Supplementary Methods

Chronic Social Defeat/Overcrowding Procedure

The chronic stress protocol started after 2 weeks of single housing, animals were daily handled and weighted. Prior to the first stress day, all mice were weighted and based on body weight, and were balanced and randomly assigned to either the control group or chronic unpredictable stress group (n = 10 each). Pre-weighed food pellets (2018S Teklad Global 18% Protein Rodent Diet) were placed on the floor of the cage and food intake was recorded daily (precision 0.1 g). All CD-1 mice were screened for aggressive behavior on 3 individual days as described.[1] Briefly, the CD-1 mouse was exposed to a training male C57BL/6J mouse (n = 8) until the first attack followed by submissive behavior of the defeated mouse occurred for a maximum of 10 minutes, those mice with the shortest attack latencies were included for the social defeat procedure. For each social defeat session, stress mice were pseudo-randomly assigned to a different aggressor-CD-1 mouse. For the CD-1 aggressor cage, existing food was removed and bedding was replaced with a clean one before introduction of an experimental animal. During the social defeat procedure, an interaction between experimental and aggressor mice was permitted until the first aggression occurred. Mice were then separated for 2 hours by a perforated plexiglass wall to allow visual, auditory and olfactory but not physical contact, and each mouse received a food pellet. Subsequently, the separator was removed and, after another defeat occurred. Stress mice were transferred back to their home cage. Social defeat sessions were carried out once daily (on days 1, 2, 4-7, 9, 12, 17, and 19) or twice daily (on days 8 and 16). Timing of daily sessions varied, most sessions were carried out across the light cycle and one session (on day 9) starting at the onset of the dark cycle. The stress protocol included overcrowding sessions: 8 stressed mice were placed into a standard holding cage for 24 hours (on days 3, 10, and 18) or 48 hours (on day 13). Mice were observed in intervals during overcrowding sessions and dominance or fighting behavior did not occur during these sessions. The behavior observed in mice in this situation was that all mice grouped in one corner of the cage. Last defeat session of the stress protocol was carried out on day 19 and started at 12 PM.

Social Interaction Test

Twenty-four hours after the last defeat session, the social avoidance test was carried out. The test mouse was placed into a plastic box (31 × 39 cm^2) containing an empty ("no target") wired mesh cage (9.5 × 7.5 cm^2) and allowed to roam around for 2.5 minutes. Then, the mouse was removed and transferred back to its home cage for 1 minute, while an unfamiliar CD-1 mouse ("target") was placed into the mesh cage. After the inter-trial-interval the test mouse was placed back into the social interaction box with the wire-mesh contained a CD-1 aggressive mouse and the time that the test mouse spent in its proximity was measured for another 2.5 minutes. At the end of the test, both mice were transferred back to their home cages and the arena and mesh cage were cleaned with 70% ethanol. All testing was carried out between 9 AM and 11 AM and was carried out under red light to reduce interference of general anxiety-like behavior with social interaction behavior. Trials were recorded by a camera mounted on the ceiling above the box and tracked and evaluated using Ethovision 3.1 (Noldus, Netherlands). Changes in interaction activity were measured by comparing the time spent in the interaction zone (area around the mesh cage) in the presence (CD-1 mouse) and the absence (empty mesh cage) of a social target, results obtained are expressed as a percentage.

Corticosterone Assay

Mice were taken from their cage and rapidly euthanized by decapitation 2 hours after the last stress session. No anesthesia was used to prevent interference with the veracity of biological samples collected. Trunk blood was collected in EDTA tubes, subsequently, placed on ice and centrifuged at 3000 g for 15 minutes at 4℃ to obtain plasma. Plasma was stored at −80℃ until analysis. Briefly, frozen plasma was thawed to room temperature and diluted 40-fold in the steroid displacement reagent and assay buffer. Samples were placed in the plate and blue conjugate solution and yellow antibody solutions were added to each well. Plates were incubated at room temperature with rotation for 2 hours. Wells were emptied and washed 3 times with the wash solution, p-nitrophenyl phosphate buffer was then added to each well and samples were incubated for an additional hour. Reactions were stopped with the addition of the stop solution and absorbance of samples at 455 nm were measured on an iMark Plate Reader (Bio-Rad, Hercules, CA, USA). A standard curve of known concentrations of corticosterone was used to convert the average absorbance of each sample to serum concentration of corticosterone (ng; mL).

Monamines Measures

In brief, full thickness colon and terminal ileum tissues were collected, weighed and placed in 1.5-mL tubes, which contained 500 mL of chilled homogenizing high-performance liquid chromatography (HPLC) buffer (0.1 M citric acid, 0.1 M sodium dihydrogen phosphate monohydrate, 5.6 mM octane sulphonic acid, 10 μM EDTA in 10% [v/v] methanol solution, pH 2.8 with n-methyl serotonin as internal standard). Each homogenate were further disrupted by a short sonication (Sonopuls HD 2070, Bandelin, Germany) during 6-8 seconds until suspended in the tube. The samples were then centrifuged at 14 000 g for 15 minutes at 4℃, and the supernatant was stored at −80℃ until the analysis. The sample supernatants were used for measuring the tissue monoamine content. Sample supernatants were diluted 1:10 in mobile phase immediately prior to analysis on the HPLC system. 20 μL of the diluted supernatant was injected onto the HPLC system, which consisted of a SCL-10Avp system controller, LC-10AS pump, SIL-10A auto-injecter, CTO-10A oven, and an LECD 6A electrochemical detector. A reverse phase column (Kinetex, 2.6u C18 100 × 4.6 mm; Phenomenex, UK) was utilized to facilitate separation with a flow rate set at 0.9 mL/min. Chromatograms were generated using Class-VP 5 software. Analyte:internal standard peak height ratios were measured and compared with standard injections, and results were expressed as ng of neurotransmitter per gram of tissue.[2]

Fecal Output and Water Content

Briefly, fecal pellets were collected from each mouse cages at 15-minute intervals during dark phase (under red light, between 4:30 PM and 6 PM) in order to minimize the risk for water evaporation and coprophagia. Pellets were counted and weighed (wet weight, in mg), then placed into 50℃ oven (16 hours) and weighed again (dry weight, in mg). Fluid content was calculated as follows: fluid content (%) = 100 (wet weight - dry weight)/wet weight.

Whole Intestinal Transit

Mice from cohort 1 (n = 20) were gavaged with 0.3 mL of the carmine solution 2 hours after the last stress session (between 3:35 PM and 3:50 PM). Carmine red (Sigma-Aldrich, Wicklow, Ireland) was prepared as a 6% (w/v) solution in 0.5% methylcellulose (Sigma-Aldrich). After administration of the solution, mice were returned without food deprivation to their individual white bedding cage to facilitate detection of carmine in feces. Feces were collected under red light during dark cycle every 10 minutes after dye administration. GI transit time was considered as the time period between gavage and first observance of red fecal pellet.

Cecum Transepithelial Ion Transport Ex Vivo

The cecum was removed (n = 6) and place in ice-cold Krebs buffer (1.2 mM NaH_2PO_4, 117 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, and 11 mM glucose). Specimens were mounted in Ussing chambers with exposed tissue area of 0.12 cm^2. Tissue was cut along the mesenteric border, cleaned, and pinned flat to silicon rubber-lined Petri dishes with the Krebs solution. The smooth muscle layer of the tissue was removed using microscissors under a stereomicroscope, paired mucosa–submucosa preparations from each animal were mounted in Ussing chambers (Harvard Apparatus, Kent, UK). The cross-sectional area of the exposed preparation was 0.12 cm^2, both sides maintained in Krebs solution at 37℃ and gassed with 95% O2/5% CO_2. Short-circuit current (Isc, μA/cm^2) was recorded in zero voltage clamp mode; transepithelial electrical resistance (Ω·cm^2) was measured by discharging a 2 mV pulse across the tissue and measuring the change in Isc as described.[3] All measurements were continuously recorded on a computer using LabTrax data acquisition hardware and analysed using DataTrax software (World Precision Instruments, Inc, Sarasota, FL, USA).

Tissues were clamped at 0 mV by applying a short-circuit current (*I*sc, μA/cm^2) using an automatic voltage-clamp apparatus (DVC-1000/EVC-4000; World Precision Instruments, Inc) to measure transepithelial ion transport. Basal *I*sc, peak change in *I*sc (delta (Δ)*I*sc), and transepithelial resistance were reported and calculated using Ohm's law. Tissues were allowed a minimum of 30-minute stabilization and the maximum (Δ)Isc that occurred within every 15 minutes of addition to the tissue of the test reagents or vehicles: apical amiloride (10 μM; dimethyl sulfoxide) to inhibit sodium channel absorption, followed by serosal bethanechol (100 μM; dH_2O) and serosal forskolin (10 μM; dimethyl sulfoxide) to initiate the calcium-mediated and cAMP-mediated ion secretion, respectively.

Western Blot

Two hours after the last stress session mice were sacrificed without anesthesia, and full thickness terminal ileum, proximal and distal colon snap-frozen and stored at −80℃. Homogenized tissues from proximal colon were prepared and total protein was determined using the Quan-iT protein assay kit (Invitrogen, Co Dublin, Ireland). Equal amounts of protein were subjected to electrophoresis on 4-12% gradient gels (NuPAGE, Invitrogen)

and transferred to a polyvinylidene difluoride membrane (Bio Rad, Westmeath, Ireland). Membranes were then incubated with goat anti-GFAP (1:500; Merck Millipore LTD, Cork, Ireland); mouse anti-S100B (1:1000; Sigma-Aldrich); rabbit anti-PGP9.5 (1:1000; Merck Millipore LTD), a general neuronal marker; and mouse anti β-actin (1:15 000; Sigma-Aldrich). Spinal cord lysates were used as a positive control for GFAP, and S100B antibody detection. Inmunoreactivity was detected with Pierce ECL detection reagent (Thermo Scientific, Rockford, IL, USA) and visualized using a luminescent image analyzer (LAS-3000; Fugifilm, Dublin, Ireland). Optical density of the immunoreactive bands was quantified using ImageJ software and normalized to mouse anti-β actin (Sigma-Aldrich).

List of antibodies

Symbol	Name	Supplier
GFAP	Anti-glial fibrillary acidic protein antibody, clone GA5 MAB3402	Chemicon
S100B	Anti-S100 beta protein antibody	Sigma-Aldrich
PGP9.5	Protein gene product 9.5 antibody AB1761	Millipore

Spleen Cytokine Assays

Two hours after the last stress session, mice were sacrificed without anesthesia and the spleens were dissected under sterile conditions and placed in 15 mL culture medium (RPMI 1640; Sigma-Aldrich, Ireland) with 10% FCS. Spleen cells were added to 96-well plates and cultured in a 37℃ incubator with 5% CO_2. Each sample was cultured in triplicate with 1 μg of lipopolysaccharide (LPS, Sigma-Aldrich), phorbol 12-myristate 13-acetate , and lipoteichoic acid or saline (untreated) for 24 hours. Spleen culture supernatant samples were analyzed for IL-6 and TNF-α using custom mouse Multi-spot 96-well plates (MSD, Gaithersburg, MD, USA). Samples were added to pre-coated wells in duplicate and incubated for 2 hours. Wells were then washed using phosphate buffered saline-Tween 20 and incubated with antibody mix (anti-IL-6 and anti-TNF-α antibodies) in diluent for 2 hours. ELISA plates were analyzed using the Sector imager 2400 from Meso Scale Discovery. This is an ultra-sensitive method, which has a detection limit for IL-6 of 0.3 pg/mL and TNF-α of 0.3 pg/mL.

Quantitative Real-time Polymerase Chain Reaction

Twenty-four hours after the last stress session, mice were sacrificed without anesthesia and the dissected terminal ileum, proximal and distal colon snap-frozen and stored at −80℃. RNA was extracted using the Qiagen RNeasy Lipid Mini Kit (QIAGEN, Valencia, CA, USA). DNase treatment subsequently removed any DNA and was carried out using the Ambion Turbo DNase kit (Ambion, Warrington, UK) according to manufacturer's instructions. RNA was quantified using NanoDrop spectrophotometer according to the manufacturer's instructions. RNA quality was assessed using the Agilent Bioanalyzer (Agilent, Stockport, UK) according to the manufacturer's procedure and an RNA integrity number was calculated. RNA with RNA integrity number value > 7 was used for subsequent experiments. RNA was reverse transcribed to cDNA using the Applied Biosystems High Capacity cDNA kit (Applied Biosystems, Warrington, UK) according to manufacturer's instructions. Briefly, Multiscribe Reverse Transcriptase (50 U/μL) was added as part of Reverse Transcription master mix, incubated for 25℃ for 10 minutes, 37℃ for 2 hours, 85℃ for 5 minutes and stored at 4℃. Quantitative polymerase chain reaction was carried out using probes (6-carboxy fluorescein) designed by Applied Biosystems to mouse specific probes listed below while using β-actin as an endogenous control. Amplification reactions contained 2 μL cDNA, 5 μL of the 2× PCR Master mix (Roche, Basel, Switzerland), 900 nM of each primer and were brought to a total of 10 μL by the addition of RNase-free water. All reactions were performed in duplicate using 384-well plates on the LightCycler480 System. Thermal cycling conditions were as recommended by the manufacturer (Roche) for 55 cycles. To check for amplicon contamination, each run contained no template controls in triplicate for each probe used. Cycle threshold (Ct) values were recorded. Data was normalized using β-actin and transformed using the $2^{-\Delta Ct}$ method.

Primers used for Taqman protocols

Encoding gene	Primer reference
Th	Mm00447557_m1
Gfap	Mm01253034_m1
Gdnf	Mm00599849_m1
Htr4	Mm00434129_m1
Bdnf	Mm04230607_s1
Slc6a4	Mm00439397_m1
Tlr4	Mm00445273_m1
Tlr2	Mm00442346_m1
β-Actin	Mm00607939_s1
p11	Mm00501457_m1

References

1. Berton O, McClung CA, Dileone RJ, et al. Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. Science 2006;311:864-868.

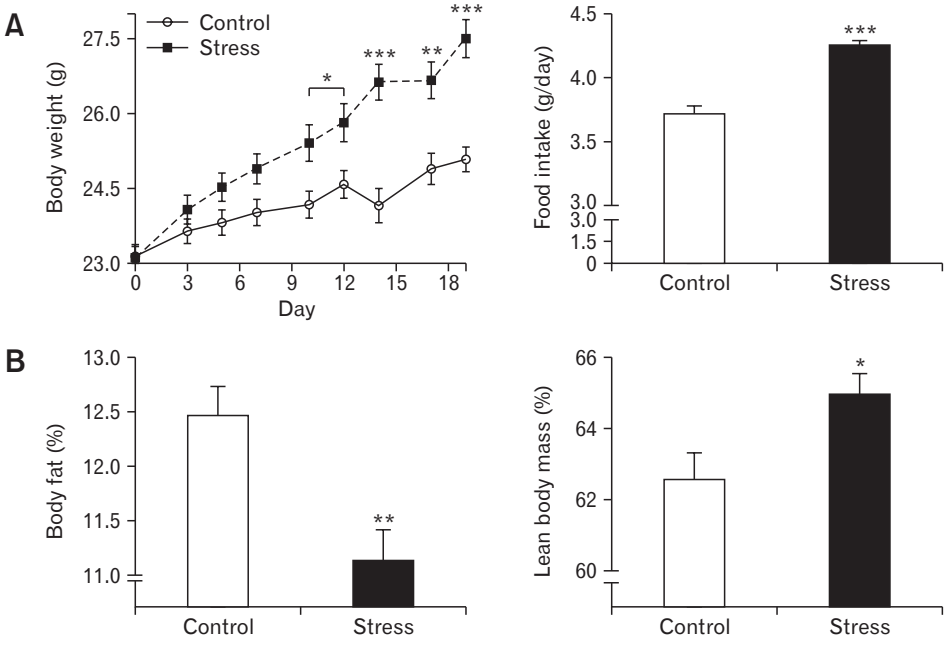
2. Julio-Pieper M, O'Mahony CM, Clarke G, Bravo JA, Dinan TG, Cryan JF. Chronic stress-induced alterations in mouse colonic 5-HT and defecation responses are strain dependent. Stress 2012;15:218-226.

3. Golubeva AV, Crampton S, Desbonnet L, et al. Prenatal stress-induced alterations in major physiological systems correlate with gut microbiota composition in adulthood. Psychoneuroendocrinology 2015;60:58-74.

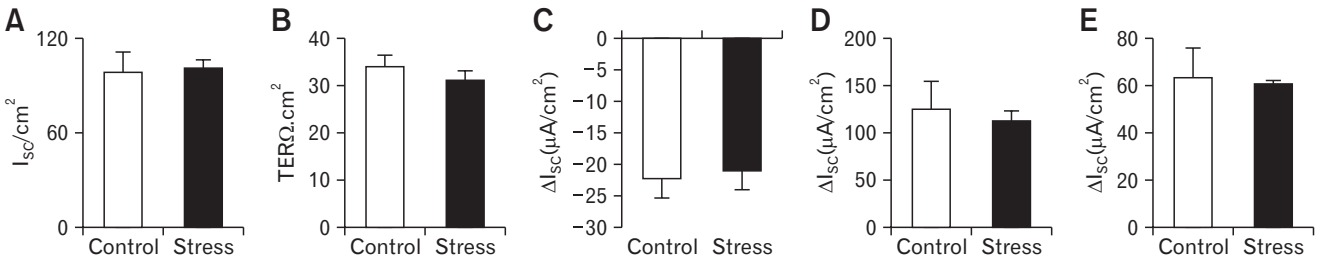
Supplementary Table. Comparison of Cytokine Levels Between Groups After Lipopolysaccharide Spleen Stimulation (n = 6/group)

Cytokine	Control	Stress
IFN-γ	0.8 ± 0.3	38.1 ± 14.4
IL-12	0.1 ± 2.0	7.2 ± 1.8[a]
IL-10	153.8 ± 39.3	177.4 ± 23.4
IL-6	272.0 ± 68.9	1056 ± 166.7[b]
TNF-α	31.0 ± 18.9	179.0 ± 58.0[a]
IL-1β	5.3 ± 1.8	12.5 ± 3.5
mKC	6.6 ± 3.5	27.9 ± 7.2[b]

[a]$P < 0.02$.
[b]$P < 0.01$ by Mann Whitney test.
IFN-γ, interferon gamma; IL, interleukin; TNF-α, tumor necrosis factor; mKC, mouse keratinocyte-derived.
Data represent mean ± SEM.



Supplementary Figure 1. Social defeat (SD)/overcrowding (OC) procedure induces changes in metabolism. During procedure for each mice body weight and food intake were monitored daily. (A) Exposure to chronic stress caused an increase in body weight over the procedure, and an increase in food intake (n = 20/group). (B) Chronic stress changes body composition and causes a decrease in the percentage of body-fat mass, an increase in the percentage of lean mass in a sub-group of animals (n = 10/group). Data represent mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by 2-way analysis of variance with Bonferroni post-test or (A) and by *t* test (B).



Supplementary Figure 2. No changes on ex vivo cecum ion transport following chronic psychological stress. Two adjacent cecum specimens were dissected, mucosal submucosal segments from each stress and control mice (n = 6/group) were mounted in Ussing chambers. (A) Baseline short-circuit current (Isc) values were comparable between groups. (B) Epithelial barrier integrity assessed using transepithelial resistance (TER) was not affected following chronic stress. No significant differences in Isc response to: (C) amiloride, (D) bethanechol, and (E) forskolin, was similar in both experimental groups. Data represent mean ± SEM. Intergroup comparisons were performed by *t* test.