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Colorectal cancer cells hijack a brain-gut polysynaptic circuit from the lateral septum to enteric neurons to sustain tumor growth V.2

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Abstract

The bidirectional interaction between the brain and peripheral tumors is critical but poorly understood. Here we show GABAergic neurons in the lateral septum (LS), a key brain region implicated in emotional regulation, connect via a polysynaptic circuit to enteric cholinergic neurons that send nerve fibers into the tumor microenvironment (TME), which were then hijacked by colorectal cancer (CRC) cells to sustain tumor growth in mice. Functionally, activation of this septo-enteric circuit induces GABA release from enteric cholinergic neurons, which in turn activates epsilon-subunit-containing GABAA receptors on tumor cells. Notably, chronic restraint stress potentiates activity within this circuit, exacerbating tumor progression. Clinically, CRC patients exhibiting elevated neuronal activity in the septal region present with larger primary tumors. Collectively, our findings uncover a stress-sensitive septo-enteric polysynaptic pathway exploited by cancer cells to promote tumor growth, underscoring the previously unrecognized role of LS-mediated neural circuitry and psychological stress in cancer progression

Guidelines

- All procedures involving animals or human samples must strictly follow the ethical and operational guidelines set by the relevant institutional committees.
- Only personnel trained in animal handling, surgical procedures, behavioral stress paradigms, and tissue collection should perform the protocol.
- Carefully review the entire protocol and prepare all reagents, animals, and instruments before starting.

Materials

	Material	Supplier	Catalog #
	Neurobasal-A	Gibco	10888022
	B-27	Gibco	17504044
	GlutaMAX	Gibco	35050061
	Antibiotic-Antimycotic	Gibco	15240062
	puromycin	MP Biomedicals	58-58-2
	CCK-8	Dojindo	CK04
	azoxymethane	MP Biomedicals	25843-45-2
	dextran sulfate sodium	MP Biomedicals	9011-18-1
	GABRE	Abcam	ab35971
	GAD67	Sigma-Aldrich	MAB5406
	GAPDH	Proteintech	60004-I-Ig
	β-actin	Proteintech	66009-1-Ig
	PrimeScript™ RT Reagent Kit	TaKaRa	RR037A
	the Universal Multiplex RNA In Situ Hybridization Kit	Pinpoease	PIF2000
	GAD65 probe hybridization solution	144171-B2	144171-B2
	collagenase I	MP Biomedicals	9001-12-1

Troubleshooting

Safety warnings

- This protocol may involve biohazardous materials, anesthetics, and procedures that induce psychological stress in animals.
- Appropriate personal protective equipment (PPE), including lab coat, gloves, and eye protection, must be worn at all times.
- All procedures should be performed in approved biosafety-level environments, and all waste should be disposed of following institutional biosafety protocols.

Ethics statement

- Animal Studies: All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committees (IACUC) of Southern Medical University. Experiments were conducted in accordance with national guidelines for the ethical treatment of laboratory animals.
- Human Samples: Human colorectal cancer samples and clinical data were obtained from Sun Yat-sen Memorial Hospital, Sun Yat-sen University. Informed consent was obtained from all participants. The study was reviewed and approved by the Institutional Review Board (IRB) of Sun Yat-sen University and conducted in accordance with the Declaration of Helsinki. All samples and data were anonymized before use in research.

Analysis of tumors from human CRC patients

1 Tumor samples along with adjacent normal tissues were collected from CRC patients at Sun Yat-sen Memorial Hospital between April 2012 and November 2017, following approval from the Medical Ethics Committee of Sun Yat-Sen Memorial Hospital. Tumor tissues were subsequently analyzed using immunohistochemistry (IHC) and immunofluorescence techniques. The tumor volume for each patient was calculated using the formula: (longest diameter × vertical diameter × depth of invasion) / 2 (cm^3).
For the assessment of NF-L+ and ChAT+ nerve fibers, brown-stained structures representing nerve fibers were quantified, and the corresponding areas were measured. Consecutive brown-stained structures were counted as a single nerve fiber. The number of nerve fibers was normalized to a density metric, calculated as the number of nerve fibers per tumor area of 1 × 108 μm^2. The density of nerve fibers was determined by dividing the total area occupied by nerve fibers by the total tumor area, with the result multiplied by 100%. To classify tumors based on nerve fiber density, the median value was used as a cut-off, separating patients into high (> median) and low (≤ median) nerve fiber density groups.
For the evaluation of GAD65/67+ and NF-L+ co-expressed nerve fibers, GAD65/67 was visualized using green fluorescence while NF-L was marked with red fluorescence. The number and area of co-expressed nerve fibers were quantified, with consecutive fluorescence signals counted as a single nerve fiber. The normalization and density calculations followed the same method as described for NF-L+ and ChAT+ nerve fibers.
To analyze TSPAN1 expression, the IHC score was derived from both staining intensity and the percentage of positive tumor cells

Citation

Jiang SH, Zhu LL, Zhang M, Li RK, Yang Q, Yan JY, Zhang C, Yang JY, Dong FY, Dai M, Hu LP, Li J, Li Q, Wang YH, Yang XM, Zhang YL, Nie HZ, Zhu L, Zhang XL, Tian GA, Zhang XX, Cao XY, Tao LY, Huang S, Jiang YS, Hua R, Qian Luo K, Gu JR, Sun YW, Hou S, Zhang ZG
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. Staining intensity was scored as follows: no staining (0), faint yellow (1), claybank (2), and dark brown (3). The percentage of positive tumor cells was assessed on a scale of 0

to 100%. The final IHC score was calculated by multiplying the staining intensity score by the percentage of positive tumor cells, yielding a final score range of 0 to 300.

Primary culture of mouse DRG neurons

2 Male C57BL/6J mice, aged 6 to 8 weeks, were used for this experiment. After anesthetizing the mice with pentobarbital sodium (60 mg/kg, intraperitoneally), the spinal column was excised from the cervical to the caudal vertebrae

Citation

Sleigh JN, Weir GA, Schiavo G (2016)
. A simple, step-by-step dissection protocol for the rapid isolation of mouse dorsal root ganglia..

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Citation

Maurel P (2018)
. Preparation of Neonatal Rat Schwann Cells and Embryonic Dorsal Root Ganglia Neurons for In Vitro Myelination Studies..

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. The spinal cord was carefully dissected, and the DRG on both sides were removed using fine forceps under a stereomicroscope. The ganglia were then incubated in a solution of 0.5% collagenase I (CAS No. 9001-12-1, MP Biomedicals) and 0.05% trypsin at a volume ratio of 1:1 at 37°C for 1 hour. Digestion was halted by the addition of DMEM/F12 containing 5% FBS. The resulting solution was filtered through a 70-µm cell strainer and centrifuged at 136 xg for 5 minutes. The cell pellet was resuspended in DMEM/F12 supplemented with 5% FBS and plated onto culture dishes pre-coated with poly-D-lysine (0.1 mg/mL). After 24 hours, the medium was replaced with a neuronal culture medium composed of Neurobasal-A (Cat. No. 10888022, Gibco), B-27 (1:50, Cat. No. 17504044, Gibco), GlutaMAX (1:100, Cat. No. 35050061, Gibco), and Antibiotic-Antimycotic (1:200, Cat. No. 15240062, Gibco). Following 5 days of neuronal growth,

tumor cells intended for co-culture were introduced, or the culture medium was harvested. To identify potential synapses, SW480-GFP or CMT93-GFP cells were added to the culture on Day 3, followed by a co-culture period of 48 hours before analysis. To block the nerve fibers, BTXA was introduced into the culture on Day 4 at a final concentration of 0.5 U/mL, and the cells were subsequently analyzed or the culture medium collected after another 24 hours. Additionally, to inhibit GAD activity, ChA was added on Day 4 at a final concentration of 50 µM, with culture medium collected after 24 hours.

Construction of cell lines with stable knockdown of GABRE/GabreUntitled section

3 Lentivirus and control viruses containing RNA interference fragments for GABRE/Gabre were procured from Guangzhou FulenGen Co., Ltd. The target sequences for the human GABRE gene included one control sequence and three distinct shRNAs: ACAGAAGCGATTGTTGATC, TCCTATCCTGAGAATGAGATG, AGCAGGCGGTTTGGCTATGTT, and GTCTCCTATATCACAGCCTTG. For the mouse Gabre gene, the corresponding sequences were similarly categorized into one control and three shRNAs: ACAGAAGCGATTGTTGATC, CCTGACCTACAATAATATTAA, GCGTCTTCGTTACAATGACAC, and GCTTCTCGAATCCTTAACACC. Each vector incorporated a puromycin resistance gene and the reporter gene EGFP. Cells exhibiting optimal growth conditions were seeded into 24-well plates 24 hours prior to viral infection, achieving approximately 50% confluency on the day of infection. Following the manufacturer's instructions, the viral solution was introduced into fresh culture medium. The plates were subsequently incubated in a humidified environment with 5% CO2 at 37°C. After 48 hours, the medium was refreshed with culture medium supplemented with puromycin at a concentration of 10 µg/mL (CAS No. 58-58-2, MP Biomedicals). One week later, upon confirming the absence of dead cells in the medium, the cells were digested and subsequently transferred into 10-cm dishes for further culture.

Proliferation assay in vitro

4 Following digestion, 2,000 cells were seeded into each well of a 96-well plate. The plates were incubated in a humidified environment with 5% CO2 at 37°C for 24 hours. After incubation, the culture medium was removed, and 110 µL of fresh culture medium containing CCK-8 (dilution 1:10, Cat. No. CK04, Dojindo) was added to each well. The plates were then incubated at 37°C for an additional 2 hours. The optical density (OD) at 450 nm was subsequently measured using a microplate reader (PerkinElmer). For experiments involving drug treatment, the drugs were added to the culture medium and incubated for 24 hours. After this incubation period, 110 µL of fresh culture medium containing CCK-8 was added to each well, followed by another 2-hour incubation at 37°C. The OD values were again measured at 450 nm to assess cell viability. Cell viability was calculated using the formula:

$$\text{Cell viability} = \frac{\text{ODobserved} - \text{ODblank}}{\text{ODcontrol} - \text{ODblank}} \times 100\%$$

PDX tumor model

5 Fresh human CRC tissues were aseptically collected and cut into 5–10 mm^3 fragments using a scalpel and forceps. Six-week-old male B-NDG mice were anesthetized via intraperitoneal injection of pentobarbital sodium (60 mg/kg). The right dorsal skin was elevated with forceps, and a 1-cm incision was made, followed by blunt dissection with scissors to create a subcutaneous pocket. Carefully, the tumor fragments were placed into the subcutaneous notch located in the axillary region, ensuring minimal contact between the tumor fragments and the skin surface. After the placement of the fragments, the incision was sutured closed, and the mice were returned to an electric blanket until fully recovered from anesthesia. Four weeks post-implantation, the mice were euthanized, and the resulting subcutaneous tumors were harvested for analysis.

Tumor cells implanted on the sciatic nerve

6 CMT93 cells were utilized for this model. The cells were digested, washed three times with PBS, resuspended in PBS, and placed on ice prior to injection. The procedure for exposing the sciatic nerve followed established protocols

Citation

Richner M, Bjerrum OJ, Nykjaer A, Vaegter CB (2011)
. The spared nerve injury (SNI) model of induced mechanical allodynia in mice..

https://doi.org/pii:3092.10.3791/3092 LINK

. Eight-week-old male C57BL/6J mice or ChAT-Cre/Ai14 mice were anesthetized via intraperitoneal injection of pentobarbital sodium (60 mg/kg). The mice were positioned on a clean foam board, and their feet were secured with surgical tape. Hair from the knee to the hip area was removed using a shaving blade, and the surgical field was disinfected with 70% ethanol. A 1-cm longitudinal incision was made in the thigh using a scalpel. The incision was deepened through blunt dissection with sterile scissors to access the underlying muscle. The muscle adjacent to the femur was separated, allowing for visualization of the sciatic nerve. Subsequently, 4×10^5 CMT93 cells were injected into

the sciatic nerve using a microsyringe at a volume of 20 µL. The muscle layer was closed with absorbable sutures, followed by suturing of the skin. The mice were then returned to their clean home cages. After a period of 4 weeks, the tumors that had formed on the sciatic nerve were excised and weighed. Tumor size was measured using vernier calipers, with tumor volume calculated using the formula: longest diameter × vertical diameter × height / 2 (in mm^3). To inhibit nerve activity, BTXA was injected into either the sciatic nerve or tumor at a concentration of 0.5 U/mL, with each mouse receiving 0.05 U of BTXA in a volume of 0.1 mL.

AOM/DSS model of colitis-associated cancerUntitled section

7 Six-week-old male C57BL/6J mice were administered an intraperitoneal injection of azoxymethane (AOM) at a dosage of 10 mg/kg (CAS No. 25843-45-2, MP Biomedicals) once a week for the initial three weeks. Following this, the mice were subjected to a 1% dextran sulfate sodium (DSS) solution (CAS No. 9011-18-1, MP Biomedicals) for one week at 4, 7, and 10 weeks. Approximately 22 weeks post-treatment, the mice were euthanized, and colorectal tissues were harvested for analysis. The number of tumors present was quantified, and the diameter of each tumor was measured using vernier calipers. The area of round tumors was calculated as π x $(\text{diameter}/2)^2$. For oval tumors, the area was calculated as π x $(\text{longest diameter} + \text{its vertical diameter}/2)^2$.

ApcMin/+ mice

8 Male $Apc^{Min/+}$ mice were utilized in this study and bred under standard conditions. At 24 weeks of age, the mice were euthanized, and intestinal tumors were examined. The total number of tumors was recorded, and the diameter of each tumor was measured using vernier calipers. The area of round tumors was calculated as π x $(\text{diameter}/2)^2$. For oval tumors, the area was calculated as π x $(\text{longest diameter} + \text{its vertical diameter}/2)^2$.

Western blotting

9 Protein samples were mixed with SDS loading buffer, boiled for 10 minutes, placed on ice for 2 minutes, and subjected to SDS-PAGE gel electrophoresis (5% stacking gel, 8%-12% separating gel). Following electrophoresis, proteins were electrotransferred from the PAGE gel to a PVDF membrane (Millipore) using the Bio-Rad micro electrotransfer system at 4°C and 200 mA for 1.5 hours. The PVDF membranes were then incubated with 20 mL blocking solution (TBST containing 5% skim milk powder) for 1 hour at room temperature, followed by incubation with primary antibody at 4°C for 20 hours, and HRP-conjugated secondary antibody (BOSTER) for 1 hour at room temperature. After allowing the PVDF membrane to slightly dry, chemiluminescence reagent was added and incubated for 1 minute. The membrane was quickly wrapped in plastic and placed on the inner plate of the chemiluminescence imaging system. Images were scanned and

captured using a Bio-Rad chemiluminescence instrument. The average density of gray values was analyzed with ImageJ software(version 1.53c), and the ratio of target gene gray values to reference gene gray values was calculated for statistical analysis. The antibodies used in this experiment included: GABRE (1:500, ab35971, Abcam), GAD67 (1:500, MAB5406, Sigma-Aldrich), GAPDH (1:5000, 60004-I-Ig, Proteintech), and β-actin (1:5000, 66009-1-Ig, Proteintech).

Fluorescence quantitative PCR

10 Total RNA was extracted from cultured cells, and reverse transcription was performed according to the protocol provided in the PrimeScript™ RT Reagent Kit (RR037A, TaKaRa). β-actin was used as an internal control, and cDNA amplification was conducted using a 7500 Real-Time PCR system (ABI) to detect the Ct value of each template. The fold change in expression levels of the target gene between the experimental group and control group was calculated using the formula: Folds = $2^{-\Delta\Delta Ct}$, where ΔΔCt = (Ct(target gene) - Ct(β-actin)) experimental group - (Ct(target gene) - Ct(β-actin)) control group. The sequences of the primers are provided in Supplementary Table 2.

Transcriptome sequencing

11 Total RNA was extracted using the TRIzol reagent (Invitrogen) in accordance with the manufacturer's protocol. RNA purity and quantification were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific), while RNA integrity was evaluated with an Agilent 2100 Bioanalyzer (Agilent Technologies). Libraries were constructed using the VAHTS Universal V6 RNA-seq Library Prep Kit, following the manufacturer's instructions. Transcriptome sequencing and subsequent analysis were conducted by OE Biotech Co., Ltd. (Shanghai, China). The libraries were sequenced on a Illumina NovaSeq 6000 platform, generating 150 bp paired-end reads. Clean reads were mapped to the reference genome using HISAT2, and fragments per kilobase of transcript per million mapped reads (FPKM) for each gene were calculated. The read counts for each gene were obtained using HTSeq-count. Principal component analysis (PCA) was performed in R (version 3.2.0) to evaluate the biological reproducibility of the samples. Differential expression analysis was carried out using DESeq2, with a significance threshold set at a Q value < 0.05 and a fold change > 2. A total of 852 differentially expressed genes (DEGs) were identified. Hierarchical clustering analysis of DEGs was conducted using R (version 3.2.0) to illustrate the expression patterns of genes across different groups and samples. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of DEGs were performed based on the hypergeometric distribution to identify significantly enriched terms, also using R (version 3.2.0). Additionally, Gene Set Enrichment Analysis (GSEA) was conducted using GSEA software (mh.all.v2023.2.Mm.symbols.gmt).

Quantification analysis of neurons and nerve fibers

12 To quantify the prevalence of PRV or HSV-infected neurons in various brain regions and the rectum of infected mice, whole brains and spinal cords were sectioned into 40 µm slices, with one slice collected every three sections. These slices were imaged using fluorescence confocal microscopy (Nikon). The number of infected neurons in each brain region was quantified, with the highest counts from each region presented in the figures and Supplementary Table 1.
For rectal tumors, whole tumors were also sectioned into 40 µm slices, and three slices from the right-central region were stained for NF-L or ChAT. The percentage of infected nerve fibers within the tumors was calculated by dividing the area occupied by infected nerve fibers by the total area of NF-L or ChAT-positive nerve fibers. This percentage was averaged across the three slices per mouse and presented in the figures.
In rectal tissue, segments approximately 5-10 mm from the tumor were sectioned into 40 µm slices and stained for NF-L or ChAT. Random 1 mm segments were selected from each slice, and the percentage of infected neurons was calculated as described above. The average percentage from three 1 mm segments per mouse was also presented in the figures.
To assess tumor innervation in tumors treated with GW441756, whole tumors were sectioned into 40 µm-thick slices. Representative slices from the right-central region of each tumor were stained with NF-L antibody. The percentage of NF-L-positive nerve fibers was calculated by dividing the area occupied by these fibers by the total tumor area on each slice. Percentages from at least three slices per mouse were used for further analysis.
To quantify the percentage of ChAT-positive cells in colons and rectums of mice treated with CRS, paraffin sections (4 µm thick) were prepared. The myenteric plexus within a randomly selected 1 mm segment of the colon and rectum was analyzed. For each mouse, at least four 1 mm segments were selected for analysis, and the area of the muscular layer within the same region was measured. The percentage of ChAT-positive cells was calculated by dividing the area occupied by these cells by the total area of the muscular layer.

Methodology for RNA in situ hybridization and immunohistochemistry in mouse brain sections

13 Mice were anesthetized with pentobarbital sodium (60mg/kg) and subjected to cardiac perfusion with 1×PBS. Fresh brain tissue was dissected and fixed in 4% paraformaldehyde for 24 hours. Following fixation, the tissue was rinsed with wate and dehydrated in a 30% sucrose solution until it sank. The brain sections with a thickness of 10 µm were washed twice with PBS for 2 minutes each. The sections were then mounted onto adhesive-coated glass slides. After air-drying, the sections were incubated in 4% pre-chilled paraformaldehyde at 4°C for 30 minutes for fixation. The slides were

subsequently washed twice with PBS for 2 minutes each. To dehydrate, the sections were sequentially immersed in 50%, 70%, and 100% ethanol solutions, with each step lasting 5 minutes at room temperature. Following the manufacturer's protocol for the Universal Multiplex RNA In Situ Hybridization Kit (Cat. No. PIF2000, Pinpoease), the slides were placed in a humidified chamber. Pre-treatment solution A (50 µL) was applied to each section, and the slides were incubated at room temperature for 30 minutes. After incubation, the sections were washed three times with ultrapure water for 1 minute each. Next, the slides were immersed in boiling pre-reaction solution B for 3 minutes, followed by a quick transfer to ultrapure water at room temperature for washing (three times, 1 minute each). After removing excess liquid, 50 µL of pre-warmed digestion enzyme solution was applied to the sections, and the slides were incubated in a hybridization chamber at 2 minutes. The slides were then washed three times with ultrapure water, each wash lasting 1 minute. To maintain moisture, reaction solutions were added immediately to the hydrophobic barrier. First, 50 µL of pre-warmed GAD65 probe hybridization solution (Cat. No. 144171-B2, Pinpoease) was applied and incubated at 40°C for 2 hours. After incubation, the probe solution was discarded, and the slides were washed in a washing buffer for 3 minutes, repeating this step three times. Following this, 50 µL of pre-warmed reaction solution 1 was added to each section and incubated at 40°C for 25 minutes, followed by three washes with washing buffer for 2 minutes each. This was followed by the addition of 50 µL of pre-warmed reaction solution 2, and incubation at 40°C for 15 minutes, with three subsequent washes for 2 minutes each. Next, 50 µL of warmed HRP inhibitor solution was added to the sections and incubated at 40°C for 15 minutes, followed by three 2-minute washes. The slides were then incubated with 50 µL of pre-warmed reaction solution 3 at 40°C for 15 minutes, followed by three washes, each for 2 minutes. After washing, 50 µL of freshly prepared 570 fluorescence reaction solution was applied to the tissue sections within the hydrophobic circle. The slides were incubated in the dark at room temperature for 30 minutes, followed by three washes with washing buffer, each lasting 2 minutes. The sections were then washed three times with 1× PBS, each for 2 minutes, before being air-dried. Subsequently, 50 µL of a 0.3% Triton X-100 and 5% BSA solution was applied within the hydrophobic circle, and the slides were incubated at 40°C for 1 hour. After washing three times with 1× PBS, the slides were incubated with 50 µL of diluted GFP primary antibody for 1 hour at 40°C, followed by three washes with PBS. Next, 50 µL of anti-rabbit 488 secondary antibody was applied to the slides, and incubation was carried out for 1 hour at 40°C. After washing the slides three times with PBS, they were air-dried, and a DAPI mounting medium was added to cover the sections. Finally, images were captured using a laser scanning confocal microscope.

Protocol references

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2. Sleigh, J.N., Weir, G.A. & Schiavo, G. A simple, step-by-step dissection protocol for the rapid isolation of mouse dorsal root ganglia. BMC Res Notes 9, 82 (2016).
3. Maurel, P. Preparation of Neonatal Rat Schwann Cells and Embryonic Dorsal Root Ganglia Neurons for In Vitro Myelination Studies. Methods Mol Biol 1739, 17-37 (2018).
4. Richner, M., Bjerrum, O.J., Nykjaer, A. & Vaegter, C.B. The spared nerve injury (SNI) model of induced mechanical allodynia in mice. J Vis Exp (2011).

Citations

Step 1

Jiang SH, Zhu LL, Zhang M, Li RK, Yang Q, Yan JY, Zhang C, Yang JY, Dong FY, Dai M, Hu LP, Li J, Li Q, Wang YH, Yang XM, Zhang YL, Nie HZ, Zhu L, Zhang XL, Tian GA, Zhang XX, Cao XY, Tao LY, Huang S, Jiang YS, Hua R, Qian Luo K, Gu JR, Sun YW, Hou S, Zhang ZG. GABRP regulates chemokine signalling, macrophage recruitment and tumour progression in pancreatic cancer through tuning KCNN4-mediated Ca(2+) signalling in a GABA-independent manner.
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Step 2

Maurel P. Preparation of Neonatal Rat Schwann Cells and Embryonic Dorsal Root Ganglia Neurons for In Vitro Myelination Studies.
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Step 2

Sleigh JN, Weir GA, Schiavo G. A simple, step-by-step dissection protocol for the rapid isolation of mouse dorsal root ganglia.
https://doi.org/10.1186/s13104-016-1915-8

Step 6

Richner M, Bjerrum OJ, Nykjaer A, Vaegter CB. The spared nerve injury (SNI) model of induced mechanical allodynia in mice.
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