Supplemental Information for

Reduction of neuropathic and inflammatory pain through inhibition of the tetrahydrobiopterin pathway.

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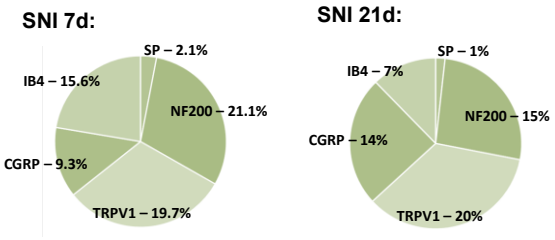
Supplemental Figure 1

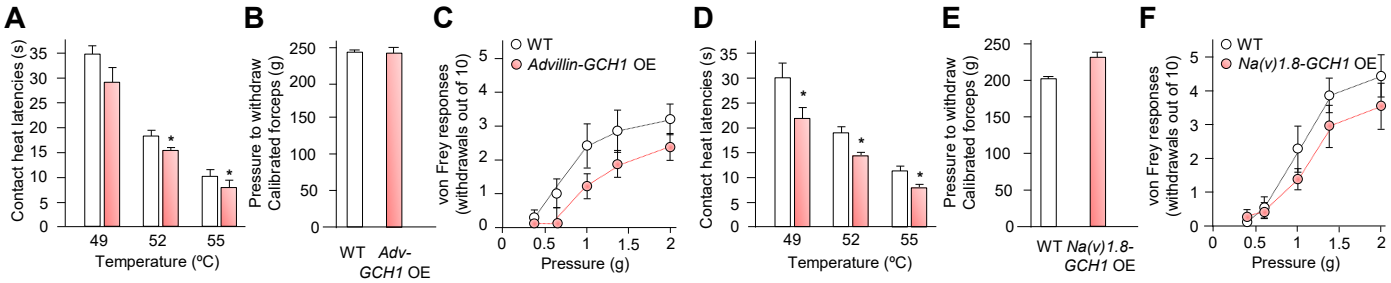
A GCH1-eGFP mice:



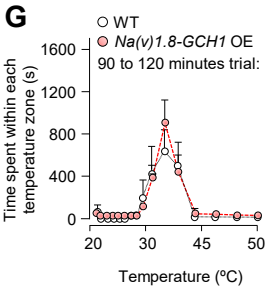
Supplemental Figure 2

Advillin-GCH1-HA OE mice:

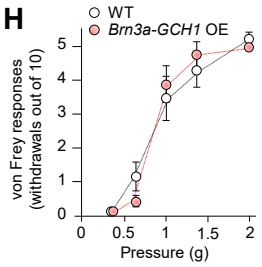
$Na(v)1.8_{Tg}$-*GCH1*-HA OE mice:



Thermal gradient (22ºC to 55ºC) in $Na(v)1.8_{Tg}$-*GCH1*-HA OE mice :

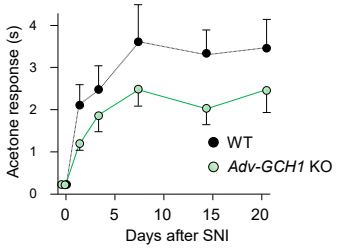


Brn3a-GCH1-HA OE mice:

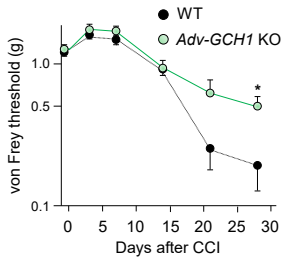


Supplemental Figure 3

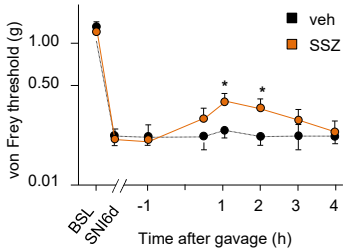
A Response to acetone in *Advillin-Gch1* KO SNI mice:



B Mechanical allodynia in *Advillin-Gch1* KO CCI mice:

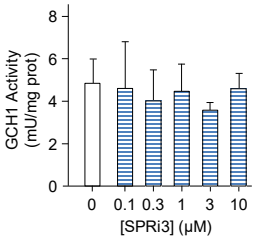


C Sulfasalazine reduces neuropathic pain in mice



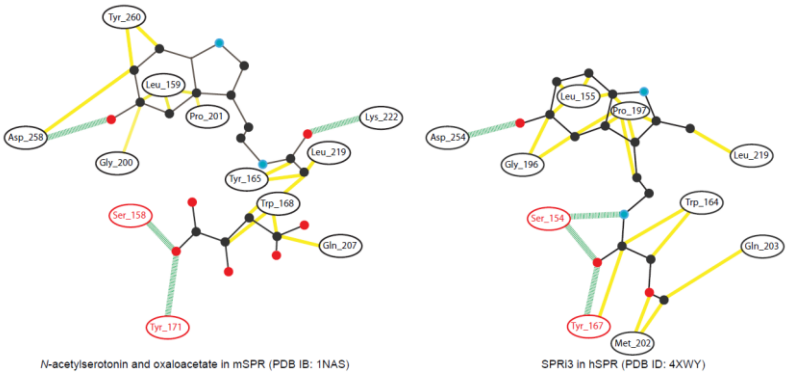
Supplemental Figure 4

A GCH1 activity in SPRi3-treated DRG neurons

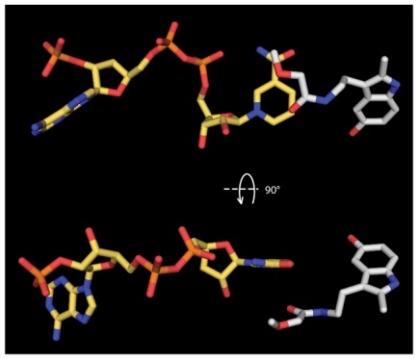


Comparison between NAS and SPRi3 interaction with SPR (A) and of SPRi3 with NADPH (B)

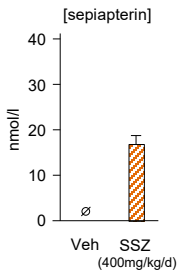
B



C

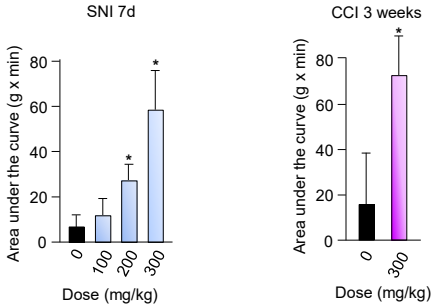


D Plasma sepiapterin levels after SSZ (400 mg/k/d p.o. for 4 days)



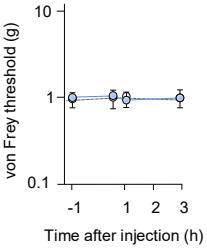
Supplemental Figure 5

A **Areas under the curve after SPRi3 administration 7 days after SNI and 3 weeks after CCI in mice:**



Nociceptive pain after SPRi3 administration (300 mg/kg ip)

B



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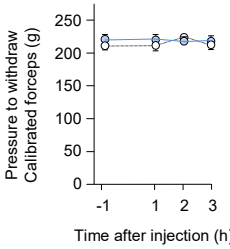

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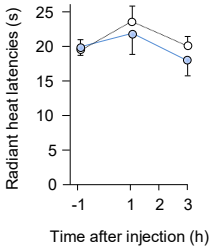
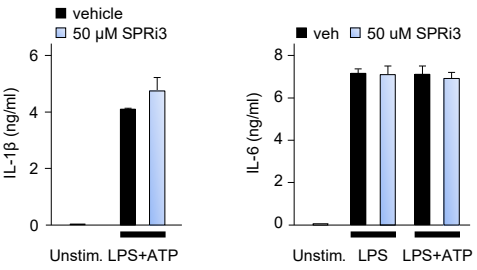


SPRi3 (50 uM) does not change IL6 production by peritoneal macrophages

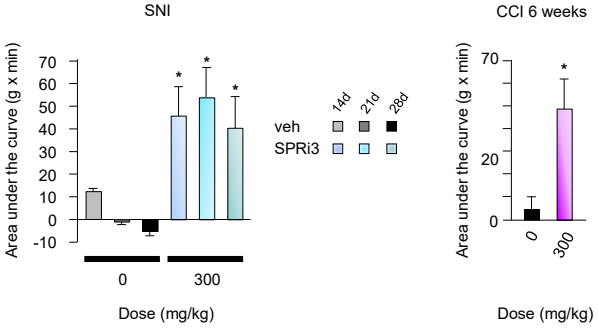
E



Supplemental Figure 6

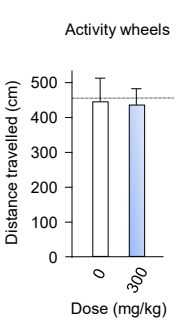
Area under the curve of SPRi3 administered in mice 2, 3 or 4 weeks after SNI and 6 wks after CCI

A



Holeboard , activity wheels and tail suspension after SPRi3 administration (300 mg/kg ip)

B



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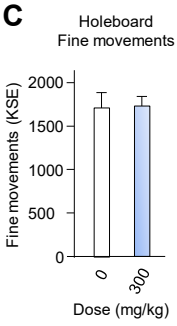

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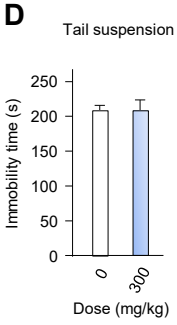
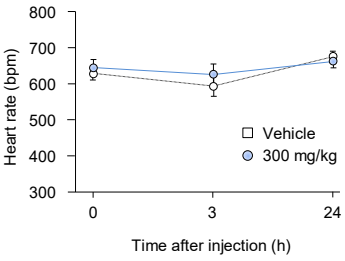


Heart rate and plasma biopterins levels after SPRi3 administration (300 mg/kg ip)

E



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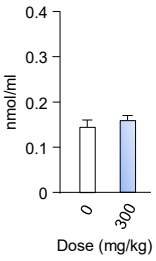

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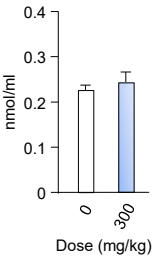
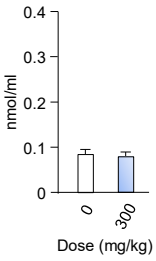
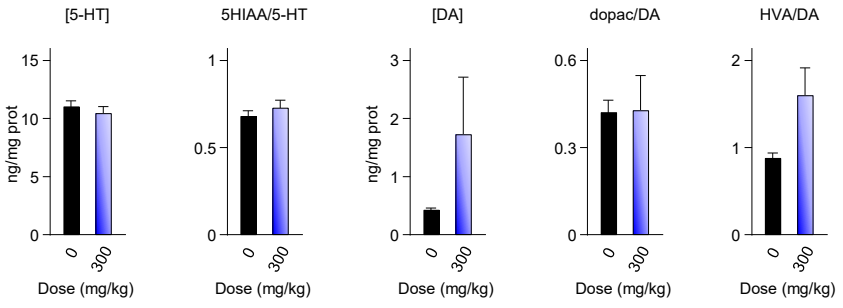


H



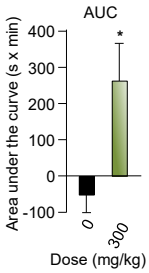
DA and 5-HT levels in the hippocampus after repeated administrations of SPRi3 (300 mg/kg ip bi-daily for 3 days)

I

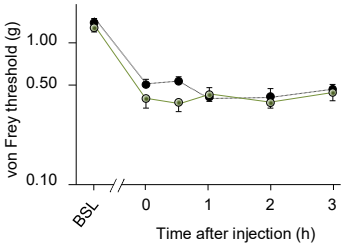


Supplemental Figure 7

A **Area under the curve of SPRi3 administered in mice injected with CFA**



B **Mechanical allodynia in mice injected with CFA after SPRi3 administration (300 mg/kg ip)**



C **Pre-emptive SPRi3 treatment (300 mg/kg ip 1hr before CFA and 2 injections after every 4 hours):**

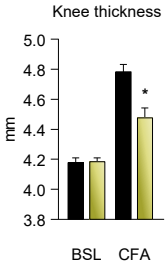


FIGURE S1, related to Figures 1. Populations of sensory neurons expressing *Gch1* after SNI are similar 7 days and 21 days after injury

A) Distribution of eGFP-positive neurons with various sensory neuron markers seven (left) or 21 days after SNI (right).

FIGURE S2, related to Figure 2. Behavioral phenotyping of sensory neuron-specific *GCH1* overexpressing mice

A, D) Contact heat withdrawal latencies in *Advillin-GCH1*-HA OE (A) and *Na(v)1.8$_{Tg}$-GCH1*-HA OE (D) mice (n= 8-12). B, E) Calibrated forceps pressure to elicit withdrawal response in grams in *Advillin-GCH1*-HA OE (B) and *Na(v)1.8$_{Tg}$-GCH1*-HA OE (E) mice (n= 8-12). C, F) Number of brisk withdrawals out of 10 stimulations at various pressures applied onto the plantar surface of the hindpaw in *Advillin-GCH1*-HA OE (C) and *Na(v)1.8$_{Tg}$-GCH1*-HA OE (F) mice (n= 7-12). G) Thermal place preference of *Na(v)1.8$_{Tg}$-GCH1*-HA OE mice and littermates tested on 22-50°C thermal gradient. Results are presented as the time spent (in seconds) in zones set at specific temperature (in Celsius degrees, x-axis) per 30 minutes periods (n= 7-9). H) Number of brisk withdrawals out of 10 stimulations at various pressures applied onto the plantar surface of the hindpaw in *Brn3a-GCH1*-HA OE mice (n= 7-12). For all graphs: mean ± SEM. *: $p<0.05$ two-tailed unpaired Student's t test.

FIGURE S3, related to Figure 3. Effects of *Gch1* deletion and SSZ treatment on neuropathic pain hypersensitivity

A) Time spent licking the paw in Advillin-hGCH1-HA OE mice after SNI (n= 12-15). B) Mechanical hypersensitivity assessed by von Frey filaments in *Advillin-Gch1* KO mice and their littermates in the CCI model (n= 5-7). *: $p<0.05$ two-way ANOVA followed by Bonferroni's multiple comparisons post-hoc test. C) Effects of sulfasalazine treatment (50mg/kg/p.o. bi-daily for 3 days) on mechanical hypersensitivity assessed with von Frey filaments 10 days after SNI. Mice were tested immediately after the last gavage (n= 8). *: $p<0.05$ two-way ANOVA followed by Bonferroni's multiple comparisons post-hoc test.

FIGURE S4, related to Figure 4. Characterization of SPRi3

A) GCH1 activity measured by spectrophotometry in primary sensory neuron cultures after 24h incubation with various doses of SPRi3 (n= 3-5; 1 unit (U) = µmol consumed sepiapterin/min). B) Schematic representation of the interactions of NAS and oxaloacetate with mSPR (left), and SPRi3 with hSPR (right). The ligands are indicated in wire frame with the oxygen and nitrogen atoms indicated as red and blue dots, respectively. Hydrogen bonds (green lines) and hydrophobic interactions (yellow lines) between ligands and amino acids (ellipse with residue number and 3-letter abbreviation), with the catalytic Ser and Tyr residues highlighted in red, as identified by Ligand Explorer (http://www.pdb.org/pdb/static.do?p=help/viewers/ligandExplorer_viewer.html) using as threshold for hydrogen bonds and hydrophobic interactions a distance cut off of 3.3 and 3.9 Å, respectively. C) The orientation of SPRi3 (carbon atoms in gray; right side of image) in the substrate binding site of hSPR with respect to NADPH (carbon atoms orange; left side of the image). D) Plasma sepiapterin levels from mice treated with sulfasalazine (400 mg/kg/d p.o. for 4 days) or vehicle (n= 5-6).

FIGURE S5, related to Figure 5. SPRi3 reduces mechanical allodynia in two models of neuropathic pain without altering nociceptive pain and pro-inflammatory cytokines production by macrophages

A) Areas under the curves of the anti-allodynic effects of SPRi3 injected in mice after SNI (left) or CCI (right; n= 7-8). *: $p<0.05$ one-way ANOVA followed by Dunnett's multiple comparisons post-hoc test (SNI) and two-tailed unpaired Student's t test (CCI). B-D) Acute administration of SPRi3 in naïve C57BL6j mice does not affect mechanical sensitivity assessed by von Frey filaments (B), calibrated forceps (C) or radiant heat (D; n= 8). E) SPRi3 (50 µM) does not change IL-1β (left) or IL-6 (right) production in purified peritoneal macrophages activated by LPS or LPS+ATP.

FIGURE S6, related to Figure 6. SPRi3 reduces mechanical allodynia in chronic pain stages without causing any side effects

A) Areas under the curve of the anti-allodynic effects of SPRi3 injected in mice 2, 3 or 4 weeks after SNI (left; n=7-9) or 6 weeks after CCI (right; n= 7-8). B-D) Effects of SPRi3 on total distance travelled in activity wheels over a 2 hours period (B), fine movements assessed in the holeboard test (C) and immobility time in the tail suspension test (D). All tests were run 30 min to 1h after injection. (KSE stands for Kinder Scientific Enhanced; n= 10). E) Heart rate of C57BL6j mice 3 and 24h after injection of SPRi3. F) Plasma levels of BH2 measured 3h after injection of SPRi3. G, H) Plasma levels of BH4 and BH2 measured 24h

after injection of SPRi3. I) Amine levels and ratio with their metabolites in the hippocampus of mice one hour after the last injection of a 3 days regime of bi-daily injections of SPRi3 (300 mg/kg i.p.; n=7-9).

FIGURE S7, related to Figure 7. SPRi3 reduces inflammatory heat pain hypersensitivity and knee thickness

A) Areas under the curve of the anti-allodynic effects of SPRi3 injected in mice one day after intraplantar CFA injection. B) Effects of injections of SPRi3 on mechanical hypersensitivity in mice 1 day after intraplantar injection of CFA (n= 8). C) Effects of a SPRi3 pre-emptive treatment on CFA-induced knee thickness (n= 9). *: $p<0.05$. For A: two-tailed unpaired Student's t test; For C: two-way ANOVA followed by Bonferroni's multiple comparisons post-hoc test.

Supplemental Experimental Procedures, related to Experimental procedures

Generation of *brn3a*-CreERT2 mice: A construct composed of an 11kb *brn3a* enhancer and a 440bp minimal promoter, which has been shown to be selectively active in the majority of DRG and trigeminal sensory neurons (Eng et al., 2001), is used to drive the expression of *Cre*ER^{T2}. A transgenic mouse line was generated by pronuclear injection of the plasmid construct. The functionality of this inducible selective DRG deleter line was verified by breeding it with *Rosa26lacZ* and *LSL-TdTomato* reporter lines (O'Donovan et al., 2014).

F10–F15 generations of offspring were used for *Na(v)1.8Tg-GCH1*-HA OE, *Na(v)1.8Tg-Gch1* KO, *Advillin-GCH1*-HA OE, *Advillin-Gvh1* KO mice. F5-7 generation *brn3a*-CreER^{T2}-*GCH1*-HA OE and *brn3a-Cre*ER^{T2}-*Gch1* KO mice.

Behavioral studies:

All animal procedures were approved by the Boston Children's Hospital Animal Care and Use Committee and conducted in a blinded fashion in a quiet room (temperature 22±1°C) from 9 AM to 6 PM. Because no gender specificity was observed in this study male and female mice were pooled by genotype to limit the number of animals used. Mice were housed with their littermates (2 to 5 mice per cage based on the litters) in OptiMICE cages with food and water ad libitum. All animals were maintained under the same conditions (22±1°C, 50% relative humidity, 12-h light/dark cycle). For behavioral experiments involving

transgenic mice, randomization was achieved through the breeding: at the time of weaning mice were separated based on their sex and placed in their new home cage. Only cages with a mixed representation of transgenic mice and their littermates were used for behavioral experiments. All experiments used at least 2 independent litters and were duplicated. Behavioral assays and genotyping were performed by different experimenters. **For pharmacological experiments** C57Bl6j male mice were obtained from Jackson Laboratory at 10-12 weeks of age. After one week of acclimation in their new home environment mice were used. Randomization was achieved by computer software (http://www.3rs-reduction.co.uk/html/5__avoiding_bias.html) and if baseline values were not significantly different between groups the mice were injected. In the case randomization led to significant baseline differences the process was repeated. Injections typically were performed between 10 and 11 am. Drug injection and behavioral assays were performed by different experimenters.

Determination of pain responses/thresholds:

Von Frey filaments (static punctate mechanical stimuli):

Before testing, each mouse was habituated in a small plastic (7.5 x 7.5 x 15 cm) cage for 1 h. Then mechanical sensitivity was determined with a graded series of eight von Frey filaments that produced a bending force of 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1 and 2 g, respectively. The stimuli were applied within the sciatic nerve territory (lateral part of the hindpaw). Each filament was tested 10 times in increasing order starting with the filament producing the lowest force. Von Frey filaments were applied at least 3 s after the mice had returned to their initial resting state. For baseline mechanical sensitivity test: all filaments were applied and the number of withdrawals was recorded. For tactile allodynia: the minimal force filament for which animals presented either a brisk paw withdrawal and/or an escape attempt in response to at least 5 of the 10 stimulations determined the mechanical response threshold.

Calibrated forceps: Before testing, each mouse was habituated to be handled within a cloth for several minutes. The day of the test, mice were gently handled and a calibrated forceps (Bioseb, France) was applied at the base of the tail until a brisk withdrawal reflex occurred. 3 measurements were performed per mouse.

Capsaicin: 20 microliters of 1 µg capsaicin diluted in 1% DMSO and saline were injected into the plantar surface of the hindpaw and the mouse was placed onto a surface set at 30°C within an acrylic container and the time spent licking or biting the paw was measured.

Contact heat pain (Hot plate test): mice were placed on a metallic plate heated to a set temperature (30, 49, 52 or 55°C) within an acrylic container (Bioseb, France), and the latency for flinching, licking one of the hindpaws, or jumping was measured. Mice were sequentially tested for 30, 52, 55 and 49°C. One temperature was tested per day.

Radiant heat pain (Hargreaves test): Before testing, each mouse was placed on an elevated glass surface set at 30°C and habituated in their individual cage for 1 h. Then a radiant heat source (beam intensity set to cause baseline latencies in C57Bl6/j mice~15s) was targeted at the paw and the latency to withdraw was measured. Radiant heat apparatus from IITC Life Science Inc. (USA).

Thermal Gradient test: A continuous temperature gradient (4 to 55°C and 20-50°C) was established along a metallic base plate, on which the mice walked freely while being video-recorded from above (Bioseb, France). After an exploration period of 2 hours the animal chooses a distinct preference or comfort zone, indicating the best temperature range tolerated by the animal. Data are presented by time spent on zones set at specific temperatures.

Acetone test (cold allodynia): Mice were habituated in a small plastic (7.5 x 7.5 x 15 cm) cage and a small volume of acetone (5 µl) was applied onto the plantar surface of the hindpaw. The time spent flinching or licking the paw was recorded for one minute.

Other behavioral assays:

Accelerating rotarod: mice were placed on an accelerating (5-45rpm in 5 min) rotarod (IITC Life Science Inc. (USA)) and the latency to fall was recorded. Mice were habituated twice before the test run.

Tail suspension test: mice were suspended by the tail (taped onto a suspension hook so that the animal hangs with its tail in a straight line) for a test duration of 6 minutes about 30-50 cm above the surface of a table covered with soft padding material and the duration of immobility is scored.

Holeboard test: A single mouse was placed in the holeboard test area to explore for up to 30 minutes Infra-red beams were used to monitor activity (number of holes explored and distance travelled).

Dynamic weight bearing test: Mice were placed in a plexiglas cage (11 x 11 x 20 cm) and onto a on a calibrated weight transducer pad (Bioseb, France). Animals could move freely in the chamber, and the transducer measured the repartition of the mouse average body weight on each paw over a 2 min time period. The weight-bearing difference was calculated as the absolute value of the difference between the weights on the two hindpaws.

Knee thickness: Mice were gently handled and knee thickness was measured a knee diameter by Digital Micrometer (Marathon, Canada).

Drug solution and routes of injection:

All drugs and their vehicle were freshly prepared. SPRi3 was dissolved with 2-ydroxypropyl-β-cyclodextrin (50% w/v) in sterile saline. L-NAME was dissolved in sterile saline. Sulfasalazine was prepared in sterile saline with 5% DMSO. Tamoxifen was prepared in sunflower oil with 5% ethanol. All drugs were administered by intraperitoneal (i.p.) injection at a concentration of the respective solution of 30 mg/ml (except tamoxifen: 10 mg/ml). Drug administered per os were used at a concentration of 10 mg/ml.

Surgery: SNI surgery was performed under 3% induction / 2% maintenance with isoflurane on adult mice (8 to 12 weeks old). The tibial and common peroneal branches of the sciatic nerve were tightly ligated with a 5.0 silk suture and transected distally, while the sural nerve was left intact (Decosterd and Woolf, 2000). After injury, incision was sutured and mice were allowed to recover on heated pads before being returned to their homecage. The surgeon who performed the SNI was blinded to the genotype.

Injection of Complete Freund's adjuvant: For intraplantar injection of CFA mice were gently restrained with a cloth then 20 µl of CFA (20 µg) were injected into the plantar surface of the paw and mice were returned to their homecage. For injection into the knee, CFA injection was performed as previously described (Wilson et al., 2006). Briefly, mice were anesthetized (3% induction / 2% maintenance isoflurane) shaved at the knee level and a 30G1/2 syringe was inserted into the knee joint. 5 µl of CFA (20 µg) were injected and the mice were returned to their homecage.

Immunohistochemistry: Mice were perfused with 4% paraformaldehyde dissolved in PBS, and L3/L4 DRGs, spinal cord, and sciatic nerve were dissected, postfixed, cryoprotected, and frozen in OCT (Tissue-Tek). Ten-µm (sciatic nerve, DRG) or twelve-µm (spinal cord) thick cryosections were blocked with 1% bovine serum albumin (Sigma-Aldrich)/ 0.1%Triton X-100 in 0.1 M phosphate buffered saline (PBS) and then incubated with primary antibodies overnight at 4°C. After 3 washes in PBS for 10 minutes each, sections were incubated with secondary antibody for 1 hour at room temperature, washed 3 times in PBS (10 minutes each) and mounted using Vectashield (H1200). For 3,3´-diaminobenzidine tetra hydrochloride (DAB) stainings, Vectastain Elite ABC Kit (Vector laboratories) was used.

Primary antibodies used: Rabbit anti-GFP (Life technologies, A11122), 1:1000; Chicken anti-NF200 (Millipore, AB5539), 1:2000; Goat anti-CGRP (Santa-Cruz, sc-8857) 1:500; Rabbit anti-ATF3 (Santa-Cruz sc-188), 1:200; Rabbit anti-$TRPV_1$ (Alomone, ACC-030), 1:500; Guinea-pig anti-SP (A Gift from John Maggio,

University of Cincinnati College of Medicine, OH, USA), 1:20000; Rat anti-CD68 (Serotec, MCA1957), 1:10000; Rabbit anti-Cre (novagen, 69050); Isolectin B4 (IB4) DyLight 594-conjugated (Vector laboratories FL-1207), 1 mg/ml. Secondary antibodies used: Alexa Fluor 488 anti-rabbit (Jackson Immunoresearch laboratories), 1:500; Alexa Fluor 594 anti-rabbit, anti-chicken, anti-goat, anti-rat, anti-guinea-pig (Jackson Immunoresearch laboratories), 1:500.

Semi-quantitative real-time PCR: The proximal stump of ipsilateral sciatic nerves, L3/L4 DRGs and corresponding dorsal horn tissue were harvested and RNA extracted by acid phenol extraction (TRIzol reagent, Invitrogen). First-stranded cDNA synthesis (1 µg of total RNA per reaction) was performed with SuperScript III Reverse Transcriptase per the manufacturer's instructions (Invitrogen). Quantitative real-time PCR was performed using the Sybr green detection system with primer sets designed on Primer Express. Specific PCR product amplification was confirmed using dissociation protocol. Transcript regulation was determined using the relative standard curve method per the manufacturer's instructions (Applied Biosystems). Relative loading was determined before RT with RNA spectrophotometry followed by gel electrophoresis and after RT by amplification of glyceraldehyde-3-phosphate dehydrogenase. Primers used for this study were: GCH1: FWD: ACAAGCAAGTCCTTGGTCTCA; REV: GTGAGGCGCTCTTGAACTTG; SPR: FWD: CATCAACAACGCAGCCACTCT; REV: CCAGTAGTTGTTCACCTCAGCTA; QDPR: FWD: AGCATCGATGTGGTGGAGAAC; REV: GTCAGCCTGCTCTGTGAATGAA; Akr1b3: FWD: TGGACACTTGGACGGCTATG; REV: GAAGAGGGTTGAAGTTGGAGACA; Cbr1: FWD: GCATCGCCTTCAAGGTCAAT; REV: CGGGTACCAAAAAAGTTCGTTT; Cbr2: FWD: CCAGATGGTAGCCAGGGACAT; REV: CCACCATGCTGGAGACATTG; Cbr3: FWD: GCGTGGCCCTGGTAACTG; REV: ACGTCCCCGGAGAATTTCC; Cbr4: FWD: TCTTGGTAAATGCAGCCGGTAT; REV: GTGCAGCTGAGAGATCATGTCTTC; DHFR: FWD: CGGCAATCCTAGCGTGAAG; REV: ACGGCGACGATGCAGTTC; PTPS: FWD: GGCCACGGGCACAACTATAA; REV: GGTCAAATTCATAACCATTCCTGTAA; Akr1e1: FWD: GCTACCGGCACTTCGATTGT; REV: CGCCCTCCTTGATCTTCTCA.

Biopterin concentrations: Concentrations were as described previously (Pickert et al., 2013). Briefly, after acidic oxidation of homogenized tissue with iodine pteridines were obtained by solid phase extraction using Oasis MCX extraction cartridges (Waters GmbH, Eschborn, Germany). Concentrations of total biopterin and the internal standard rhamnopterin were determined by liquid chromatography coupled to tandem mass spectrometry. HPLC analysis was done under gradient conditions using a Gemini C18 5 µm,

150 x 2 mm column (Phenomenex, Aschaffenburg, Germany). MS/MS analyses were performed on an API 4000 Q TRAP, a hybrid triple quadrupole/linear ion trap mass spectrometer with a turbo ion spray source. Precursor-to-product ion transitions of m/z 236 to 192 for biopterin and m/z 266 to 192 for rhamnopterin were used for the MRM. Quantification was done with Analyst software 1.5 (AB Sciex). Linearity of the calibration curve was proven from 0.1-100 ng/ml. The coefficient of correlation for all measured sequences was at least 0.99. The intra-day and inter-day variability was <10%.

Sepiapterin concentrations: Sepiapterin concentrations were determined by liquid chromatography coupled to electrospray ionization tandem mass spectrometry. The method was adapted from (Tegeder et al., 2006) and the following optimized multiple-reaction-monitoring (MRM) parameters 237.9 > 177.2 and 237.9 > 178.2 were used for sepiapterin identification and quantification.

SNAP-based competition time-resolved FRET (TR-FRET) assay:

Purified SNAP-SPR and SNAP-mEGFP were prepared as described in (Haruki et al., 2013). Fusion proteins were labeled with 2-fold excess of benzylguanine-Terbium cryptate conjugate (K2-benzylamide-BG, Cisbio) or benzylguanine-sulfasalazine (BG-SSZ, (Chidley et al., 2011)) respectively, and purified with NAP5 columns (GE healthcare) to remove excess of labeling reagents. Final reaction mixture contained 1.0 nM Tb-SNAP-SPR, 180 nM SSZ-SNAP-mEGFP, 10 µM NADPH, 10 µM NADP+ in buffer A (50 mM HEPES-NaOH pH7.4, 0.15 M NaCl, 0.5 µg/µl BSA, 0.05% Triton X-100, 1 mM DTT). Signal was measured after 3 h of incubation with compounds using Infinite F500 (TECAN). Excitation wavelength was 320 nm, emission wavelength were 485 nm and 520 nm respectively.

Measurements of cellular biopterins levels in SK-N-BE(2) neuroblastoma cells:

Measurement of intracellular total biopterin level was performed as described previously (Bonafe et al., 2001) with some modifications as specified below. Human SK-N-BE(2) cells were purchased from ATCC. Cells were seeded in 12-well dishes (5×105 cells/well) and incubated for 2 days. Then, culture supernatant was replaced with 1 ml of fresh medium to which compounds were added as DMSO solution (2µl). After 24h incubation cells were washed, detached using 300µl of trypsin/EDTA, suspended in the same volume of PBS + 2% FBS and transferred to 1.5 ml tubes. Samples were centrifuged at 1 krpm for 1 min at 4°C to discard supernatant. Cells were washed twice with PBS and finally suspended in 0.15 ml 50 mM Tris-HCl pH 7.4. 50 µl of suspension of cells was mixed with 25 µl of freshly prepared iodine solution (10 mg I2 and 20 mg KI in 1 ml of 1 M HCl) and incubated 1 h at room temperature in the dark. Then 46.5 µl of ascorbic acid (10.75mg/mL in water) was added to the samples to neutralize. Samples were left on ice for ~30 min

and centrifuged at 15 krpm for 10 min at 4°C to collect clear supernatant into glass vials. Samples were analyzed by HPLC with fluorescence detection (Shimadzu UFLC). Excitation and emission wavelengths were 350 nm and 450 nm, respectively. Neopterin, biopterin and pterin were resolved using gradient of methanol in 15 mM K2HPO4 pH 3.6 (adjusted with HCl) on Ascentis RP-Amide C18, 5 µm (15 cm × 4.6 mm) column (Sigma) with flow of 1 ml/min. Remaining cell suspension in 50 mM Tris-HCl was subjected to 3 cycles of freeze and thaw. After spinning of the tubes, protein concentration of the supernatant was measured with Bradford reagent using bovine serum albumin as a standard. Measured biopterin levels were normalized to protein content. Experiments were performed at least in triplicate.

Plasma levels of SPRi3: Plasma levels of SPRi3 were determined by LC/MS. The chromatographic system consisted of a Zorbax Eclipse C18 column run with a mobile phase of methanol: formic acid 0.1% (1:4) at a flow rate of 1mL/min and a temperature of 38ºC. Detection was carried in single ion mode at 263.2 and 174.2 m/z. Calibration curves were prepared freshly with each batch of samples. The method passed linearity, repeatability and reproducibility tests (CV% <5%) and a lower limit of quantification of 1ng/ml.

Plasma levels of Phenylalanine (Phe): Levels of Phe were assessed fluorimetrically by using a commercial kit (MAK005; Sigma; MO, USA) and following the technical recommendations of the manufacturer.

Blood pressure: Systolic blood pressure and heart rate were measured using an automated computerised tail-cuff system in conscious mice following five consecutive training periods (Visitech BP2000, Visitech Systems, Inc., USA). All recordings were performed between 08:00 and 12:00 h.

Kinetic assay of GCH1 activity: The enzyme activity was measured spectrophotometrically (SpectraMax M5 Multi-Mode Microplate Reader, Molecular Devices, USA), according to Kolinski and Gross 2004 (Kolinsky and Gross, 2004), with some modifications. Briefly, tissues or cultured neurons were homogenized (three times at 6,000 rpm during 30 sec; Bertin Tech homogenizer, USA) in 150 µL of freshly prepared 50 mM Tris-HCL buffer pH 7.5, containing 1.0 mM dithioerythritol and protease inhibitor cocktail (1 tablet / 10 mL buffer; cOmplete, ULTRA, Mini, EDTA-free, EASYpack; Roche, USA). After centrifugation (10,000 x g for 5 min at 4ºC) and in order to remove salts and pteridins, sample supernatants were transferred to Zeba spin desalting columns (40k MWCO, Thermo Sci, IL, USA). Then, three freeze-thaw cycles were applied to the extracts, in order to allow the complete dissociation of the GTPCH feedback regulatory protein from GCH1. The activity was assessed by following the formation of 7,8-

dihydroneopterin triphosphate (NH2) in the presence of 1 mM GTP, 5 mM zinc, 0.1% Triton X-100 and 250 µg/mL extract protein at 340 nm. The extinction coefficient of ϵ340 = 1820 M^{-1}. cm^{-1} was used for the calculations after correction for multi-well plates. Activities are depicted as mU / mg protein, and 1U corresponds to 1 µmol formed NH2 / min at 37°C.

Kinetic assay of SPR activity: The enzyme activity was measured spectrophotometrically (SpectraMax M5 Multi-Mode Microplate Reader, Molecular Devices, USA) as previously described (Sueoka and Katoh, 1982), with some modifications. Briefly, the activity in the extracts (prepared as indicated for GCH1 activity) was measured following the disappearance of sepiapterin at 420 nm in a medium containing 50 mM Tris-HCL buffer pH 7.5, 0.1% Triton X-100, 200 µM sepiapterin, 600 µM NADPH and 25 µg/mL extract protein. The extinction coefficient of ϵ420 = 10.4 mM–1. cm^{-1} was used for the calculations after correction for multi-well plates. Activities are depicted as U / mg protein, and 1U corresponds to 1 µmol consumed NADPH / min at 37°C.

Macrophage activation assay: Thioglycollate-elicited macrophages were isolated and plated at a concentration of 10^6/ml. After 24 hours the macrophages were stimulated with LPS (5µg/ml) with or without ACS Inhibitor (50µM). After 24hour of stimulation the supernatant was collected and nitrite levels were measured using Greiss Reagents (RnD Systems) as well as IL-6 using ELISA (Biolegend). Additionally, a second signal was introduced (ATP; 1mM) for 1 hour to induce IL-1β secretion which was measured in the supernatant using an IL-1β ELISA kit (Abcam).

Structure determination of SPRi3e bound to hSPR: hSPR carrying an N-terminal hexa-His-tag followed by a TEV protease cleavage site was expressed in BL21(DE3) E. coli. Upon cell lysis, hSPR was captured by a sulfapyridine-modified Affigel-10 resin (BioRad) in the presence of 1 mM NADP. After washing away non-specifically bound protein, hSPR was eluted with 20 mM MES, 100 mM NaCl (pH 6.5), 1 mM NADPH and 1 mM SPRi3, and concentrated by ultrafiltration to a protein concentration of 27 mg/ml. Crystallization was performed at 4 °C by the hanging drop method. One volume of protein was combined with one volume of 100 mM Hepes (pH 7.5), 1.7 M ammonium sulphate and 2 % PEG 1000. Crystals were harvested after 12 days and cryo-protected with 25 %(v/v) glycerol and flash-frozen in liquid nitrogen. Diffraction data were collected at the Swiss Light Source (Villigen, CH) using the X10SA beam line. XDS (Kabsch, 2010) was used for indexing, Scala (Evans, 2006) for scaling, MolRep (McCoy et al., 2007) for molecular replacement using the hSPR structure PDB ID 4HWK (Haruki et al., 2013), and RefMac (Murshudov et al., 1997) and Coot (Emsley and Cowtan, 2004) for final refinements, using the SPRi3 structural parameter file

obtained from PRODRG (Schuttelkopf and van Aalten, 2004) modified to allow both cis and trans conformations of the side chain amide group.

Statistical analyses: All values are expressed as means +/- SEM. Two-tailed unpaired Student's T-test was used to compare a single measurement between two groups. one-tailed Mann Whitney test was used for non-paramatric comparisons of a single measurement between two groups. one-way ANOVA followed by Dunnett's multiple comparisons post-hoc test was used for QPCR analyses and dose-dependent enzymatic activity experiments. two-way ANOVA followed by Bonferroni's multiple comparisons post-hoc test were used for the analyses of time courses experiments. Areas under the curves (AUCs) were calculated using the linear trapezoidal rule and comparison between doses were performed by one-way ANOVA followed by Dunnett's multiple comparisons post-hoc test. In all tests $P \leq 0.05$ was considered significant.

General Synthetic Procedures

Commercially available starting materials, reagents and dry solvents were used as supplied. Preparative HPLC was conducted using a Phenomenex Luna column (5 µm, 250 x 21.2 mm, C18, Phenomenex, Torrance, USA) using a Gilson GX-281 Liquid Handler system combined with a Gilson 322 HPLC pump (Gilson, Middleton, USA), over a 15 minute gradient elution from 10:90 to 100:0 MeOH:water (both modified with 0.1% formic acid) at a flow rate of 20 mL/min or over a 15 minute gradient elution from 40:60 to 100:0 MeOH:water (both modified with 0.1% formic acid) at a flow rate of 20 mL/min. ^{1}H NMR spectra were recorded on a Varian 400Mhz instrument. ^{13}C NMR spectra were recorded on a Bruker Avance-500 instrument at 125Mhz. Samples were prepared as solutions in a deuterated solvent and referenced to the appropriate internal non-deuterated solvent peak or tetramethylsilane. Chemical shifts were recorded in ppm (δ) downfield of tetramethylsilane. IR analysis was performed on Shimadzu FT-IR. MS analysis was performed on a Waters SQD-2 instrument. HRMS analysis was performed on an Agilent 1200 series HPLC and diode array detector coupled to a 6210 time of flight mass spectrometer with dual multimode atmospheric pressure CI/ESI source. Analytical separation was carried out at 30 °C on a Merck Purospher STAR column (RP-18e, 30 x 4 mm) using a flow rate of 1.5 mL/min in a 4 minute gradient elution with detection at 254 nm. The mobile phase was a mixture of MeOH (solvent A) and water (solvent B) both containing formic acid at 0.1%. Gradient elution was: 1:9 (A/B) to 9:1 (A/B) over 2.5 min, 9:1 (A/B) for 1 min, and then reversion back to 1:9 (A/B) over 0.3 min, finally 1:9 (A/B) for 0.2 min. The following references masses were used for HRMS analysis: caffeine $[M+H]^{+}$ 195.087652; (hexakis(1*H*,1*H*,3*H*-

tetrafluoropentoxy)phosphazene $[M+H]^+$ 922.009798) and hexakis(2,2-difluoroethoxy)phosphazene $[M+H]^+$ 622.02896 or reserpine $[M+H]^+$ 609.280657.

Synthetic Procedures

5-Methoxy-2-methyl-1*H*-indole-3-carbaldehyde

$POCl_3$ (1.67 mL, 13.64 mmol) was added dropwise to DMF (8.0 mL) at 0 °C-10 °C. The resulting mixture was stirred for 30 minutes, cooled to 0 °C, and a solution of 5-methoxy-2-methyl-1*H*-indole (2.0 g, 12.4 mmol) in DMF (8.0 mL) was added over 15 minutes. After the addition was complete, the reaction mixture was stirred at ambient temperature for 2 hours. The reaction mixture was quenched with ice (25 g), poured into water (30 mL), and basified (to pH~10) using a 1N NaOH solution. The mixture was extracted using ethyl acetate (3 × 20 mL), washed with water (2 × 10 mL) and brine (15 mL), and dried over anhydrous Na_2SO_4. Removal of the solvent afforded the title compound as a pale brown solid (1.7 g, 72%). ^{1}H NMR (400MHz, $CDCl_3$): δ 10.15 (s, 1H), 8.35 (bs, 1H), 7.77 (s, 1H), 7.21 (d, *J* = 8.78 Hz; 1H), 6.88 (dd, 1H), 3.88 (s, 3H), 2.72 (s, 3H). Mass (M+H): 189.9.

(*E*)-5-Methoxy-2-methyl-3-(2-nitrovinyl)-1*H*-indole

A suspension of 5-Methoxy-2-methyl-1*H*-indole-3-carbaldehyde (1.7 g, 8.99 mmol) and ammonium acetate (2.3 g, 30.85 mmol) in nitromethane (120.0 mL) was stirred at 90 °C for 7 hours. The reaction mixture was concentrated under reduced pressure. The resulting crude product was dissolved in ethyl acetate (100 mL), washed with water (2 × 30 mL) and brine solution (25 mL), dried over anhydrous Na_2SO_4, and the solvent was removed to afford the title compound as yellow solid (2.1 g, 98%). ^{1}H NMR (400MHz, $CDCl_3$): δ 8.52 (bs, 1H), 8.33 (d, *J* = 13.42 Hz; 1H), 7.71 (d, *J* = 13.42 Hz; 1H), 7.27-7.25 (m, 1H), 7.11 (d, 1H), 6.91- 6.89 (m, 1H), 3.90 (s, 3H), 2.62 (s, 3H). Mass (M+H): 203.9.

5-Methoxy-2-methyl-3-(2-nitroethyl)-1*H*-indole

To a cold (0 °C) solution of (*E*)-5-Methoxy-2-methyl-3-(2-nitrovinyl)-1*H*-indole (1.0 g, 4.74 mmol) in methanol (40.0 mL) was added portionwise $NaBH_4$ (360 mg, 9.48 mmol) over 20 minutes. After the addition was complete, the reaction mixture was allowed to stir at 10 °C for 2 hours. The reaction mixture was quenched with water (10 mL) and concentrated under reduced pressure. The resulting aqueous

residue was diluted with water (20 mL) and extracted with ethyl acetate (2 × 30 mL). The combined ethyl acetate layers were washed with water (2 × 20 mL) and brine solution (20 mL), dried over anhydrous Na_2SO_4, and the solvent was removed to afford the crude product (1.1g) as a pale brown gum that was used directly in the next step. ^{1}H NMR (400MHz, $CDCl_3$): δ 7.75 (bs, 1H), 7.17 (d, *J* = 8.70 Hz; 1H), 6.90 (s, 1H), 6.81- 6.78 (m, 1H), 4.57 (t, *J* = 7.25 Hz; 2H), 3.86 (s, 3H), 3.40 (t, *J* = 7.46 Hz, 2H), 2.37 (s, 3H). Mass (M+H): 235.1.

2-Methyl-3-(2-nitroethyl)-1*H*-indol-5-ol

To a cold (-70 °C) solution of 5-methoxy-2-methyl-3-(2-nitroethyl)-1*H*-indole (600 mg, 2.56 mmol) in dichloromethane (30 mL) was added slowly BBr_3 (0.74 mL, 5.12 mmol). After the addition was complete, the reaction mixture was allowed to reach 0 °C and stirred for 4 hours. The reaction mixture was diluted with dichloromethane (25 mL), washed with water (2 × 10 mL) and brine (20 mL), dried over anhydrous Na_2SO_4, and the solvent removed to afford the crude product which was purified by column chromatography (silica gel 100-200 mesh using 20% ethyl acetate in petroleum ether as the eluent) to give the title compound as a pale brown gum (100 mg, 18%). ^{1}H NMR (400MHz, $CDCl_3$): δ 7.75 (bs, 1H), 7.12 (d, *J* = 8.78 Hz; 1H), 6.86 (s, 1H), 6.71- 6.68 (m, 1H), 4.73 (bs, 1H), 4.54 (t, *J* = 7.31 Hz; 2H), 3.35 (t, *J* = 7.31 Hz, 2H), 2.36 (s, 3H). Mass (M+H): 221.0.

3-(2-Aminoethyl)-2-methyl-1*H*-indol-5-ol

A suspension of 2-methyl-3-(2-nitroethyl)-1*H*-indol-5-ol (100 mg, 0.454 mmol) and 10% Pd/C (60 mg) in MeOH (20.0 mL) was hydrogenated (40 psi H_2 pressure) at 26 ºC for 2 hours. The reaction mixture was filtered, the cake was washed with methanol (3 × 5mL), and the combined filtrates were concentrated under reduced pressure to afford the title compound as a pale brown gum (80 mg). Mass (M+H): 191.1. This material was used directly in the next step.

Methyl-2-((3-(2-(2-methoxyacetamido)ethyl)-2-methyl-1*H*-indol-5-yl)oxy)acetate

A cold (0 °C) solution of 3-(2-aminoethyl)-2-methyl-1*H*-indol-5-ol (80 mg, 0.42 mmol) and Et_3N (0.06 mL, 0.46 mmol) in dichloromethane (15.0 mL) was treated with methoxyacetyl chloride (0.04 mL, 0.42 mmol) added slowly over 5 minutes. After the addition was complete, the reaction mixture was allowed to reach room temperature and stirred for 2 hours. The reaction mixture was diluted with dichloromethane (25 mL), washed with water (2 × 10 mL) and brine (20 mL) and dried over anhydrous Na_2SO_4. The solvent was removed to give a pale brown gum which was purified by column chromatography (silica gel 100-200 mesh, using 20% ethyl acetate in petroleum ether as the eluent) to give the title compound (46 mg, 33%).

^{1}H NMR (400MHz, $CDCl_3$): δ 7.87 (bs, 1H), 7.23-7.21 (m, 2H), 6.87-6.84 (m, 1H), 6.58 (bs, 1H), 4.31 (s, 2H), 3.85 (s, 2H), 3.56 (s, 3H), 3.53-3.48 (q, 2H), 3.31 (s, 3H), 2.90-2.86 (m, 2H), 2.38 (s, 3H). Mass (M+H): 335.1.

_N_-(2-(5-Hydroxy-2-methyl-1_H_-indol-3-yl)ethyl)-2-methoxyacetamide (SPRi3)

Methyl-2-((3-(2-(2-methoxyacetamido)ethyl)-2-methyl-1*H*-indol-5-yl)oxy)acetate (46 mg, 0.14 mmol) was treated with K_2CO_3 (20 mg, 0.15 mmol) in MeOH (3.0 mL) and stirred at 26 °C for 2 hours. The reaction mixture was concentrated and the residue diluted with water (20 mL) and extracted with ethyl acetate (2 × 20 mL). The combined ethyl acetate layers were washed with brine (2 × 10 mL), dried over anhydrous Na_2SO_4, and concentrated to afford the crude product, which was purified by PREP-TLC using 70% ethyl acetate in petroleum ether as the eluent to afford the title compound as a pale brown gum (15 mg, 36%). ^{1}H NMR (400MHz, $CDCl_3$): δ 7.65 (bs, 1H), 7.13 (d, *J* = 8.70 Hz; 1H), 6.94 (s, 1H), 6.70-6.68 (m, 1H), 6.62 (bs, 1H), 4.79 (bs, 1H), 3.86 (s, 2H), 3.54-3.49 (m, 2H), 3.32 (s, 3H), 2.86 (t, *J* = 6.84 Hz; 2H), 2.35 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 11.7, 24.3, 39.3, 59.1, 72.0, 102.7, 108.0, 110.6, 110.8, 129.3, 130.4, 133.0, 149.7, 169.7; IR (cm^{-1}): 3382, 2923, 1649, 1200, 1114, 799. HPLC purity (%): 97.01 (Max plot), 95.28 (254 nm), 97.22 (215 nm).

ESI-HRMS: Found 263.1398, calculated for $C_{14}H_{19}N_2O_3$ ($M+H^+$): 263.1390.

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