Supplementary Information for

Galanin receptor 3 attenuates inflammation and influences the gut microbiota in an experimental murine colitis model.

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Supplementary methods

Experimental animals

Mice with an early termination mutation in the intron of the GAL-R gene on the C57BL/6J background were kindly provided by Marina Picciotto’s lab (Yale University, New Haven, CT, USA) [1]. GAL-R-KO mice were initially obtained from the European Mouse Mutant Archive (EMMA). They were generated by homologous recombination targeting both coding exons. The mouse line was backcrossed to the C57BL/6N lineage for at least 7 additional generations. The mouse line underwent a thorough phenotypical characterization in our lab [2].

WT and KO animals used in this study were bred at the animal facility of the Paracelsus Medical University, Salzburg, Austria, and originated from homozygous breeders. To avoid genetic drift, all genotypes were crossed back twice to the C57BL/6N or J background (Charles River, Germany) every 8-10 generations. All animals were genotyped before being used in experiments as described previously [2, 3]. At the age of about 6 weeks, experimental animals were transferred to the animal facility of the Medical University of Graz, Austria, where the experiments were conducted. Before the start of the experiments, the mice were allowed to habituate to the animal facility for at least one week.

Evaluation of disease-related parameters and sample collection

On treatment day 7, a disease activity score (DAS) was calculated, which combined the scores of three parameters: weight difference between start and end of DSS treatment (0 - weight increase ≥ 1 g, 1 - weight increase < 1 g, 2 - weight decrease < 1 g, 3 - weight decrease ≥ 1 g), stool consistency (0 - normal stool, 1 - soft but formed stool, 2 - loose stool), and presence of blood in feces and/or the perianal region (0 - no trace of blood, 1 - positive hemoccult test, 2 - trace of blood or bloody perianal region) [4, 5]. Presence of blood in feces was determined with the hemoccult test (Beckman Coulter, Krefeld, Germany).

At the end of the treatment period, unfasted mice were deeply anesthetized with pentobarbital (150 mg/kg i.p.) and blood was collected by cardiac puncture in a syringe containing 100 µl sodium citrate (3.8% w/v) as anticoagulant. Blood was centrifuged at 2,500 g for 10 min at 4°C. Plasma was collected and stored at -70°C until analysis. Mice were euthanized by cervical dislocation and the large intestines, including cecum, colon, and rectum, were dissected and colon length (from end of the cecum to the rectum) was measured. Subsequently, the colon was flushed with ice-cold phosphate-buffered saline (PBS), tapped dry with a paper towel and weighed. The colon was cut into pieces. The most distal part of the colon was fixed in 4% paraformaldehyde for a maximum of 3.5 hours, washed with PBS and then embedded into paraffin (FFPE). Additional pieces were snap-frozen in liquid nitrogen and stored at -70°C. The contents of the cecum were stored at -70°C. The spleen was excised and weighed.

Evaluation of intestinal inflammation

Longitudinal sections (5 µm) of FFPE colon tissue were cut and stained with hematoxylin and eosin (HE). Digital micrographs were taken with a Moticam 5+ camera using Motic Images Plus 2.0 software (Motic, Wetzlar, Germany). Inflammatory alterations in the colon tissue were evaluated independently by a researcher and a pathologist blinded to information on treatment or genotype. Semiquantitative scoring was performed according to Erben et al. [6] and is described in detail in Supplementary Table S2.

Colonic myeloperoxidase content

The tissue levels of MPO were used as an index of inflammation-associated infiltration by neutrophils into the colon tissue. Murine colon samples were homogenized and MPO levels were measured by ELISA (Hycult Biotechnology, Uden, The Netherlands) [7].

Real-time quantitative PCR (qPCR)

RNA was extracted from colon samples using the RNeasy Mini Kit (Qiagen, Hilden, Austria). Two micrograms of RNA were submitted to a reverse transcription reaction using random hexamer primers and Maxima reverse transcriptase (Thermo Fisher, Vienna, Austria). In colon, mRNA expression levels of the various members of the galanin system [ppGAL, GAL-R, GAL-R, and GAL-R], inflammatory cytokines [interleukin (IL) 1β, IL-5, IL-6, IL-10, IL-17A, IL-22 and IL-23, interferon γ (IFNγ), transforming growth factor β (TGFβ) and tumor necrosis factor α (TNFα)] and chemokines [chemokine (C-X-C motif) ligand 1 (CXCL1) and chemokine (C-C motif) ligand 2 (CCL2)] were measured in duplicates by qPCR
using the SYBR Green iQ Supermix (Bio-Rad, Vienna, Austria) on a qTower 2.0 system (Analytik Jena, Vienna, Austria). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) served as a housekeeping gene. Specific primer sequences are shown in Supplementary Table S3 and cycling conditions are listed in Supplementary Table S4. Quantification cycles (Cq) >32.0 were rated as negative expression. Relative mRNA expression levels of target genes were calculated relative to expression of HPRT by the ΔΔCq method [2^-ΔΔCq of gene of interest-Cq of HPRT].

**Plasma cytokine levels**

Concentrations of IL-1β, IL-6, IL-10, IL-21, TNFα, IFNγ, CXCL1 and CCL2 were quantified in plasma in duplicates by using the ProcartaPlex™ Multiplex immunoassay (eBioscience, Thermo Fisher, Vienna, Austria) on a Bio-Plex 200 system with Bio-Plex 5.0 Software (Bio-Rad, Hercules, CA, USA). Values below the detection limit were set to zero [5].

**Immunohistochemistry**

For IHC studies, human colon sections were stained with hGAL1R (TX108207, 1:400; Genetex, Irvine, CA, USA), hGAL3R (customized: S4510-1, 1:400; PTG, Manchester, UK), and hGAL5R (TX108163, 1:500; Genetex, Irvine, CA, USA). Antibodies against GALRs were extensively validated, recently [8]. Sections from mouse colon were stained with mNIMP-R14 (ab2557, 1:100, Abcam, Cambridge, UK) as published previously [9]. Primary antibodies were diluted in Dako Antibody Diluent with Background Reducing Components (DAKO, Glostrup, Denmark). FFPE human and mouse colon tissue was sectioned to 4-5 μm, mounted onto SuperFrost Plus Microscope glass slides (Thermo Fisher) and dried at 60°C for 1 h. After deparaffinization and rehydration, heat-induced epitope retrieval was performed with 10 mM Tris-HCl, pH 8, 1 mM EDTA, 0.05% Tween-20 for 40 min at 95°C for human tissue or with 10 mM Tris-HCl, pH 9, 1 mM EDTA at 60°C overnight for murine tissue. Immunohistochemical staining was performed using the Envision+ System-HP (DAB) Kit (DAKO) according to the manufacturer’s instructions, including blocking of endogenous peroxidases. Slides were incubated with primary antibody for 60 min at room temperature (RT) or 40 min at 37°C. After washing with 1x PBS containing 0.5% Tween-20, human tissue was incubated with the Envision+ HRP-labeled polymer (anti-rabbit, second antibody, ready-to-use, DAKO) and murine tissue with rabbit anti-rat immunoglobulins/HRP (P0450, 1:50, Dako) for 30 min at RT. Another washing step was followed by visualization with Envision+ Liquid DAB+ Chromogen (diaminobenzidine solution) for 10 min at RT. Slides were washed in tap water and counterstained in Mayer’s hemalum solution (Merck KGaA, Darmstadt, Germany) for 3-5 min. Slides were briefly rinsed in 3% hydrochloric acid in ethanol and rinsed for 10 min under running tap water. After dehydration with 2-propanol, the slides were incubated in xylene and mounted in Histokit (Karl Hecht GmbH & Co. KG, Sondheim, Germany). Digital micrographs were taken with a Moticam 5+ camera using Motic Images Plus 2.0 software (Motic, Wetzlar, Germany).

For each round of IHC staining (human colon only), appropriate control sections were included as quality control. The cell line SH-SYSY transfected with either human GAL1R or GAL5R was used as a positive control for GAL1R and GAL5R staining, respectively [8]. Human skin sections were used as a positive control for GAL5R staining (positive blood vessels) [9]. Furthermore, as a control, the primary antibody was omitted (human and murine colon).

Quantification of IHC staining in human sections was performed independently by a researcher and a pathologist blinded to information regarding the disease group. Per colon specimen the total number of neutrophil granulocytes and the number of GAL1R- and GAL5R-positive neutrophils were counted in three high power fields (HPF). Neutrophils were determined by their characteristic neutrophil morphology. Quantification of IHC staining in murine colon was performed independently by two researchers blinded to information on treatment and genotype. The number of infiltrated neutrophils was determined by counting NIMP-R14+ cells in the colonic mucosa and submucosa. NIMP-R14+ cells inside blood vessels were disregarded as well as positively stained cells lacking characteristic neutrophil morphology. Up to four sections per mouse were evaluated. Neutrophil numbers were normalized to the total evaluated area of colon mucosa and submucosa. The area was assessed in mm² using Image J software.
Figure S1. Representative images of IHC staining of GAL3R in human colon (case 13, a) and of a negative control with second antibody only staining (b). Blood vessels are GAL3R-positive (a). Scale bar: 50 μm.
Figure S2. Disease-related parameters analyzed on day 7 of DSS treatment in GALαR-KO, GALαR-KO and corresponding WT mice. Colon weight (a), colon length (b), colon weight to length ratio (c) and spleen weight (d), cumulative disease activity score (DAS) (e) and DAS single scores (f-h). Data represent means ± SEM. n=9-11. Data were analyzed with two-way ANOVA, followed by Tukey’s test (main effect treatment: §§§<p0.001; main effect genotype: &p<0.05), or by Kruskal-Wallis-Test followed by Mann-Whitney-U Test, as appropriate (*p<0.05, **p<0.01, ***p<0.001 vs. corresponding control group).
Figure S3. Relative mRNA expression levels of IL-5 (a), IL-10 (b), IL-23 (c) and TGFβ (d) in colon of GAL2R-KO, GAL3R-KO and corresponding WT mice determined relative to the housekeeping gene HPRT. Data represent means ± SEM. n=7-11. Data were analyzed by Kruskal-Wallis test followed by Mann-Whitney U test. *p<0.05, **p<0.01 vs. corresponding controls or as indicated.
Figure S4. Relative mRNA expression levels of pre-pro-galanin (ppGAL, a), GAL1-R (b), GAL2-R (c) and GAL3-R (d) in distal colon of GAL3-R-KO, GAL2-R-KO and corresponding WT mice determined relative to the housekeeping gene HPRT. Data represent means ± SEM. n=8-11. Data were analyzed with two-way ANOVA, followed by Tukey’s test. Main effect treatment: §p<0.05.
**Supplementary tables**

**Supplementary Table S1:** Detailed information on human colon samples.

| Case No. | Age (years) | Sex | Study group       | Reason for endoscopy       |
|----------|-------------|-----|-------------------|---------------------------|
| 1        | 16.6        | m   | Healthy           | Exclusion of celiac disease|
| 2        | 18.0        | m   | Healthy           | Abdominal pain            |
| 3        | 20.9        | f   | Healthy           | Diarrhea                  |
| 4        | 24.6        | f   | Healthy           | Exclusion of Crohn's disease|
| 5        | 18.7        | f   | Healthy           | Chronic obstipation       |
| 6        | 22.5        | f   | Healthy           | Exclusion of Crohn's disease|
| 7        | 10.1        | f   | Healthy           | Abdominal pain            |
| 8        | 17.5        | f   | Healthy           | Abdominal discomfort      |
| 9        | 19.7        | f   | Healthy           | Hematochezia              |
| 10       | 10.9        | m   | Crohn's disease   | Diagnostic endoscopy      |
| 11       | 15.5        | m   | Crohn's disease   | Diagnostic endoscopy      |
| 12       | 18.0        | f   | Crohn's disease   | Diagnostic endoscopy      |
| 13       | 19.1        | m   | Crohn's disease   | Diagnostic endoscopy      |
| 14       | 24.8        | m   | Crohn's disease   | Diagnostic endoscopy      |
| 15       | 26.4        | f   | Crohn's disease   | Diagnostic endoscopy      |
| 16       | 26.7        | m   | Crohn's disease   | Diagnostic endoscopy      |
| 17       | 23.5        | m   | Crohn's disease   | Diagnostic endoscopy      |
| 18       | 12.6        | m   | Crohn's disease   | Diagnostic endoscopy      |
| 19       | 23.9        | m   | Crohn's disease   | Diagnostic endoscopy      |
| 20       | 13.8        | f   | Ulcerative colitis| Diagnostic endoscopy      |
| 21       | 17.7        | m   | Ulcerative colitis| Diagnostic endoscopy      |
| 22       | 20.4        | f   | Ulcerative colitis| Diagnostic endoscopy      |
| 23       | 25.5        | f   | Ulcerative colitis| Diagnostic endoscopy      |
| 24       | 23.6        | m   | Ulcerative colitis| Diagnostic endoscopy      |
Supplementary Table S2: Scoring scheme for DSS-induced colonic inflammation.

**INFLAMMATORY CELL INFILTRATE**

| Severity                        | Extent                  | Score 1 |
|---------------------------------|-------------------------|---------|
| Minimal (<10%)                  |                         | 0       |
| Mild (10-25% / scattered immune cells) | Mucosa                 | 1       |
| Moderate (26-50%)               | Mucosa and submucosa    | 2       |
| Marked (>51% / dense infiltration) | Transmural              | 3       |

**INTESTINAL ARCHITECTURE**

| Epithelial changes | Mucosal architecture                  | Score 2 |
|--------------------|---------------------------------------|---------|
| Intact epithelium  |                                       | 0       |
| Focal erosions     |                                       | 1       |
| Erosions           | ± Focal ulcerations                   | 2       |
|                    | Extended ulcerations ± granulation tissue ± pseudopolyps | 3       |

**TOTAL SCORE**

Sum of score 1 and 2 0-6
**Supplementary Table S3:** Primer sequences for quantification of mRNA levels of inflammatory cytokines and chemokines and galanin system components in mouse colon.

| Gene   | Forward (5’-3’)                     | Reverse (3’-5’)                     |
|--------|-------------------------------------|-------------------------------------|
| CCL2   | AAGCCAGCAGCTCTCTTCTCTCCTACC        | TGAGTACGAGCAGGATGAGTGGG            |
| CXCL1  | TGACCCTTGAAGCTCCCTTTGT            | TCAGAAGCAGCCTGACAGGAGTGGG          |
| ppGAL  | ACCAGGAAGTGTGGTGTGTCCTCC         | TCTAGGTCTCTTGAGGAGTGG              |
| GAL₁R  | CTGCCCTTACTGTCTATCTGC            | TACAAGGACACACGAGGAGC              |
| GAL₂R  | TCTGGCTGGACAGGTATCTGG          | CTGACTGTAGTACTGACTGAGG             |
| GAL₃R  | GGGCTGCTCGAGGATAGGG        | AGCTTAGGATGAGGCCGGA               |
| HPRT¹  | GTCCCAGCAGCTGTCAGTGGG            | GAGCAAGCTTTTACGCTCTGTCC           |
| IL-1β  | AAAGACGGGCACACACCACTCTA         | CCAGTGGGGAATACTCTTGAGAC           |
| IL-5   | AGCATGAGGCCCTCTGCTCCCT          | CATTTCACAGTACCCCCACGG             |
| IL-6   | CCGGAGAAGGAGACCTCACAGG         | TCTGCAAGTGACATCAGTTGT             |
| IL-10  | ACAGGCGGGAAGGACATAACT          | TTCCGATAAGGCTGCAAC                |
| IL-17A | ACCGGTTCAGCTGCACCTCTG          | GTCCAGTTCTCCCTCCGAA              |
| IL-22  | AGGTGTGTGCTCTCTCTGACCA         | GCGAGTCCAGTCCCCAACGT             |
| IL-23  | CCAGCGGAGACATGATCAGCTCTAA      | TGAAGCGAGAATGGCTGGTT             |
| IFNγ   | TAGCTCTGAGCAATGAACG         | CACATCTATGCACTTGGAGT             |
| TGFβ   | ACCCTGGTAAAGGGGTGCTGA         | GCGCTGAATCGAAAGGCCCTGT            |
| TNFα   | GGTCCCCAAAGGGATGAGAA        | TCTAGCCACTCCAGCTGCTC             |

Legend:

¹HPRT served as the housekeeping gene.
**Supplementary Table S4:** Cycling conditions used in quantitative real-time PCR analysis.

| Gene          | Cycling conditions                                                                 |
|---------------|------------------------------------------------------------------------------------|
| **Standard program**¹ | 95°C, 3 min<br>(95°C, 15 sec; 64°C, 30 sec; 72°C 10 sec) 40x<br>95°C, 1 min<br>70°C, 1 min<br>Melt curve: 75>95°C, 5 sec per 0.5°C |
| **GAL₃R**     | 95°C, 3 min<br>(97°C, 15 sec; 63°C, 30 sec; 72°C 10 sec) 45x<br>95°C, 1 min<br>70°C, 1 min<br>Melt curve: 75>97°C, 5 sec per 0.5°C |

Legend:

¹The standard program is used for mRNA analysis of all gene targets listed in Supplementary table S3. Gene targets with deviating cycling programs are listed separately.
**Supplementary Table S5:** Detailed F statistics to statistical analyses of data sets on body weight, food intake and water intake in GAL3R-KO, GAL3R-KO and corresponding WT mice.

| Dependent variable | Groups | Type of test | Subject effects | Main effects or Interactions | df | Error | F value | P value |
|--------------------|--------|--------------|-----------------|-----------------------------|----|-------|---------|---------|
| Body weight        | GAL3R-KO/-WT | 2-way RM\(^1\) ANOVA | Within | D\(^2\) | 2.123 | 82.801 | 31.855  | <0.001  |
| Body weight        | GAL3R-KO/-WT | 2-way RM ANOVA | Within | D\(^2\)G\(^3\) | 2.123 | 82.801 | 4.622   | 0.011   |
| Body weight        | GAL3R-KO/-WT | 2-way RM ANOVA | Within | D\(^2\)T\(^4\) | 2.123 | 82.801 | 37.987  | <0.001  |
| Body weight        | GAL3R-KO/-WT | 2-way ANOVA, day 4 | - | G\(^*\)T | 1 | 39 | 4.518  | 0.040   |
| Body weight        | GAL3R-KO/-WT | 2-way ANOVA, day 5 | - | G\(^*\)T | 1 | 39 | 5.384  | 0.026   |
| Body weight        | GAL3R-KO/-WT | 2-way ANOVA, day 6 | - | G\(^*\)T | 1 | 39 | 7.938  | 0.008   |
| Body weight        | GAL3R-KO/-WT | 2-way ANOVA, day 7 | - | G\(^*\)T | 1 | 39 | 9.072  | 0.005   |
| Body weight        | GAL3R-KO/-WT | 2-way RM ANOVA | Within | D | 2.190 | 78.827 | 10.719 | <0.001  |
| Body weight        | GAL3R-KO/-WT | 2-way RM ANOVA | Within | D\(^*\)T | 2.190 | 78.827 | 16.028 | <0.001  |
| Body weight        | GAL3R-KO/-WT | 2-way RM ANOVA | Between | T | 1 | 36 | 5.286 | 0.027   |
| Body weight        | GAL3R-KO/-WT, controls | 1-way RM ANOVA | Within | D | 2.475 | 47.027 | 3.398 | 0.033   |
| Body weight        | GAL3R-KO/-WT, DSS-treated | 1-way RM ANOVA | Within | D | 1.771 | 30.100 | 19.314 | <0.001  |
| Food intake        | GAL3R-KO/-WT | 2-way RM ANOVA | Within | D | 1.696 | 27.136 | 56.293 | <0.001  |
| Food intake        | GAL3R-KO/-WT | 2-way RM ANOVA | Within | D\(^*\)T | 1.696 | 27.136 | 8.054  | 0.003   |
| Food intake        | GAL3R-KO/-WT, controls | 1-way RM ANOVA | Within | D | 2.340 | 18.717 | 86.570 | <0.001  |
| Food intake        | GAL3R-KO/-WT, DSS-treated | 1-way RM ANOVA | Within | D | 1.464 | 11.710 | 21.058 | <0.001  |
| Food intake        | GAL3R-KO/-WT | 2-way RM ANOVA | Within | D | 2.678 | 37.493 | 10.256 | <0.001  |
| Food intake        | GAL3R-KO/-WT | 2-way RM ANOVA | Within | D\(^*\)T | 2.678 | 37.493 | 7.280  | 0.001   |
| Food intake        | GAL3R-KO/-WT | 2-way RM ANOVA | Between | T | 1 | 14 | 4.433 | 0.054   |
| Food intake        | GAL3R-KO/-WT, DSS-treated | 1-way RM ANOVA | Within | D | 6 | 42 | 17.536 | <0.001  |
| Dependent variable | Groups | Type of test | Subject effects | Main effects or Interactions | df  | Error  | F value  | P value   |
|-------------------|--------|--------------|-----------------|-----------------------------|-----|--------|----------|-----------|
| Water intake      | GAL3R-KO/-WT | 2-way RM ANOVA | Within          | D                           | 1.712 | 27.389 | 8.218    | 0.002     |
| Water intake      | GAL3R-KO/-WT | 2-way RM ANOVA | Within          | D*T                         | 1.712 | 27.389 | 10.628   | 0.001     |
| Water intake      | GAL3R-KO/-WT, controls | 1-way RM ANOVA | Within          | D                           | 6    | 48     | 8.443    | <0.001    |
| Water intake      | GAL3R-KO/-WT, DSS-treated | 1-way RM ANOVA | Within          | D                           | 1.337 | 10.698 | 9.872    | 0.007     |
| Water intake      | GAL3R-KO/-WT | 2-way RM ANOVA | Within          | D                           | 2.039 | 28.540 | 30.256   | <0.001    |
| Water intake      | GAL3R-KO/-WT | 2-way RM ANOVA | Within          | D*T                         | 2.039 | 28.540 | 6.695    | 0.004     |
| Water intake      | GAL3R-KO/-WT, controls | 1-way RM ANOVA | Within          | D                           | 6    | 42     | 9.935    | <0.001    |
| Water intake      | GAL3R-KO/-WT, DSS-treated | 1-way RM ANOVA | Within          | D                           | 1.710 | 11.967 | 21.654   | <0.001    |

Legend:
1 RM: repeated measures
2 D: Days
3 G: Genotype
4 T: Treatment
SI references

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