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Supplemental Information

GINIP, a $G_{\alpha i}$-Interacting Protein, Functions as a Key Modulator of Peripheral $GABA_B$ Receptor-Mediated Analgesia

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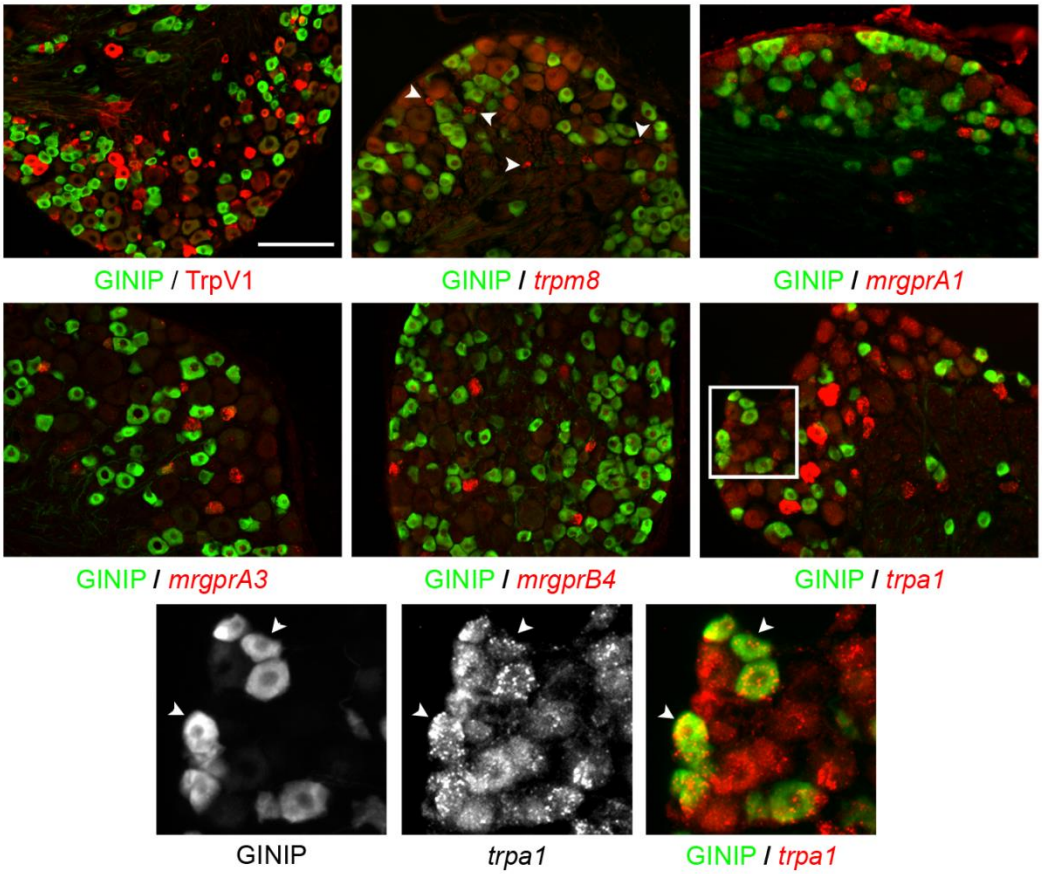
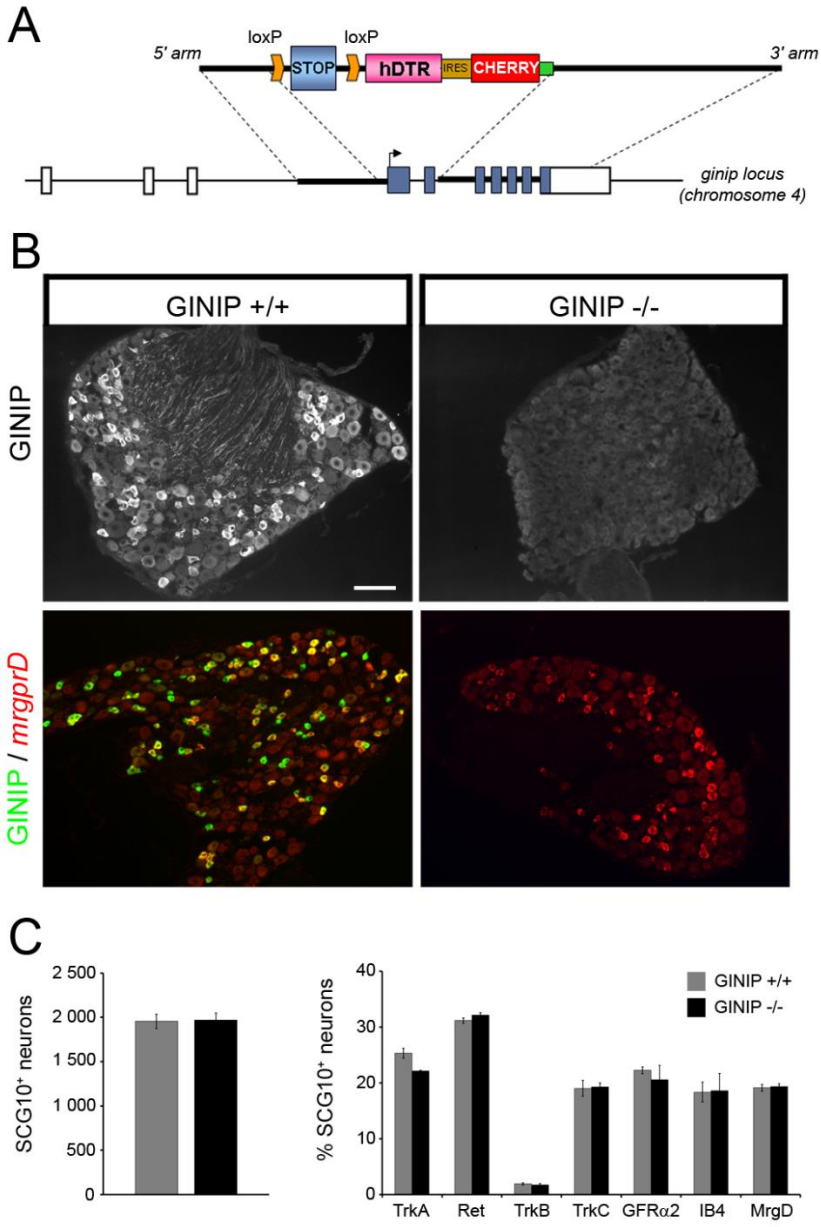
SUPPLEMENTAL FIGURES

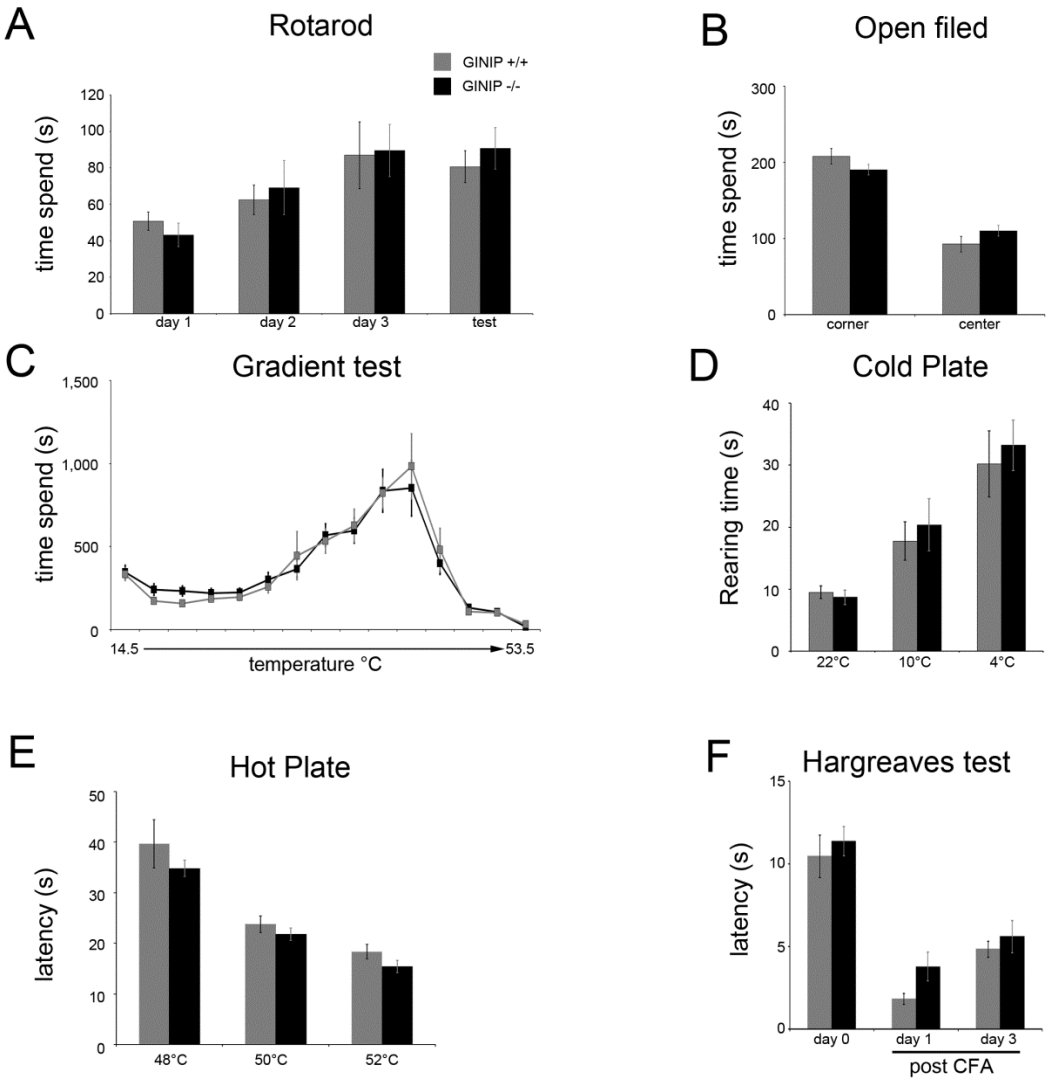
Supplemental Figure 1, related to Figure 2



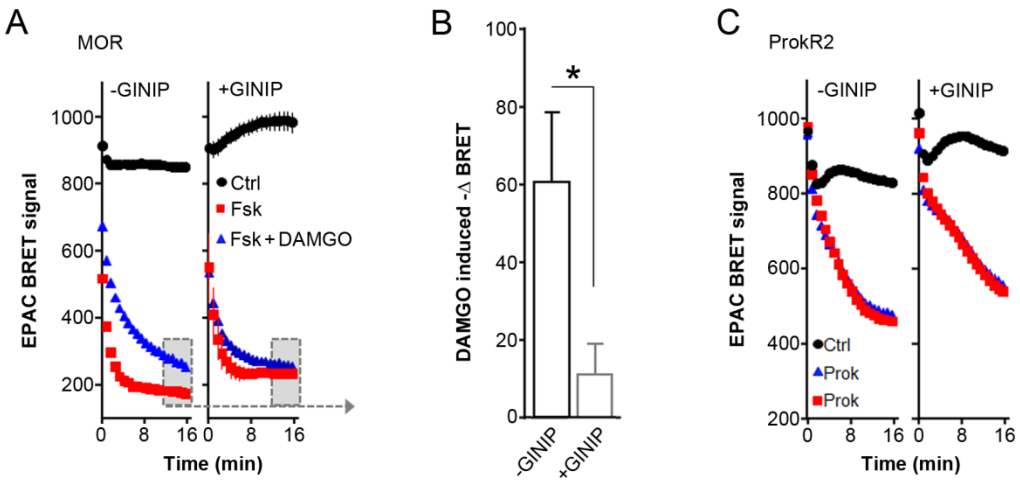
Supplemental Figure 2



Supplemental Figure 3, related to Figure 3



Supplemental Figure 4, related to Figure 6



SUPPLEMENTARY FIGURE LEGENDS

Figure S1: Characterization of GINIP positive population in adult mouse DRGs.

In-situ hybridization of DRGs using antisense probes for, *trpm8, mrgprA1, mrgprA3, mrgprB4 or trpa1,* or immunostaining using rabbit anti-TRPV1 antibody, followed by immunostaining using rat anti-GINIP antibody. Scale bar: 100µm.

Figure S2: Characterization of GINIP knock-out mice.

A. Schematic representation of the construct used to target the *ginip* locus. Two LoxP sites flanking a transcriptional stop cassette is followed by a second cassette that will ensure CRE recombinase-mediated expression of hDTR and the cherry protein under the transcriptional control of the *ginip* locus. GINIP$^{-/-}$ mice were obtained by crossing GINIP^{flx} line with cre deleter mice.

B. *In-situ* hybridization using *mrgprD* antisense probe followed by immunostaining using rat anti-GINIP antibody in WT or GINIP$^{-/-}$ DRG validates loss of GINIP expression in GINIP$^{-/-}$ mice without affecting maturation of the corresponding population. Scale bar: 100µm.

C. Survival of neurons is unaffected in the absence of GINIP. The total number of SCG10+ neurons in L4 DRGs is not different between GINIP$^{+/+}$ and GINIP$^{-/-}$ adult mice (1954 ±79 and 1970 ±78, respectively; n=3). Correct maturation of sensory neurons in absence of GINIP. Percentage of TrkA^{+} (25.3 ±0.89 in WT vs 22.1 ±0.11 in knock-out), cRet^{+} (31.1 ±0.49 vs 32.1 ±0.44), *trkB*$^{+}$ (1.9 ±0.23 vs 1.7 ±0.24), TrkC^{+} (19 ±1.42 vs 19.2 ±0.71), *gfrα2*$^{+}$ (22.2 ±0.61 vs 20.6 ±2.5), IB4^{+} (18.3 ±1.77 vs 18.6 ±3.05) and *mrgprD*$^{+}$ (19.1 ±0.61 vs 19.3 ±0.46) neurons in L4 DRG show no difference between GINIP$^{+/+}$ and GINIP$^{-/-}$ adult mice (n=3). $p>0.1$ one-way ANOVA followed by unpaired t test. Error bars represent SEM.

Figure S3: Behavioral characterization of GINIP knock-out mice.

A. Behavior of GINIP-/- and their WT littermates in the Rotarod test (n=11 and 13, respectively).

B. Behavior of GINIP$^{-/-}$ and their WT littermates in the open field test (n=11 and 10, respectively).

C to F. Behavior of GINIP$^{-/-}$ and their WT littermates in the thermal gradient test (**C**, n=14 and 17 for WT and knock-out respectively), in cold test (**D**, n=9 for each genotype), in the hot plate test (**E**, n=8 and 9, respectively) and in response to CFA-induced thermal hyperalgesia (**F**, n=8 and 11, respectively). Error bars represent SEM.

Figure S4: GINIP modulates cAMP levels in response to DAMGO

A. DAMGO-induced changes in the BRET ratio determined in tsA201 cells expressing MOR receptors in the presence or absence of GINIP.

B. Bar graph of the changes in BRET ratio determined in experiments as in A.

C. BRET ratio determined in tsA201 cells expressing a Gαs coupled receptor ProkR2 in the presence or absence of GINIP.

Data are presented as the mean ± SEM and compared with a t-test.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice

Mice were maintained under standard housing conditions (23°C, 40% humidity, 12 h light cycles, and free access to food and water). Special effort was made to minimize the number as well as the stress and suffering of mice used in this study. All protocols are in agreement with European Union recommendations for animal experimentation.

Generation of GINIP versatile mouse model

The targeting construct was designed to ensure *ginip* gene inactivation, $GINIP^{+}$ neurons genetic marking and tissue specific neuronal ablation. The targeting vector was built in the plasmid vector pl452AM (a kind gift from A. Patapoutian) as follow: The 5' homology arm consisting of a 1.32 kbp up stream of the ATG containing exon of *ginip* locus was fused to a two loxP sites flanked transcriptional stop cassette to ensure premature abortion of *ginip* transcripts. The stop cassette was then followed by a second cassette encoding the human diphtheria toxin receptor and the fluorescent protein mCherry. An FRT flanked PGK-Neo positive selection cassette was introduced downstream of the SV40 polyA of mCherry and up-stream of the 3'ARM homology arm consisting of 5840 bp located upstream of the the third coding exon. Exon 3 encodes the PHD zinc finger domain. Both 5' and 3' homology arms were PCR amplified from CK35 embryonic stem cells DNA as a template using "high fidelity" Taq Phusion polymerase (Finnzymes). The final targeting construct was linearized using SalI restriction enzyme and electroporated into 129/SV-derived embryonic stem cells. Homologous recombinant clones were identified by PCR using a 5' external primer (5'-TACCTTGCCTCTTACCCACTGT-3') and an internal primer inside the stop cassette (5'-ACCGCTGAAAGGTCTCAGTAAG-3'). PCR-positive clones were validated by Southern-blot using a 3'-external probe and a neomycin probe. One targeted clone was injected into C57Bl6/J derived blastocysts at the SEAT transgenic facility (Villejuif, France). Germ line

transmitting chimeras were mated to C57Bl6/J females to generate GINIP$^{fl/+}$ mice. GINIP$^{fl/+}$ males were then crossed with an Actin-Flipase mouse line in order to remove the two FTR sites flanked PGK-Neo cassette. To generate mice in which GINIP-expressing neurons are genetically marked by the mcherry protein, GINIP$^{fl/+}$ mice were crossed with a cre-deleter mouse line to produce GINIP$^{+/mcherry}$ mice. GINIP$^{+/mcherry}$ mice were then intercrossed to generate GINIP$^{mcherry/mcherry}$ and wild type littermates (hereafter GINIP$^{-/-}$ and WT mice respectively). A multiplex PCR genotyping strategy was designed for tail DNA using the following primers: P1 5'-AGCCATGGGGGTGTTGATGTCC-3' and P2 5'-CTTCAACCACACCTAGCTTTCC-3' for the WT allele, P3 5'-AACAACGGGGTCACGTAGAG-3' and P4 5'-GATATCCATG GCTCTGTGGACAAAATCAG-3' for the recombinant allele before CRE mediated deletion of the Stop cassette and P5 5'-TATGAGATTGTGTGCCTGTGTG-3' and P6 5'-CTCC TTCACATATTTGCATTCTCC-3' for the recombinant allele after CRE-mediated excision of the stop cassette.

In situ hybridization and immunofluorescence

In situ hybridization and immunofluorescence were carried out following standard protocols (Moqrich et al., 2004). To obtain adult tissues, animals were deeply anesthetized with a mix of ketamine/xylazine and then transcardially perfused with an ice-cold solution of 4% paraformaldehyde in PBS. After dissection, they were post-fixed ON in the same fixative at 4°C. Tissues were then transferred into a 30% (w/v) sucrose solution for cryoprotection before being frozen 24h later and stored at -80°C. Samples were sectioned at 12µm (DRG section) or 16µm (spinal cord section) using a standard cryostat (Leica).

RNA probes were synthesized using gene-specific PCR primers and cDNA templates from mouse DRG. Double fluorescent *in situ* hybridization was carried out using a combination of digoxigenin and fluorescein/biotin labeled probes. Probes were hybridized overnight at 55°C

and the slides incubated with the horseradish peroxidase anti-digoxigenin/fluorescein/biotin antibodies (Roche). Final detection was achieved using fluorescein/cy3/cy5 TSA plus kit (Perkin Elmer). For double fluorescent *in situ* experiments, the first antibody was inactivated using H_2O_2 treatment.

The following oligonucleotides were used for the nested PCRs for probe synthesis:

Gnai1-F1: TAGAGTGTCCCAGCAACTCAG,
Gnai1-R1: ATAATTGATCCAAAGGCAGGTG,
Gnai1-F2: TCCTGGACCTGACCAGAGTT,
Gnai1-R2+T7: TAATACGACTCACTATAGGGAATTGATCCAAAGGCAGGTG,
Gnai2-F1: TGCTTCTCCCATCTTTTCTCTC,
Gnai2-R1: AAAGCAAGGAAGTGTTCAACG,
Gnai2-F2: CTGTTCTCAGCTCCCCTGTC,
Gnai2-R2+T7: TAATACGACTCACTATAGGGACACGCAGACACACTCAAGG,
Gnai3-F1: GGCATGAGATGTTCAAATCAGA,
Gnai3-R1: AGACAAACAGGCAAGATTCCAT,
Gnai3-F2: GAAGTTGAGGTGCTGCCTTC,
Gnai3-R2+T7: TAATACGACTCACTATAGGGCATCTGCATCTCATTGACGG,
GINIP-F1: CAGGATAGGTGGGACAGAGAAG,
GINIP-R1: ATGTATCTCCTGCCTGCTTCAT,
GINIP-F2: TACCTGCTATGGATC,
GINIP-R2+T7: TAATACGACTCACTATAGGGTTCTCCTGAAACCAT,
gfrα2-F1: CCTTTCTCCTCCCAAATTTCTT,
gfrα2-R1: GCAACTCGCTTCCTAGTACGTT,
gfrα2-F2: TCACTGGTGTTTTCTCTCTGGA,
gfrα2-R2+T7: TAATACGACTCACTATAGGGACATTTTCGCTCATCTGTAGGG,
MrgprD-F1: GGGCATCAACTGGTTCTTACTC,
MrgprD-R1: AGGGATTGTCTTGACTGTCG,
MrgprD-F2: AACGGGATGTGAGGCTACTTTA,
MrgprA1-F1: ACATCCAGCAAGAGGAATGG,
MrgprA1-R1: GTCTTGCAGTGCATTCTGGA,
MrgprA1-F2: CAACAGCACCCACAACAACT,
MrgprA1-R2+T7: TAATACGACTCACTATAGGGCTGAAGGATCCCACGAAGAA,
MrgprA3-F1: GACCCTGATCCCAGACTTGA,
MrgprA3-R1: CAGTGGAGAGCTTTGGAAGG,
MrgpRA3-F2: ATTGTGTTCTGGCTCCTTGG,
MrgprA3-R2+T7: TAATACGACTCACTATAGGGACAGTGGTCAAGTGCAGCAG,
MrgprB4-F1: GGACCTGTGCCAGATATTCC,
MrgprB4-R1: GGACCCCTCTCTCCACTCTC,
MrgprB4-F2: CAGGAATGCCAGTGGAAAAT
MrgprB4-R2+T7 : TAATACGACTCACTATAGGGCATCGCAACCTGTGTTGTCT,
gabbr1-F1: AGATTGTGGACCCCTTGCAC,
gabbr1-R1: AAGAGGGGGATTGGAGCTTG,
gabbr1-F2: TGCCAAGGAGGAACCAAAGG,
gabbr1-R2+T7: TAATACGACTCACTATAGGGCCATCACAGCTAAGCCGGTC,
gabbr2-F1: GCGAAAACACCCACATGACC,

gabbr2-R1: CCGAGACCATGACTCGGAAG,
gabbr2-F2: CAAGGGGCTCCTCATGTTGT,
gabbr2-R2+T7: TAATACGACTCACTATAGGGCATGTTCTTGAGGGCTCGGT,
TrkB-F1: CTGAGAGGGCCAGTCACTTC,
TrkB-R1: CATGGCAGGTCAACAAGCTA,
TrkB-F2: CAGTGGGTCTCAGCACAGAA,
TrkB-R2+T7: TAATACGACTCACTATAGGGCTAGGACCAGGATGGCTCTG,
SCG10-F1: GCAATGGCCTACAAGGAAAA,
SCG10-R1: GGCAGGAAGCAGATTACGAG,
SCG10-F2: AGCAGTTGGCAGAGAAGAGG,
SCG10R2+T7: TAATACGACTCACTATAGGGGGCAGGAAGCAGATTACGAG.

TOPO-trpm8 and TOPO-trpa1 plasmids (a kind gift from A. Patapoutian) are used to synthetized *trpm8* and *trpa1* probes.

For immunofluorescence, primary antibodies were diluted in PBS-10% donkey serum (Sigma)-3% bovine albumin (Sigma)-0.4% triton-X100 and incubated overnight at 4°C. Primary antibodies used in this study are as follows: rabbit anti-TrkA 1:1000 (generous gift from Dr. L. Reichardt, University of California), goat anti-TrkC 1:500 (R&D systems), goat anti-Ret 1:500 (R&D systems), goat anti-CGRP 1:1000 (Acris antibodies), rabbit anti-PKCγ 1:1000 (Santa Cruz Biotechnology). Corresponding donkey or goat anti-rabbit, anti-rat and anti-goat Alexa 488, 555, or 647 (Invitrogen or Molecular probe antibodies) were used for secondary detection. Isolectin B4 conjugates with AlexaFluorR 488, 568 or 647 dye was used at 1:200 (Invitrogen). Acquisition of images was performed on AxioImager Z1 (Zeiss).

Cell counts and statistical analysis

We adopted a strategy that has been previously validated for DRG cell counts (Chen et al., 2006). Briefly, 12 µm serial sections of thoracic DRG were distributed on 6 slides which were subjected to different markers including the pan-neuronal marker *SCG10*. This approach allowed us to refer all countings to the total number of neurons (*SCG10*$^{+}$). For each genotype, both lumbar (L4) DRG were counted in three independent animals. All cell counts were conducted by an individual who was blind to mice genotypes. Statistical significance was set to $p<0.05$ and assessed using one way ANOVA analysis followed by unpaired t-test.

Generation of rat anti-GINIP antibody

Full length recombinant GINIP protein was expressed in *E.coli* as a GST fusion protein using pGEX-6P-1 vector (GE Healthcare). Glutathione-sepharose beads coated with GST-GINIP were incubated overnight at 4°C in TBS containing 1 mM DTT, 1 mMEDTA and 10U of PreScission Protease (GE Healthcare) per 250 ml of culture. Supernatant contained only GINIP fragment and was injected intraperitonealy into rats (200 µg per injection) four times at two weeks intervals. Rats were sacrificed one week after the last injection and sera were harvested. Serum can be directly used at dilutions ranging from 1:1000 to 1:5000.

Yeast Two-Hybrid screen

Yeast two-hybrid screening was performed by Hybrigenics Services, S.A.S. (Paris, France). The coding sequence for *mus musculus* KIAA1045 (GenBank accession number gi: 28972587) was PCR-amplified and cloned into pB27 as a C-terminal fusion to LexA (N-LexA-KIAA1045-C). The construct was checked by sequencing the entire insert and used as a bait to screen a random-primed mouse adult brain cDNA library constructed into pP6. pB27 and pP6 derive from the original pBTM116 (Vojtek and Hollenberg, 1995) and pGADGH (Bartel and Fields, 1995) plasmids, respectively. 134 million clones (13-fold the complexity of the library) were screened using a mating approach with Y187 (mat-α) and L40ΔGal4 (mat-a) yeast strains as previously described (Fromont-Racine et al., 1997). 83 His^+ colonies were selected on a medium lacking tryptophan, leucine and histidine. The prey fragments of the positive clones were amplified by PCR and sequenced at their 5' and 3' junctions. The resulting sequences were used to identify the corresponding interacting proteins in the GenBank database (NCBI) using a fully automated procedure.

Cell culture and transfection

HEK-293T cells were grown in Dulbecco's modified Eagle's medium (DMEM)+Glutamax containing 10 % foetal bovine serum supplemented with 100 µM sodium pyruvate, in 5%

CO2 humidified incubator. Cells were transfected with 3 µg of each plasmid in 6-well dishes using Promofectin (PromoKine) according to manufacturer's protocol. Plasmids containing WT-human-Gαi1-venus, WT-rat-Gαi2, WT-rat-Gαi3, WT-rat-Gαolf as well as GTPase mutant forms (Q204L for the three Gαi, Q214L for Gαolf and Q205L for rat-Gαo) were generously gift by X. Morin, ENS, Paris. Plasmids containing WT-rat-Gαs was a generously gift by Jean Pierre Vilardaga, Pittsburgh. WT and GTPase mutant forms of Gαi2 and 3, Gαolf, GTPase mutant forms of Gαo and WT form of Gαs are fused to venus thanks to the SOE method (Splicing by Overlap Extension) (Warrens et al., 1997). WT form of Gαo and GTPase mutant form of Gαs are produce with a site-specific mutagenesis by using an accurate recombinant polymerase chain reaction method (Ansaldi et al., 1996). WT form of Gαq is produce from DRG cDNA and after fused to venus thanks to the SOE method. GTPase form of Gαq is produce again with a site-specific mutagenesis by using an accurate recombinant polymerase chain reaction method. After 24h of expression, cells were harvested and lysed in 1 ml RIPA buffer.

GST-pull down assay

Expression of the GST fusion proteins in *E. coli* was induced by 3h incubation with 1mM isopropyl L-D-thiogalactoside at 37°C. Cells were lysed with 2.5 ml of BugBuster protein extraction reagent (Novagen) per 50 ml culture. Soluble fractions were purified on glutathione-Sepharose 4B beads (GE Healthcare). Equal amount of GST or GST-GINIP were re-coated on 30 µl of beads and then incubated overnight at 4°C with 500µl of HEK-293T lysate transfected with each construct. After three washes in 1.5 ml RIPA buffer for 5 min at 4°C, beads were processed directly for western-blot.

After 5 min heating at 95°C, protein samples were separated on 10% SDS polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membrane (GE Healthcare). Membranes were first saturated in 5% non-fat dry milk (Santa-Cruz) diluted in TNT solution

(50 mM Tris-HCl, pH 7.4, 150mM NaCl, 0.05% Tween-20) for 1h at RT, and then incubated with mouse anti-GFP antibody (1:1000; Roche) ON at 4°C. Membranes were rinsed three times in TNT, incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (1: 5000; Jackson ImmunoResearch Laboratories) for 1h at RT and then developed using ECL-Plus reagents (PerkinElmer).

Electrophysiology on recombinant channels Cav2.2 channels in tsA-201 cells.

tsA-201 cells were cultivated in DMEM supplemented with GlutaMax and 10% fetal bovine serum (Invitrogen). Cell transfection was performed using jet-PEI (QBiogen) in 35 mm dishes with a DNA mix containing the plasmid constructs that code for human $Ca_V2.2$ calcium channel isoform (1µg), the rat a2-d1 and b1b ancillary subunits cloned on a single dual promotor plasmid (0.5µg), either the human $GABA_B$ R1 and R2 or the rat MOR receptors (0.5µg), and either GINIP fused to GFP or GFP empty plasmid (1µg). Two days after transfection, tsA-201 cells were dissociated with Versene (Invitrogen), and plated at low density in 35 mm Petri dish for electrophysiological recordings. Macroscopic currents were recorded at room temperature using an Axopatch 200B amplifier (Molecular Devices). The extracellular solution contained the following (in mM): 135 NaCl, 20 TEACl, 5 BaCl2, 1 MgCl2, and 10 HEPES (pH adjusted to 7.25 with KOH, 330 mOsm) and the internal solution contained the following (in mM): 130 CsCl, 10 EGTA, 10 HEPES, 1 MgCl2, 4 Mg-ATP, 0,3 tris-GTP, and 2 CaCl2 (pH adjusted to 7,25 with KOH, 315 mOsm). Borosilicate glass pipettes with a typical resistance of 2–2.5 Mohm were used in voltage clamp studies. Recordings were sampled at 8 kHz and filtered at 2–5 kHz. Data were analyzed using pCLAMP10 (MolecularDevices), and GraphPad Prism (GraphPad) software. Baclofen and DAMGO were prepared as stock solutions and diluted at the distinct test concentrations in the extracellular medium supplemented with 05mg/Ml of BSA to prevent drug absorption on tubing. Control and drug containing solutions were applied by a gravity-driven homemade

perfusion device and control experiments were performed using the vehicle without drug. Results are presented as the mean +/- SEM, and *n* is the number of cells used.

Whole-Cell patch-clamp recording from spinal cord slices with attached dorsal root

Transverse spinal cord slices with attached dorsal roots from juvenile (P28 to P44) GINIP$^{-/-}$ and WT mice were prepared for whole-cell recording. Briefly, the animals were anesthetized using isoflurane for 5 min, then beheaded. The vertebral column and surrounding muscles were quickly removed and immersed in ice cold oxygenated low calcium artificial cerebro spinal fluid (ACSF) (in mM: NaCl 101; KCl 3.8; MgCl2 18.7, MgSO4 1.3;KH2PO4 1.2; HEPES 10; CaCl2 1; Glucose 1) after laminectomy, the spinal cord was gently removed and its lumbar part was placed into a small 3% agarose block. Spinal slices (300µm thick) were cut using a Leica VTS1000 vibratome, and transferred in warm (31°C) ACSF (in mM: NaCl 130.5; KCl 2.4;CaCl2 2.4; NaHCO3 19.5; MgSO4 1.3;KH2PO4 1.2; HEPES 1.25; glucose 10; pH 7.4) equilibrated with 95%O2-5%CO2 for at least one hour before starting patch clamp recordings. Spinal slices were placed in a recoding chamber bathed with warmed (31°C) ACSF Electrophysiological measurements were performed under the control of an Olympus BX51 microscope using a multiclamp 2B (Molecular devices). Patch pipettes (typically 7-11 Ω) were filled with Cs based pipette solution (in mM: CsMethaneSulfonate 120; CsCl 20; CaCl2 0.1; MgCl2 1.3; EGTA 1; HEPES 10; GTP 0.1; cAMP 0.2; Leupeptin 0.1; Na2ATP 3; D-Manitol 77; pH 7.3) to limit post synaptic effects of applied drugs. A glass suction electrode connected to a Master 8 (A.M.P.Instrument Ltd) stimulator was used to stimulate dorsal roots. Typically, a pair of high duration (500µs) high intensity stimulations (350µA) was used to recruit most primary afferent fibers in the recorded slice. 20 consecutive paired stimulations with 5s intervals were averaged. Paired pulse ratio was calculated as the ratio between the amplitude of the second and the first synaptic responses was calculated. Normalized synaptic responses were calculated by dividing the average response during drug

application by the average response measured in control. The protocol was repeated 5 times, in control and 1, 3, 5 and 7 minutes after drug application (bath application for 1 minute). The peak of the response (usually observed at 1 or 3 minutes) was used for statistical analysis. Liquid junction potentials (calculated value -16.5mV) are not corrected for. SNC80 was purchased from Tocris, DAMGO and Baclofen were purchased from Sigma-Aldrich. SNC80 and DAMGO superfusion was achieved in the presence of 1µM CGP55845A in order to prevent an indirect action on pair pulse ratio through local GABAergic interneurons. 2 way ANOVA with repeated measures was used to compare the effects of drugs in WT and GINIP$^{-/-}$ mice. Student test, and signed rank sum test were used to compare the relative decrease in EPSC amplitude between different GINIP+/+ and -/- mice.

EPAC-BRET experiments

tsA-201 cells tsA-201 cells were cultivated in DMEM supplemented with GlutaMax and 10% fetal bovine serum (Invitrogen) and plated onto 96 well plates. Cell transfection was performed using jet-PEI (QBiogen) with a DNA mix containing 40ng of the intracellular cAMP sensor EPAC-RLuc-YFP plasmid (Barak et al., 2008), 15ng of G protein coupled receptors encoding plasmids ($GABA_B$-R1 and GAB_B-R2, MOR or ProkR2), and 30ng of HIS-tagged GINIP or empty vector. Two days after transfection, BRET signal was measured using the ARPEGE pharmacological screening platform of the IGF (http://www.arpege.cnrs.fr). Wells were washed twice with PBS and 40 µl of TRIS-KREBS buffer was added to control wells followed by addition of 10 µl of a 25 µM coelenterazine solution (final concentration, 5 µM). Coelenterazine treated cells are stimulated with 50µM forskolin alone or in combination with 50 µM baclofen or 1µM DAMGO. In control experiments, Coelenterazine treated cells were stimulated with 50µM forskolin alone or in combination with 10 or 100nM Prokineticin. Plates were then placed into a Mithras LB940 instrument (Berthold Technologies, Bad Wildbad, Germany) that allowed the sequential integration of the luminescent signals detected

in the 465 to 505 nm and 505 to 555 nm windows using filters with the appropriate band pass and by using MicroWin 2000 software (Berthold Technologies). Reactions were measured during 16 minutes. The BRET signal was determined by calculating the ratio of the light emitted at 505 to 555 nm to the light emitted at 465 to 505 nm subtracted from the background: ((YFP/R-Luc)-Rluc alone)*1000).

Sindbis virus production and DRG neurons infection

The Epac-S^{H150} digested with HindIII was inserted into pSinRep5 (Invitrogen) digested with StuI and made blunt by Klenow and HpaI. Recombinant pSinRep5 constructs and helper plasmid pDH26S were transcribed in vitro into capped RNA using the Megascript SP6 kit (Ambion). Baby hamster kidney-21 (ATCC no. CCL-10) cells were electroporated with sensor-coding RNA and helper RNA (2.10^7 cells, 950 µF, 230 V) and incubated for 24 h at 37 °C in 5% CO2 in Dulbecco's modified Eagle Medium supplemented with 5% fetal calf serum before collecting cell supernatant containing the viruses. The virus titer (~10^9 infectious particles/ml) was determined after counting fluorescent baby hamster kidney cells infected using serial dilution of the virus stock. DRG neurons cultured on glass coverslip in 500µl of culture medium were infected with 5 µl virus per well and kept overnight at 37°C. The next morning, the medium was replaced. DRG neurons were labeled by incubating cells with Isolectin B4 conjugates with AlexaFluorR 568 dye (1:200, Invitrogen) prior to transfer into the recording chamber. During the experiment, cells were continuously perfused with artificial cerebrospinal fluid containing (in mM): 126 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2.5 KCl, 20 d-glucose, and 5 sodium pyruvate and saturated with 5% CO_2/95% O_2 at a rate of 2ml per minute. All the drugs were bath applied. Imaging was performed at 33°C.

cAMP imaging

Two-photon images were acquired with a custom-built 2-photon laser scanning microscope based on an Olympus BX51WI upright microscope (Olympus, Tokyo, Japan) with ×40 (0.8 NA) or ×20 (0.5 NA) water-immersion objectives and a titanium:sapphire laser (MaiTai HP; Spectra Physics, Ellicot City, MD, USA) as described earlier (Bonnot et al. 2014). Two-photon excitation for FRET imaging was performed at 850 nm for CFP excitation. A fluorescence cube containing 479/40 and 542/50 emission filters and a 506 nm dichroic beamsplitter (FF01-479/40, FF01-542/50, and FF506-Di02-25x36 Brightline Filters; Semrock) was used for the orthogonal separation of the two fluorescence signals. Two imaging channels (H9305 photomultipliers, Hamamatsu) were used for simultaneous detection of the two types of fluorescence emission. The identification of IB4 positive cells co expressing Epac S^{H150} was performed by exciting fluorophor 568 at 750 nm and using a fluorescence cube containing 525/45 Brighline and 617/73 Brightline filters (Semrock). The dichroic beamsplitter was Q570LP from Chroma

Images were analyzed with custom routines written in ImageJ (National Institutes of Health) and in IGOR Pro environment (Wavemetrics). The emission ratio was calculated for each pixel as fluorescence at 480nm over fluorescence at 535 nm for Epac-S^{H150}. To evaluate the impact of baclofen on cAMP variation induced by forskolin the amplitudes and the slope of the onset of the responses were calculated. The slope was calculated from the early region of the response onset using a linear fit with Igor Pro. The difference between the two responses was evaluated by calculating the ratio of the amplitudes in presence of baclofen and the amplitude on the control response on the same cell. The difference in onset kinetics was determined by calculating the ratio between the line slope of the baclofen response and the control response. Differences were considered significant when the p value was below 0.05 when using the student t-test.

Behavioral essays

All behaviour analyses were conducted on littermate males 8–12 weeks old. Animals were acclimated for one hour to their testing environment prior to all experiments that are done at room temperature (~22°C). Experimenters were blind to the genotype of the mice during testing. The number of tested animals is indicated in the figure legends section. Statistical significance was set to $p<0.05$ and assessed using one way ANOVA analysis followed by unpaired t-test. All error bars represent standard error of the mean (SEM). Gradient, Thermal plates, open-field, Hargreaves and Von Frey apparatus were from Bioseb instruments.

Open-field test

The Open-field test is commonly used to assess locomotor, exploratory and anxiety-like behavior. It consists of an empty and bright square arena (40x40x35cm), surrounded by walls to prevent animal from escaping. The animals were individually placed in the center of the arena and their behavior recorded with a video camera over a 5 minutes period and the time spent in the corner versus the center of the arena is recorded.

Rotarod test

A rotarod apparatus (LSI Letica Scientific Instruments) was used to explore coordinated locomotor and balance function in mice. Mice were placed on a rod that slowly accelerated from 4 rpm to 44 rpm with a constant speed of rotation over 5min and the latency to falloff during this period was recorded. The test was done 4 consecutive days. Each day, the animals were tested three times separated by at least 5 min resting period.

Temperature gradient arena

Response to temperature Gradient assay was performed as described previously (Moqrich et al., 2005). Briefly, mice were individually video tracked for 90 min in four separate arenas of the thermal gradient apparatus (Bioseb). A controlled and stable temperature gradient of 14°C to 55°C was maintained using two Peltier heating/cooling devices positioned at each end of the aluminium floor. Each arena was virtually divided into 15 zones of equal size (8 cm) with

a distinct and stable temperature. Floor temperature was measured with an infrared thermometer (Bioseb). The tracking was performed using a video camera controlled by the software provided by the manufacturer.

Hot plate test

To assess heat sensitivity, mice were placed individually on a metal surface maintained at 48°, 50°or 52°C and the latency to nociceptive responses are measured (licking, shaking of hind paws or jumping). To prevent tissue damage, mice were removed from the plate immediately after a nociceptive response or a cut-off 90s, 60s and 45s was applied respectively. Each mouse has been tested three times with a 5 min interval between each test. The withdrawal time corresponds to the mean of the three measures.

Cold plate

To test cold sensitivity, mice were placed individually into a plexiglass chamber maintained at 22°, 10°, 4°C or 0°C. The Rearing time of the mice is monitored for one minute. Each mouse is exposed three times to each temperature with a minimum of 5 min resting period between trials and one hour separating periods between temperatures.

Formalin test

Mice were housed individually into Plexiglass chambers 20 minutes before injection. Following intraplantar injection of 10µl of a 2% formalin solution (Fischer Scientific) into left hindpaw, time spent to shaking, licking or lifting the injected paw was monitored for 60min and analysed at 5 minutes intervals.

Complete Freund's Adjuvant (CFA)-induced heat hyperalgesia

To assess hind paw heat sensitivity, Hargreaves' test was conducted using a plantar test device (Bioseb). Mice were placed individually into Plexiglass chambers on an elevated glass platform and allowed to acclimatize for at least 30 minutes before testing. A mobile radiant heat source of constant intensity was then applied to the glabrous surface of the paw through

the glass plate and the latency to paw withdrawal measured. Paw withdrawal latency is reported as the mean of three measurements for both hind paws with at least a 5 minutes pause between measurements. IR source was adjusted to 20% and a cut-off of 20 s was applied to avoid tissue damage. Mice were first challenged to measure the baseline (acute response). We made an intraplantar injection of 10 µl of a 1:1 saline/CFA (Sigma, St. Louis, MO, USA) emulsion with a 30 gauge needle and measured thermal thresholds one and three days after the injection

Von Frey test of mechanical threshold

Mice were placed in plastic chambers on a wire mesh grid and stimulated with von Frey filaments (Bioseb) using two methods: the up-down method (Chaplan et al., 1994) starting with 1g and ending with 2.0 g filament as cutoff value and the two times five stimulations method (see below).

Unilateral peripheral mononeuropathy

For the chronic constriction of the sciatic nerve (CCI) model, unilateral peripheral mononeuropathy was induced in mice anaesthetized with Ketamine (40mg/kg ip) and Xylasine (5mg/kg ip) with three chromic gut (4_0) ligatures tied loosely (with about 1mm spacing) around the common sciatic nerve (Bennett and Xie, 1988). The nerve was constricted to a barely discernable degree, so that circulation through the epineurial vasculature was not interrupted (Descoeur et al., 2011). For the chronic constriction model, mechanical allodynia and hyperalgesia were assessed before the surgery and every other 5 days post-surgery using the Von Frey hair filaments of three different bending forces (0.07, 0.6 and 1.4 g). For each filament, two times five stimuli were applied with an interval of 3 to 5 seconds.

Spared nerve injury model of neuropathic pain

Spared nerve injury surgery was performed as described (Decosterd and Woolf, 2000). Briefly, mice were anesthetized with a mix of ketamine/xylazine while an incision was made

through the skin and thigh muscle at the level of the trifurcation of the sciatic nerve. The common tibial and peroneal nerve were ligated with 6.0 silk (Ethicon, Piscataway, NJ, USA) and transected, leaving the sural nerve intact. Mechanical thresholds were determined 7 days after the surgery, before and after drug administration and 30 days after the surgery. Mechanical threshold were determined using the up-down method.

Intrathecal injections of drugs

Animals were placed in habituation cages one hour prior to the administration of drugs. 10 µl of each compound were injected intrathecally in unanesthetized mice. Successful placement of the needle was confirmed by flick of the tail. Baclofen (sigma aldrich) was dissolved in H_20 solution (pH7.6), SNC80 (tocris bioscience) was dissolved in 100mM HCl solution and DAMGO (sigma aldrich) was dissolved in saline (0.9% NaCl) solution. Response to mechanical stimulations was recorded 15 minutes after drug administration using the up-down method.

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