Supplemental Methods

Animals. All experiments were approved by the Linköping Animal Care and Use Committee and followed national and international guidelines. All mice used for behavioral experiments were, if not otherwise mentioned, male mice on a C57BL/6 background and weighed 25-33 g. Mice were maintained on a 12 hour light and dark cycle and were provided with food and water ad libitum. The COX-1 knockout mice (Taconic) were generated by insertion of neo-cassette into exon 11 of Ptgs1 (1) and were on a mixed background. Mice with abrogated COX-2 activity (Jackson Laboratories) were generated by a inducing a point mutation leading to a Y385F substitution into exon 8 (2). Mice with cell type specific COX-2 deletions were generated by crossing mice with floxed Ptgs2 (3) with mice expressing Cre recombinase from the nestin promoter (Jackson Laboratories; 003771), the LysM promoter (Jackson Laboratories; 004781) or CreERT2 from the Slco1c promoter (4, 5). Gene deletion in mice with the Slco1c-CreERT2 construct was induced by intraperitoneal injection of tamoxifen (4). Tamoxifen (Sigma; 1 mg/mouse) was injected twice a day for 5 consecutive days. Mice with mPGES1 gene ablation were generated through the removal of part of exon 1 and the entire exon 2 of Ptges gene (6). Mice with global knock out of Ptger1 (EP1R)(7) and Ptger3 (EP3R)(8) were from the laboratory of Beverly H. Koller. Mice with floxed EP3-receptors (9) and mice lacking serotonin transporter were obtained from Jackson laboratories and mice with Cre-expression in serotonergic cells from GENSAT/MMRC, UC Davis, U.S.A. (B6.FVB(Cg)-Tg(Slc6a4-cre)ET33Gsat/Mmucd). The resulting crossings generated mice of mixed C57/129 background.

Drugs. 20µl of diluted formalin (2.5%) was injected below the skin on dorsal side of the hind paw to induce acute inflammatory pain. The COX-2 specific inhibitor parecoxib (Pfizer; 10 mg/kg) was dissolved in saline and administered i.p. 30 minutes before the pain paired training session. The COX-1 specific inhibitor SC560 (Tocris Bioscience; 5 mg/kg) was first dissolved in dimethyl sulfoxide (DMSO) and then in saline keeping the DMSO concentration less than 5%. It was then given i.p. 30 minutes before the training sessions. U-50488 (Tocris Bioscience; 2.5 mg/kg) was diluted in saline and injected i.p.. Lipopolysaccharide (120 microgram/kg, O111:B4, Sigma-Aldrich) was injected i.p.

Injection of viral vectors. Mice were subjected to stereotaxic surgery under isoflurane anesthesia. EP3R floxd mice were injected with 200 nl of AAV5-hSyn-Cre (2,40E+14 gc/ml; Vector Unit, Lund University) mixed with AAV5-EF1α-DIO-eYFP (for visualization of recombination; 3,52E+12 vm/ml; Gene Therapy Center, University of North Carolina) or 200 nl of AAV5-hSyn-GFP (1,82E+14 gc/ml,

(Vector Unit, Lund University). SertCre mice were injected with 250 nl of AAV8-hSyn-DIO-hM3Dq-mCherry (5,7E+12; Gene Therapy Center, University of North Carolina) or 250 nl of AAV8-hSyn-DIO-mCherry (3,8E+12; Gene Therapy Center, University of North Carolina). EP3R-SertCre mice were injected with 250 nl of AAV8-hSyn-DIO-hM4Di-mCherry (5,3E+12 vm/ml; Gene Therapy Center, University of North Carolina) or 250 nl of AAV8-hSyn-DIO-mCherry (3,8E+12; Gene Therapy Center, University of North Carolina). Vector solutions were injected in the DR (coordinates relative to bregma AP: -4,85; ML: 0; DV: -3,45), at the rate of 100 nl/min. The needle was slowly retracted 4 minutes after the injection. Post-surgical pain-relief was induced by buprenorphine (0,1 mg/kg). Behavioral experiments were performed at least 3 weeks after the surgery.

Formalin-induced conditioned place aversion. The experiments were performed with male mice in three chambered place preference boxes 46 (w) x 27 (d) x 25 (h) cm (Spatial Place Preference Box; Panlab). The two larger chambers of the apparatus contained distinct visual cues on the inner walls (diagonal grey stripes versus black dots) and distinct floor colors (grey versus black). The third, smaller chamber without any visual cues connected the two big chambers. Mice were single housed and weighed one day before the pre-test (day 0). In the pre-test (day 1), mice were allowed to freely explore the box for 15 minutes with access to all chambers, and the time spent in the striped and dotted chambers were recorded manually. Mice were excluded after the pre-test if they crossed the corridor less than 5 times or if they spent more than 10 minutes in one chamber. The initially most preferred chamber during pretest was assigned for pain-conditioning to avoid any natural bias. The training was performed on two consecutive days (day 2 and 3) and each day had two sessions, one in the morning (8-12:00) and one in the afternoon (14-18:00). In the morning session, mice were briefly anesthetized with isoflurane and confined to the non-preferred chamber for 60 minutes. In the afternoon sessions, the mice were anesthetized with isoflurane, injected with diluted formalin in the hind paw and confined to the preferred chamber for 60 minutes. In experiments involving neuronal stimulation/inhibition by DREADDs all mice were administered CNO (2 mg/kg, dissolved in saline, ENZO) i.p. 20 min before formalin injection and conditioning. The morning and afternoon sessions were separated by at least 4 hours. The mice were trained on day 3 in the same manner as on day 2 except formalin injection was in the other hind paw. The post-test was performed on day 4 and lasted for 15 minutes. During the test, mice were again allowed to explore the box freely, with access to all chambers. The time spent in both chambers were recorded and the aversion scores were calculated by subtracting the time spent in the pain-paired chamber during the pre-test from the time spent in the same chamber during test. All the experimental sessions (pre-test, training and post-test) were performed under dim illumination (25±5 lux). The experiments were performed in a

blinded manner where the experimenter was unaware about the genotype of the mice and/or the pharmacological solutions. The time-line of the protocol is shown in Supplemental Figure 2F.

Conditioned place aversion induced by thermal pain. A two chamber arena with temperature-regulated floors (Series 8, IITC Life Sciences) was used. The chambers were differentiated by distinctive patterns (checkered or striped). Day 1, a pre-test where the mouse was allowed free access to both chambers during a 15 minute period was performed. The location of the mouse was monitored with the in-built automatic, beam-brake based, detection system or by a video camera mounted above the arena. Mice were excluded if time spent in one chamber exceeded 12 minutes. A preferred chamber was defined based on the result of the pre-test. Conditioning was performed during four consecutive days (day 2-5) with two sessions (10 minute each) per day. During conditioning in the preferred chamber, the floor was 45 ºC and during conditioning in the non-preferred chamber it was 30 ºC. The sessions were performed at least 4 hours apart. Finally (day 6), a test was performed using the same protocol as the pre-test. The aversion scores were calculated by subtracting the time spent in the pain-paired chamber during the pre-test from the time spent in the same chamber during the test. Experiments were performed in a blinded manner.

Scoring nociceptive behaviors. The mice were kept in a transparent plexiglass box 20 (w) x 15 (d) x 25 (h) cm for acclimatization for 30 minutes. Formalin was then injected in the right hind paw and the behavior of the mouse was videotaped for 60 minutes with a Canon LEGRIA HF R48 camera. Nociceptive behaviors such as paw lifting (category I), licking, biting and shaking time (category II) were measured from the video and nociceptive scores were calculated for each 5 minute slot of total 60 minutes with the formula: Nociception score (N.S.) = (time of paw lifting) x1 + (time of paw shaking, biting, and licking) x2 / 300 (10).

Real-time place preference. The two chamber arena described in *Conditioned place aversion induced by thermal pain* was used. Mice were allowed to freely explore both chambers during the whole test (17 minutes). For the first two minutes the floor temperature was set to 30±0,1^{o}C in both compartments. After habituation, the temperature in one of the compartments was increased to 45±0,1^{o}C and the time spent in the two chambers was measured for 15 minutes.

Hot-plate test. The same apparatus with temperature regulated floors was used. Chambers were replaced with a transparent, bottomless plexiglass box. Floor temperature was set to 50±0,1^{o}C. Mice were placed on the heated surface and the latency to the first paw licking or jumping was measured by a blinded observer. Mice were removed from the plate immediately after licking or jumping.

Sucrose preference. Animals were single-housed 5 days before the start of the test. On day 0, two conical tubes with a rubber stopper and a metal sipper spout containing water were introduced to the cage. On day 1, one of the tubes was replaced with a tube containing 1% (w/v) sucrose. Animals were trained to consume sweet solution for 3 days. Each day the position of tubes containing water and sucrose was changed. The fluid intake measurements were taken on the day 5 and 6. Next, sucrose concentration was increased to 5% (w/v) and the intake was measured on day 7 and 8. Fluid consumption was measured by weighting tubes on an electronic scale. Values were corrected for spillage. After each measurement the position of tubes containing water and sucrose was changed and the preference was calculated as a mean from two days.

Body temperature measurements. Mice were implanted with a transmitter (ETA-F10; Data Sciences International) in the peritoneal cavity. The surgery was performed under isoflurane anesthesia and postoperative analgesia was provided (buprenorphine). The mice were allowed to recover for at least one week before the experiment. The experiments were carried out at an elevated temperature (27 - 29°C).

Immunofluorescence. Animals were perfused with saline followed by ice-cold 4% paraformaldehyde. Brains were removed, postfixed in 4% PFA for 4h at 4°C and transferred to 30% sucrose in PBS. When saturated, brains were cut to 40 µm sections on a cryostat. Additional antigen retrieval (2 min in 93°C in Antigen Retrieval Citrate Solution, BioGenex) was performed only on tissue stained with mouse anti-TpH antibody. Free floating sections were incubated for 45 min in blocking solution (1% BSA and 0.3% Triton X-100 in PBS – the same solution was used for diluting antibodies). Sections were then incubated with primary antibodies: sheep anti-TpH (Millipore, cat. No. AB1541, 1:500) and mouse anti-Cre (Millipore, cat. No. MAB3120, 1:1000) or mouse anti-TpH (Sigma, cat. No. TO678, 1:500) and chicken anti-GFP (Abcam, cat. No. ab13970, 1:10 000) or mouse anti-TpH (Sigma, cat. No. TO678, 1:500) and rabbit anti-RFP (MBL, cat. No. PM005, 1:1000) overnight at room temperature. Sections were rinsed 5 times in PBS and incubated with secondary antibodies: donkey anti-mouse Alexa Fluor

488 and donkey anti-sheep Alexa Fluor 568 (both 1:500) or donkey anti-mouse Alexa Fluor 568 and goat anti-chicken Alexa Fluor 488 (both 1:1000) or donkey anti-mouse Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 568 (both 1:1000) for 2 hours. Finally, sections were washed in PBS and mounted on object glasses. Double-labeled images were obtained by sequential scanning.

Quantitative PCR. Mice were sacrificed by cervical dislocation. Dissected brains were placed in RNAlater (Qiagen). After 7h, cerebral cortex (fragments containing anterior cingulate, motor and somatosensory areas), and thalamus together with hypothalamus were dissected and stored in RNAlater overnight at 4^{o}C. The area containing dorsal raphe was dissected after overnight incubation in RNAlater, using a brain matrix and a punch. RNA extraction was performed with RNeasy Lipid Tissue Kit (Qiagen) (cortex and thalamus+hypothalamus) or with RNeasy Mini Kit (Qiagen) (dorsal raphe). cDNA was synthesised with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was performed with Real-Time 7500 Fast apparatus (Applied Biosystems), using TaqMan Gene Expression Master Mix and TaqMan assay targeting the alpha isoform of EP3R (11). GAPDH (TaqMan assay Mm99999915_g1) was used as endogenous control. Relative quantification was done by ΔΔCT method.

Statistics. All the data are expressed as mean ± SEM. Differences between groups were analyzed using two tailed Student's t-tests, except for body temperature measurements which were analyzed with two-way ANOVA. Differences were considered significant when $P < 0.05$ (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Statistical analyses were conducted using GraphPad Prism (version 6.0; GraphPad). The number of mice (n) are specified in Supplemental Figure 1 or the figure legends.

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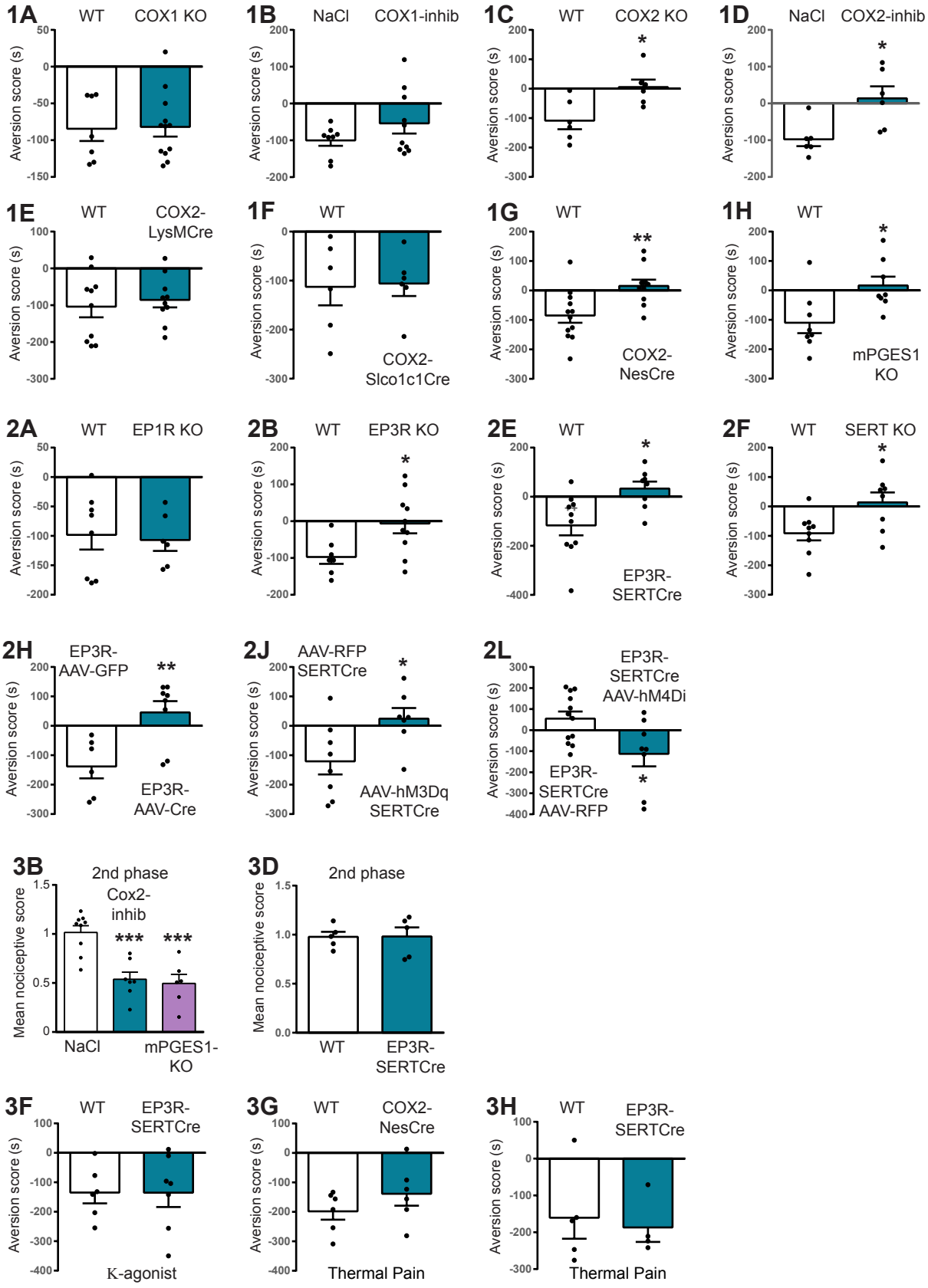
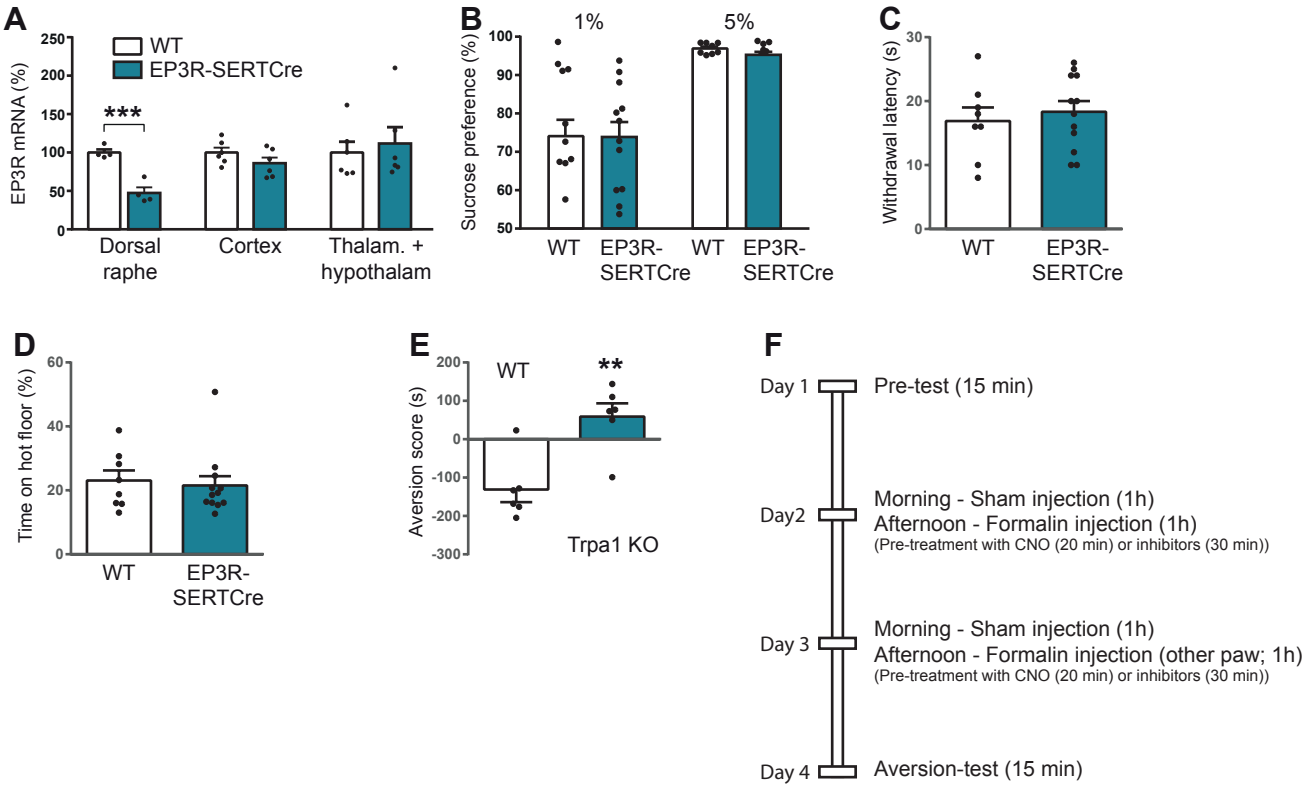


Supplemental Figure 1. Graphs showing the individual values of the mice in the behavioral experiments.



Supplemental Figure 2. **A**. Expression of EP3R-alpha mRNA in the region of the dorsal raphe, the cerebral cortex and the the thalamus/hypothalamus. **B**. Sucrose preference in WT and EP3R-SERTCre mice. **C**. Withdrawal latencies of WT and EP3R-SERTCre mice in the hot-plate test. **D**. Avoidance of a hot floor (45 degrees) compared to a floor of 30 degrees in WT and EP3R-SERTCre mice. **E**. Conditioned place aversion in response to formalin injection in the paw of WT and Trpa1 KO mice. **F**. Time-line of the protocol used to induce conditioned place preference to inflammatory pain.