGGAAGGTGGACAGTGAGG

Western blotting

Mice from Cohort 3 were humanely killed by cervical dislocation and heads were decapitated, the brain was then removed. For the dopamine pathway-related proteins the striatum was dissected and immediately placed in dry ice. Tissue was sonicated in lysis buffer containing 5mM HEPES, 300mM NaCl, 1% NP40, 10% glycerol, 0.4% DDM, 1:100 Halt(™) protease and phosphatase inhibitors (Thermo Fisher Scientific, CA, USA) and made up to volume with MilliQ water. A BCA assay (Thermo Fisher Scientific, CA, USA) was performed on the lysed tissue to determine protein concentration in each sample. Samples were loaded into 8-16% Mini-PROTEAN TGX Stain-Free Protein Gels (Bio-Rad, CA, USA). Precision Plus Protein Dual Color Standards (Bio-Rad, CA, USA) were used as molecular weight markers. Proteins were then electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon, Millipore Corp., MA, USA) overnight at 4 °C and 30 V. Membranes were blocked for one hour

at room temperature in 5% BSA in 1x PBST and then probed with primary antibodies overnight at 4 °C (Table 2). Blots were washed at room temperature three times for 5 minutes each in blocking buffer. Blots were then incubated with secondary antibody for 1 hour at room temperature. Membranes that were probed with HRP-conjugated secondary antibody were incubated for 5 minutes with Pierce enhanced chemiluminescence (ECL) Plus substrate (Thermo Fisher Scientific, CA, USA) before imaging. Blots were visualised via fluorescence using the Amersham Typhoon (GE Healthcare Bio-Sciences Corp, MA, USA) or via chemiluminescence using the Bio-Rad Chemidoc Imaging System (Bio-Rad, CA, USA). Images were analysed using Image J via densitometry. Mean grey value was measured for each protein band maintaining a consistent ROI size. Background was also measured directly above or below each band where no staining was evident. Pixel density of each ROI was inverted, and background subtracted from corresponding protein bands. Bands of interest were then normalised to loading controls (NeuN or β-actin; chosen dependent on the molecular weight of the protein of interest) and presented as fold-change with respect to WT mice.

	Primary Ab	**Secondary Ab**
AADC	(Rb) Anti-dopa decarboxylase (1:500; ab3905 Abcam, CBG, UK)	Alexa-fluor 647 anti-Rb (1:1000; A31573 Thermo Fisher Scientific, CA, USA)
	(Ms) Anti-NeuN (1:1000; MAB377 Millipore, MA, USA)	Alexa-fluor 488 anti-Ms (1:1000; A21202 Thermo Fisher Scientific, CA, USA)
DAT	(Rb) Anti-dopamine transporter (1:1000; ab184451 Abcam, CBG, UK)	HRP-conjugated anti-Rb (1:5000; AP307P Millipore, MA, USA)
	(Rb) Anti-β-actin (1:1000; 4967S Cell Signalling, MA, USA)	
MAO-A	(Rb) Anti-monoamine oxidase A (1:1000; ab126751 Abcam, CBG, UK)	HRP-conjugated anti-Rb (1:5000; AP307P Millipore, MA, USA)
	(Ms) Anti-NeuN (1:1000; MAB377	Alexa-fluor 488 anti-Ms (1:1000;

	Millipore, MA, USA)	A21202 Thermo Fisher Scientific, CA, USA)
MAO-B	(Rb) Anti-monoamine oxidase B (1:1000; ab137778 Abcam, CBG, UK)	HRP-conjugated anti-Rb (1:5000; AP307P Millipore, MA, USA)
	(Rb) Anti-β-actin (1:1000; 4967S Cell Signalling, MA, USA)	
TH	(Ms) Anti-tyrosine hydroxylase (1:1000; SAB4200697 Sigma Aldrich, MO, USA)	Alexa-fluor 647 anti-Ms (1:1000; A31573 Thermo Fisher Scientific, CA, USA)
	(Rb) Anti-β-actin (1:1000; 4967S Cell Signalling, MA, USA)	Alexa-fluor 488 anti-Rb (1:1000; A21206 Thermo Fisher Scientific, CA, USA)

Supplementary Figures

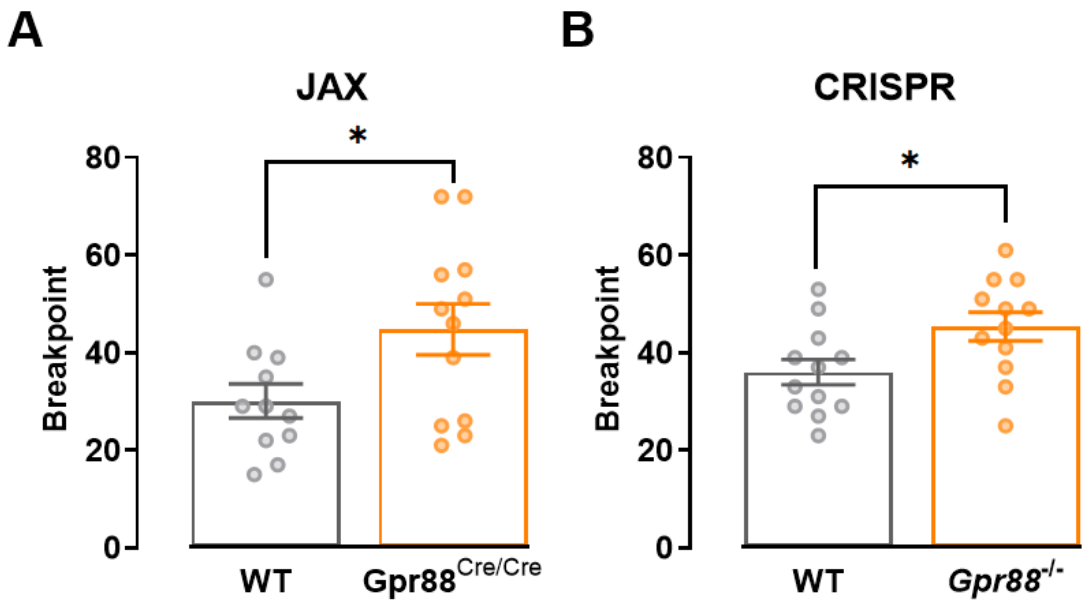


Figure S1. Progressive ratio breakpoint is increased in male **(A)** JAX *Gpr88*$^{Cre/Cre}$ and **(B)** CRISPR *Gpr88*$^{-/-}$ mice. *P<0.05 determined by unpaired t test; n=10-12. Individual data points presented with mean ± SEM.

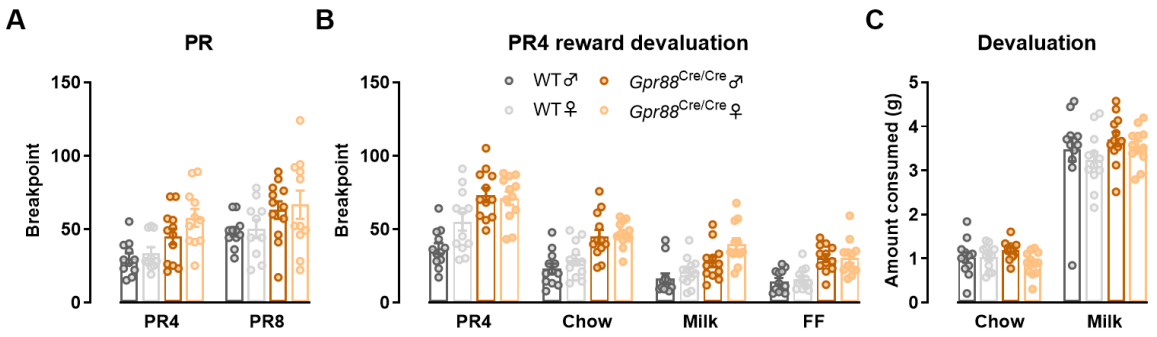


Figure S2. No sex-dependent effects of *Gpr88* deletion are found on **(A)** progressive ratio breakpoint at PR4 or PR8 (RM three-way ANOVA, genotype x sex P=0.5112), **(B)** PR4 breakpoint following reward devaluation (RM three-way ANOVA, genotype x sex P=0.4386) or **(C)** reward consumption during devaluation (RM three-way ANOVA, genotype x sex P=0.6412). Individual data points presented with mean ± SEM; n=10-12.

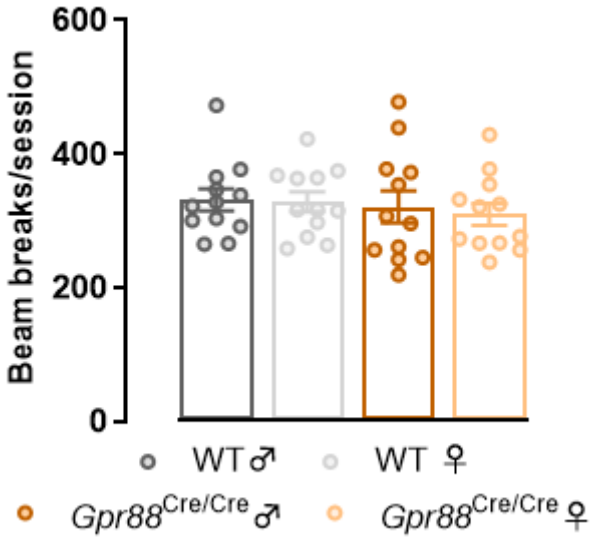
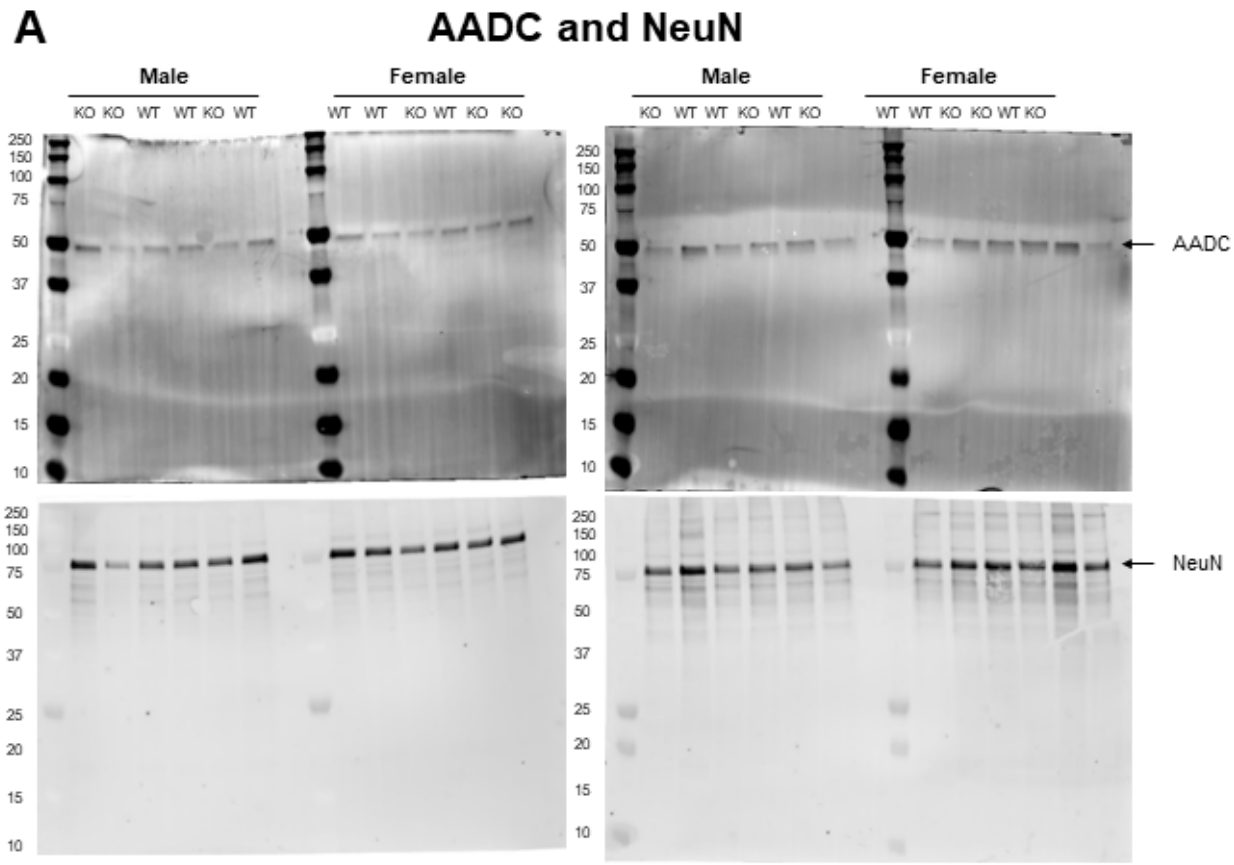
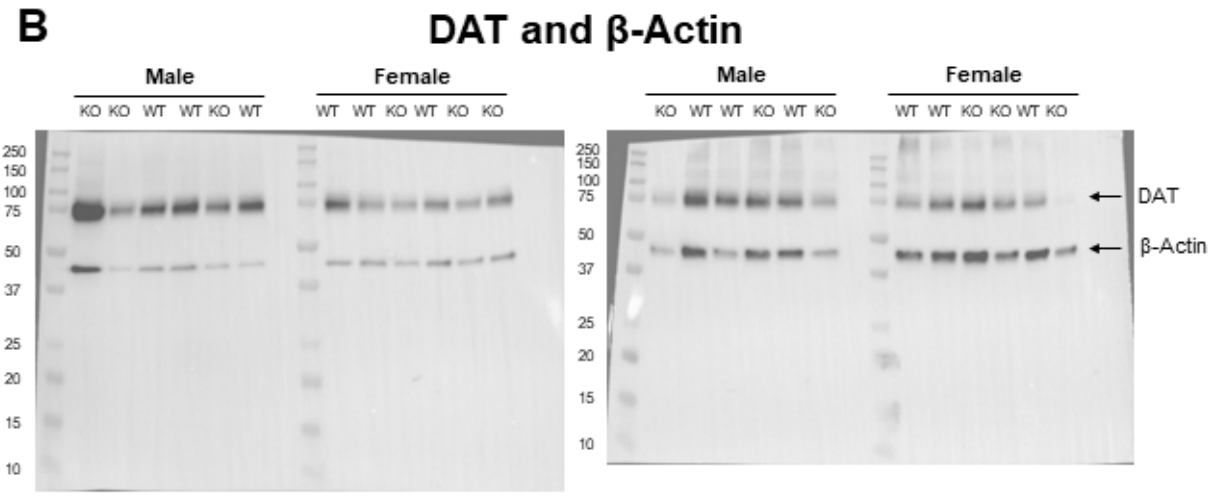
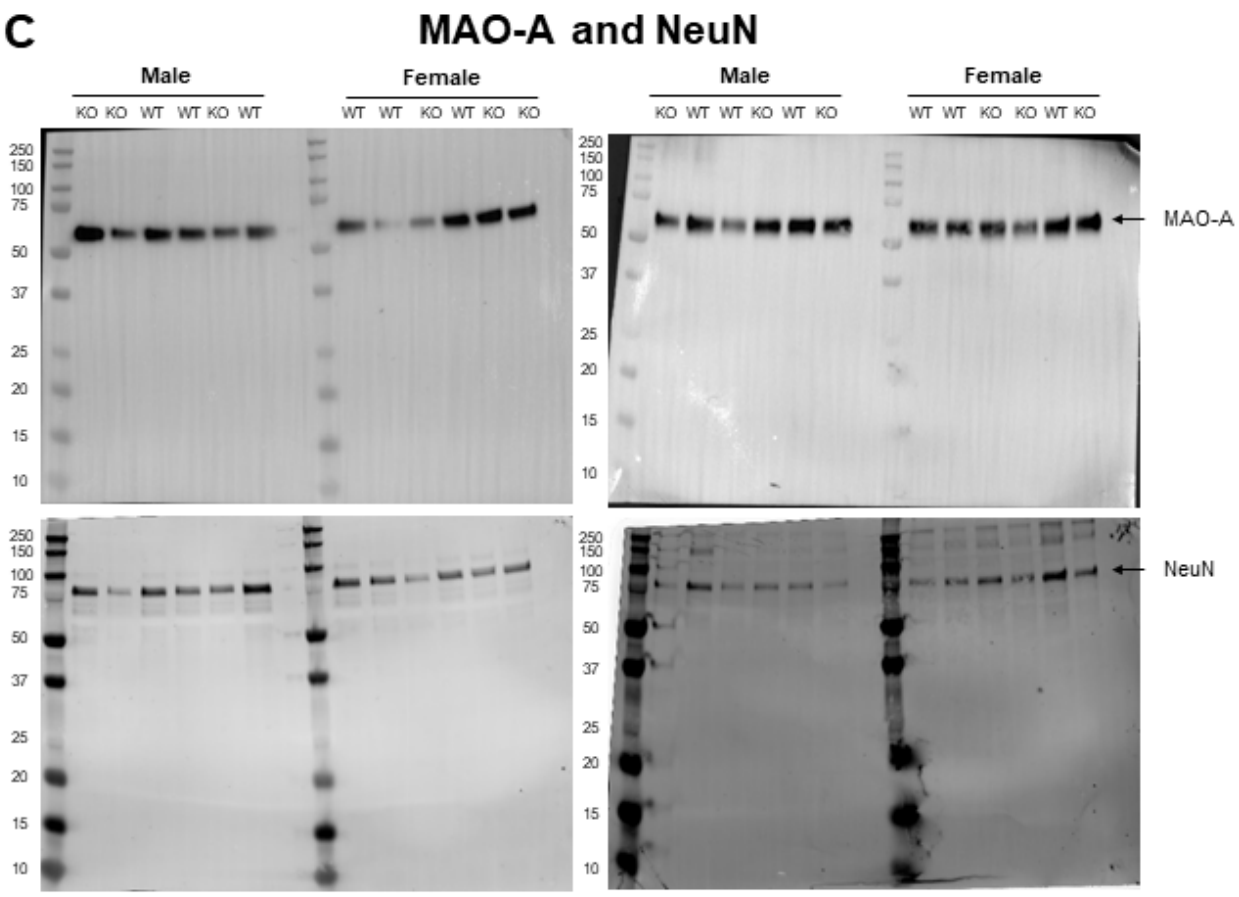
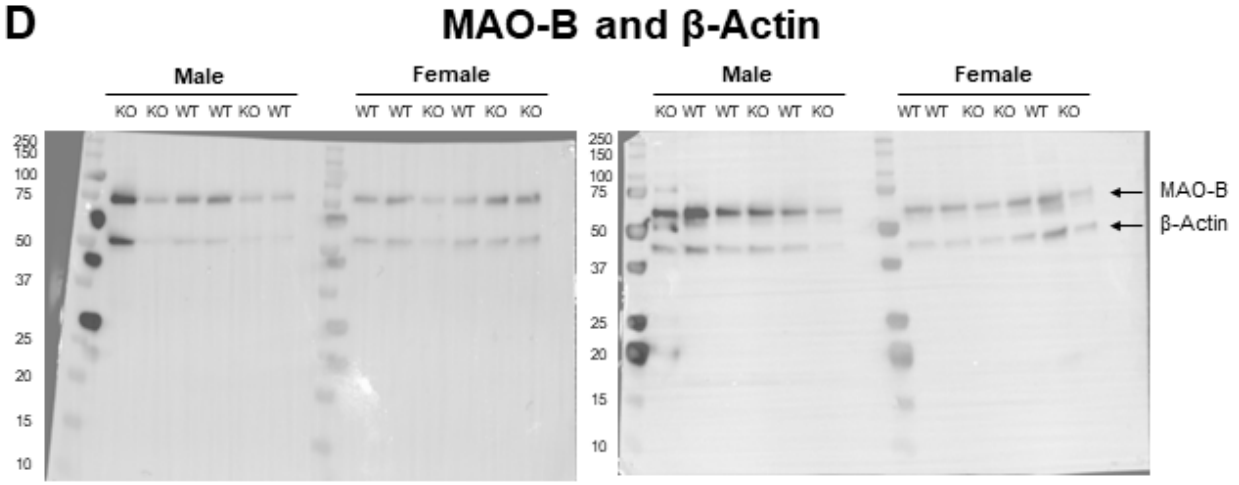


Figure S3. *Gpr88* knockout mice are not hyperactive during touchscreen habituation sessions (two-way ANOVA, genotype P=0.4301). Average IR beam breaks from two habituation sessions. Individual data points presented with mean ± SEM; n=12.


A
AADC and NeuN
Male
Female
KO KO WT WT KO WT
WT WT KO WT KO KO
250
150
100
75
50
37
25
20
15
10
AADC
NeuN


B
DAT and β-Actin
Male
Female
KO KO WT WT KO WT
WT WT KO WT KO KO
250
150
100
75
50
37
25
20
15
10
DAT
β-Actin


C
MAO-A and NeuN
Male
Female
Male
Female
KO KO WT WT KO WT
WT WT KO WT KO KO
KO WT WT KO WT KO
WT WT KO KO WT KO
250
150
100
75
50
37
25
20
15
10
MAO-A
NeuN


D
MAO-B and β-Actin
Male
Female
Male
Female
KO KO WT WT KO WT
WT WT KO WT KO KO
KO WT WT KO WT KO
WT WT KO KO WT KO
250
150
100
75
50
37
25
20
15
10
MAO-B
β-Actin

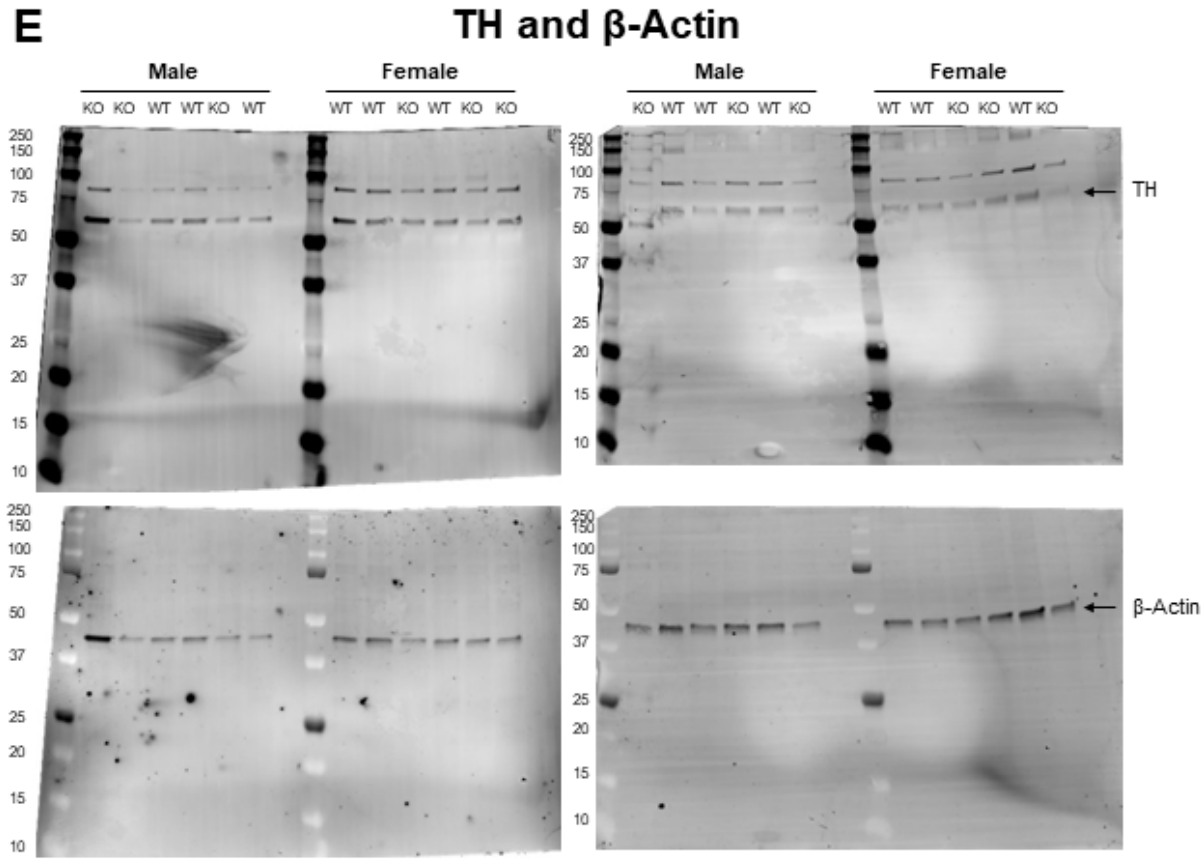


Figure S4. Western blot images from protein quantification of **(A)** AADC, **(B)** DAT, **(C)** MAO-A, **(D)** MAO-B, and **(E)** TH. Separate images of the same gel are shown where secondary antibodies for the protein of interest and loading control are different (AADC, MAO-A, TH).

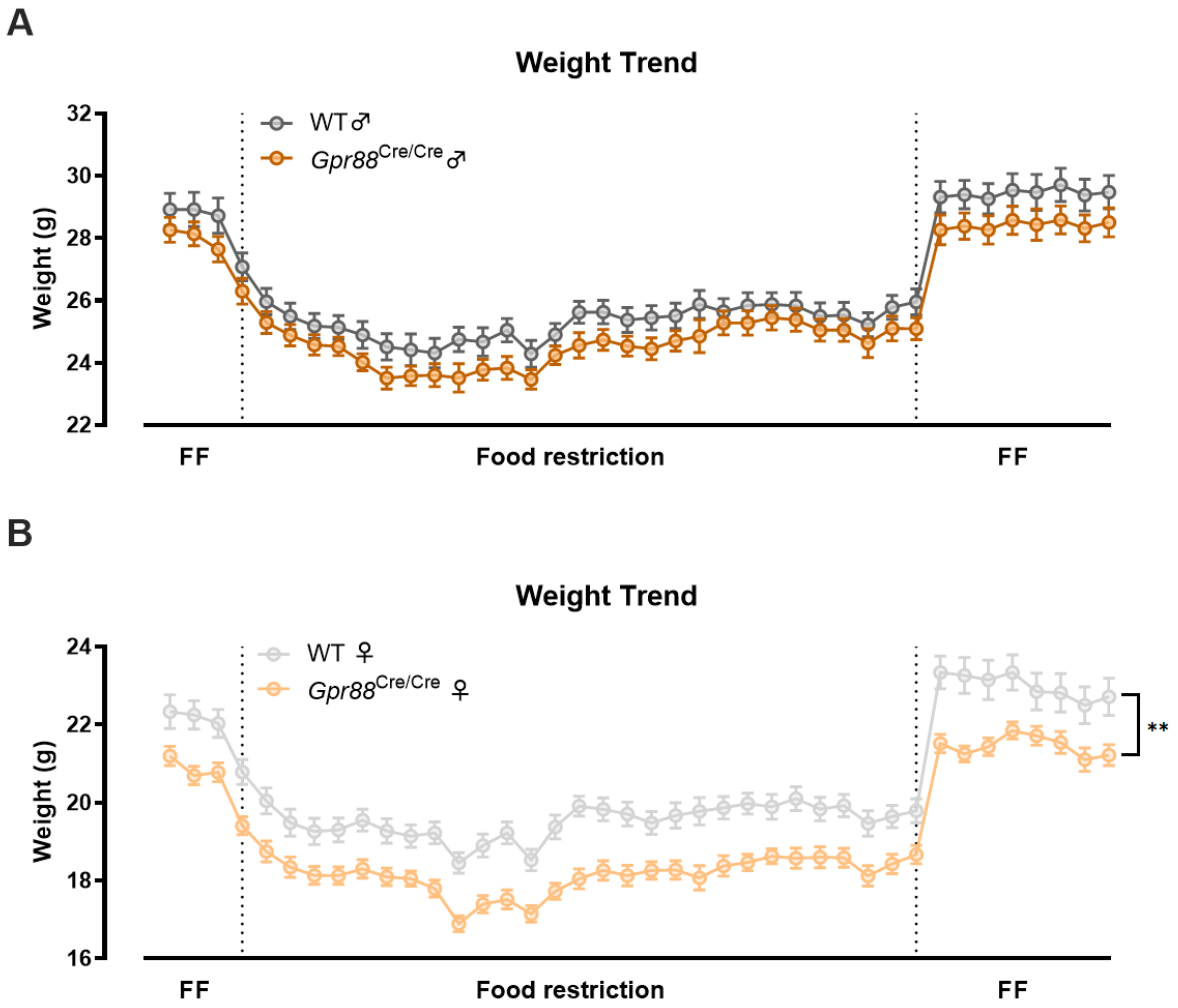


Figure S5. (A) Female *Gpr88*$^{Cre/Cre}$ mice have significantly lower body weight than WT mice across free-feeding and food restriction conditions in Experiment 2 (RM two-way ANOVA, genotype P=0.0010), while **(B)** male *Gpr88*$^{Cre/Cre}$ and WT mice do not significantly differ (RM two-way ANOVA, genotype P=0.1514). Individual data points presented with mean ± SEM; n=12.

Supplementary References

1. Horner AE, Heath CJ, Hvoslef-Eide M, Kent BA, Kim CH, Nilsson SRO, *et al.* (2013): The touchscreen operant platform for testing learning and memory in rats and mice. *Nat Protoc* 8: 1961–1984.

2. Heath CJ, Bussey TJ, Saksida LM (2015): Motivational assessment of mice using the touchscreen operant testing system: effects of dopaminergic drugs. *Psychopharmacology (Berl)* 232: 4043–4057.