NCBI Bookshelf. A service of the National Library of Medicine, National Institutes of Health.

Zhu MX, editor. TRP Channels. Boca Raton (FL): CRC Press/Taylor & Francis; 2011.

Chapter 13 Methods Used for Studying TRP Channel Functions in Sensory Neurons

Louis S Premkumar.

13.1. INTRODUCTION

Over 30 members of the transient receptor potential (TRP) family of ion channels have been cloned. Several of these TRP channels are expressed in subpopulations of primary sensory afferent neurons. TRP channels are Ca^{2+} permeant nonselective cation channels and are activated by physical (temperature and mechanical force) and chemical stimuli.[1–8]

The cell bodies of sensory neurons innervating the head and neck are located in the trigeminal ganglia (TG), and the cell bodies innervating the rest of the body are located in the dorsal root ganglia (DRG). TG neurons form their synapses at the caudal spinal trigeminal nucleus (CSTN), and DRG neurons form synapses at the dorsal horn (DH) lamina I and lamina II of the spinal cord. TRP channels are distributed in the peripheral and central terminals of sensory neurons, and they play a role in nociceptive transmission by initiating action potentials at the nerve terminals and modulating neurotransmitter release at the first sensory synapse.

Sensory TRP channels are sensitized by pro-inflammatory agents and mediate heightened pain sensitivity.[3,9–12] In order to gain insight into the process of nociception, it is first necessary to understand the characteristics of TRP channels that respond to physical and chemical stimuli. Nociceptive TRP channels are targets for next-generation analgesics. In fact, some TRP channels are specifically involved following inflammation.[4,7,12] On the basis of their distribution in nociceptors, the targets include TRPVanilloid 1, TRPVanilloid 3, TRPVanilloid 4, TRPAnkyrin 1, and TRPMelastatin 8. The methods that can be adopted to study the expression and function of TRP channels in sensory neurons are described in this chapter.

13.2. IN VITRO NEURONAL PREPARATIONS TO STUDY SENSORY NEURONS

13.2.1. DRG and TG Neuronal Cultures from Embryonic Rats or Mice

The advantages of using embryonic DRG/TG neurons in culture are that they can be used after four days and can survive for weeks. Only embryonic neurons grown in culture have been shown to readily form synaptic connections between sensory neurons and second-order neurons.

Pregnant rats/mice are deeply anesthetized with a lethal dose of Nembutal (80 mg kg^{-1}, I.P.), and embryonic day 18 (E18) embryos are removed. The embryos are decapitated in hypothermic anesthesia. The heads are used to collect TG, and the bodies are used for collecting DRG. Both TG and DRG are removed under a dissecting microscope.

For TG dissections, the head of the embryo is hemisected sagittally, and TG is exposed in the medial region. For DRG dissections, the skin on the back of the embryo is split with fine forceps, and the spinal cord is exposed. The spinal column is removed, and DRG can be located on either

side of the vertebral column. TG/DRG are removed and placed into a 15-mL conical tube with a 5-mL cold L-15 medium (Sigma, St. Louis, MO, USA) and centrifuged for 5 min at 500 × g.

After centrifugation, the supernatant is discarded, and the pellet is resuspended in 5 mL Hank's buffered salt solution (Ca^{2+} and Mg^{2+}-free, HBSS) containing 5 mg collagenase D, Worthington type 2 (0.1%) (Roche Molecular Biochemicals, Indianapolis, IN, USA), and 5 mg trypsin, type 1 (0.1%) (Sigma). DRG are digested for 45 min in a 37°C water bath with rotation at 150 rpm. After digestion, the samples are centrifuged at 500 × g for 5 min. The supernatant is discarded, and the pellet is resuspended in an 8-mL Neurobasal medium (NBM) supplemented with B-27, a serum free supplement (2 mM), L-Glutamine (10 µL/mL) (Invitrogen, Carlsbad, CA, USA), nerve growth factor (NGF 2.5S, 100 ng/mL, Sigma), and penicillin–streptomycin (Invitrogen). DRG are triturated 30–50 times with a siliconized fire-polished 9-inch Pasteur pipette and plated on glass cover slips previously coated with poly-D-lysine (10 µg/mL, Sigma) in a 24-well plate containing 0.5 mL medium per well. Samples are incubated at 5% CO_2 and 37°C. On day 2, the medium is changed. The neurons are ready to use after 4 days in culture.

13.2.2. DRG and TG Dissociated Neurons from Adult Rats or Mice

In order to address ontogenic changes and while studying disease models, it is necessary to use sensory neurons from adult animals. Rats/mice are deeply anesthetized with isoflurane (Abbott Labs, Chicago, IL, USA) and, when they no longer respond to the foot pinch, are decapitated for TG collection. The brain is removed from the head, and TG can be located in the trigeminal notch of the petrosal bone. For DRG collection, the spinal column is removed, trimmed, cut into two segments, and placed into an L-15 medium in a Petri dish. The dorsal side of the vertebral column is cut by a pair of fine scissors, and the spinal cord is exposed. DRG are located in the intervertebral foramen. TG and DRG are collected with fine forceps and placed in an ice-cold L-15 medium. The processing procedure is the same between embryonic and adult tissues. Neurons are maintained at 37°C in an incubator (humidified atmosphere of 5% CO_2) and are used within 24 hours.

13.2.3. DRG-DH Co-Cultures from Embryonic Rats or Mice

In order to study the role of TRP channels expressed in the central terminals of DRG/TG neurons, embryonic DRG/TG neurons are co-cultured with spinal DH or CSTN neurons. Embryonic neuronal cultures readily form synapses as compared to adult neurons.

Embryonic DRG/TG neurons are prepared as described above. The spinal cord is collected after the meninges are pulled away with two pairs of forceps. The spinal cord is placed on its ventral side, and the medial ventral horn part is pressed down. The DH, which is located on both lateral sides, is cut by a blade longitudinally. The DH of the spinal cord is transferred to a 15-mL tube containing HBSS with digestive enzymes, and neurons are dissociated as described above. The DH, together with the DRG neurons, is plated on poly-D-lysine coated glass cover slips. In some dishes, only DH or DRG neurons are plated to obtain DH or DRG neuronal monocultures, respectively. The co-cultures are ready to use after one week. DRG neurons and DH neurons are easily distinguished by their morphology. DRG neurons are rounded with long axons, whereas DH neurons are pyramidal shaped. In a similar manner, CSTN neurons can be isolated for TG-CSTN neuronal co-cultures.

13.2.4. Spinal Cord and CSTN Slice Preparations

Rats/mice between the ages of 2 and 10 weeks old are anesthetized with isoflurane and decapitated with a guillotine. The brain and the spinal cord are removed and placed in an ice-cold sucrose solution for 2 min containing (in mM): 209 sucrose, 2 KCl, 1.25 NaH_2PO_4, 5 $MgCl_2$, 0.5 $CaCl_2$, 26 $NaHCO_3$ and 10 D-glucose, titrated to pH 7.4 (290 mOSM). The solution/tissue is aerated with a 95% O_2–5% CO_2 gas mixture. Horizontal slices of the CSTN are obtained upon isolating a section of medulla trimmed caudally at the cervical spinal cord and rostrally at the obex.[13] Transverse spinal cord slices are obtained after hydraulic extrusion of the spinal cord. An OTS 3000 vibratome (Leica, Nussloch, Germany) or a Precisionary VF-200 vibratome (Greenville, NC, USA) is used to cut 250-µm slices of the desired tissue in ice-cold aerated sucrose. Slices are immediately harvested and placed in an incubation chamber containing oxygenated hibernate A (Brain Bits, Springfield, IL, USA) at 32°C. The temperature of the incubation chamber is allowed to fall to room temperature as slices incubate, and slices are allowed to recover for 1 hour after slicing before being placed in the recording chamber.

13.3. EXPRESSION OF TRP CHANNELS IN DRG/TG NEURONS

13.3.1. RT-PCR

The reverse transcription-polymerase chain reaction (RT-PCR) technique is used to identify the RNA of interest in a given tissue. Total RNA from DRG/TG neurons is extracted by Trizol reagent (Invitrogen) and reversely transcribed to cDNA by using a cDNA synthesis kit (Promega, Madison, WI, USA). PCR is performed by using different cDNAs as templates in 30 cycles with 30-s denaturation at 95°C, 30-s annealing at 58°C, and 30-s extension at 68°C using PCR green master mix (Promega). Appropriate primer pair sequences and orientation (F, forward; R, reverse) must be designed and obtained; for example, to identify the presence of TRPV1 mRNA, the primer pair is F: accacggctgcttactatcg, R: ctccagtgacacg gaaatagtcc. A housekeeping gene, hypoxanthine guanine phosphoribosyl transferase I (HPRT), is used as a control, and the primer pair is F: gcttcctcctcagaccgcttt, R: ctggttcatcatcgctaatcacg. Samples are treated with DNase (Ambion, Austin, TX, USA) followed by cDNA generation. Products are run in an agrose gel (1.5%) with ethidium bromide in the Tris/Borate/EDTA (TBE) buffer. The gel is imaged by the Versa Doc system (Bio-Rad, Hercules, CA, USA), and the band density is quantified by Quantity One (Bio-Rad).

Because DRG neurons consist of multiple-cell types, single-cell RT-PCR can be performed by harvesting cell contents in identified cells after recording currents using a patch-clamp technique as described by Monyer and Jonas.[14]

To quantify the amount of RNA, real-time RT-PCR is performed with SYBR Green fluorescent tag (New England Biolabs, Ipswich, MA, USA) using a BioRad Cycler. Real-time RT-PCR conditions are as follows: 95°C for 15 min, then 25 cycles of 95°C for 10 s, 62°C for 25 s, then 72°C for 30 s. Results are quantified using HPRT as a reference in the comparative C_T method. For this method, the amplification efficiencies of the target gene and the housekeeping gene have to be similar. The comparative C_T method is also known as the $2^{-\Delta\Delta C_T}$ method, where

$$\Delta\Delta C_T = \Delta C_{T,sample} - \Delta C_{T,reference}$$

Here, $\Delta C_{T,sample}$ is the C_T value for any sample normalized to the endogenous housekeeping gene, and $\Delta C_{T,reference}$ is the C_T value for the calibrator also normalized to the endogenous housekeeping gene.

13.3.2. Immuimohistochemistry

Rats/mice are anesthetized by ketamine and xylazine (85 and 5 mg/kg, respectively, I.P.) and are transcardially perfused with freshly made fixative consisting of 4% paraformaldehyde in Sörenson's K-Na phosphate buffer. To prepare the fixative, 1.78 g monobasic potassium phosphate and 14.38 g dibasic sodium phosphate are dissolved in 750 mL dH_2O, and 250 mL of 16% of paraformaldehyde stock solution is added and titrated to pH 7.4. Tissue samples of spinal cord segments, DRG, and paw skin are harvested and kept in the same fixative for 2 hours. After immersion in 15 and 30% sucrose for successive 24-hour periods at 4°C, tissues are then embedded with an embedding medium (Triangle Biomedical Sciences, Durham, NC, USA) and quickly frozen with liquid nitrogen. The spinal cord, DRG, and paw skin tissues are sectioned into 20-, 10-, and 20-μm sections, respectively, using a cryostat (Leica CM 1850, Nussloch, Germany). Immunostaining can be done either on slides or on free-floating tissues. After samples are permeabilized with phosphate buffered saline (PBS) containing 0.1% Triton X-100 for 20 min and blocked with 10% donkey serum for 30 min, the sections are incubated with polyclonal rabbit anti-TRP antibodies (Affinity BioReagents, Golden, CO, USA; Alomone Laboratories, Jerusalem, Israel; Abcam Ltd., Cambridge, UK) and monoclonal mouse antiNeuN antibody (Millipore, Billerica, MA, USA) for 1 hour at room temperature. The sections are then washed with PBS and incubated with secondary antibodies (rhodamine red-X-conjugated donkey anti-goat IgG and FITC-conjugated donkey anti-rabbit IgG) (Jackson Immuno, West Grove, PA, USA) for 1 hour at room temperature. Sections are washed, mounted onto Superfrost/Plus slides, and covered with cover slips with the use of Vectashield (Vector Laboratories, Burlingame, CA, USA). Images are obtained using a confocal microscope (Olympus Fluoview, Tokyo, Japan). The intensity of staining is determined by measuring the gray value of the stained region using Image J (Research Service Branch, NIMH, Bethesda, MD, USA). Data from at least six sections of each animal and from at least three different animals are collected and averaged. The imaging system is calibrated with a density step tablet to assure that optical densities obtained are within the linear response range of the system. The identity of all sections can be concealed/blinded to insure unbiased quantification.[15] The specificity of antibodies can be determined by pre-incubating a peptide sequence against which the antibody has been generated to prevent binding or by using knockout animals. Also, see Chapter 6 for discussion on TRP antibodies.

13.3.3. In Situ Hybridization

In situ hybridization is a powerful technique that allows researchers to determine the distribution of DNA and RNA sequences in specific cell types.[16] Radioactive and nonradioactive probes can be used for in situ hybridization. Nonradioactive probes (sense and antisense) using digoxigenin (Roche Molecular Biochemicals) are often preferred because they have high sensitivity and are easy and safe to use. Tissues of rats/mice are fixed by transcardial perfusion of 4% paraformaldehyde in PBS. DRG are dissected, postfixed for an additional 2 hours in paraformaldehyde, and cryoprotected overnight in 30% sucrose. Sections (10 μm) are treated with 1 μg/mL proteinase K (Sigma) for 5 min, acetylated for 10 min with 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine, pre-hybridized for 4 hours at 56°C, and hybridized with

probes overnight at 56°C. Following post hybridization washes and blocking, sections are incubated for 30 min in anti-DIG antibody conjugated with horseradish peroxidase (Roche Molecular Biochemicals). The signal is visualized using tyramide signal amplification (Perkin Elmer, Waltham, MA, USA).

Radioactive probes can be used in the in situ hybridization study. Selective oli-gonucleotide probes (36- to 45-mer) are synthesized and purified by Sigma Genosys (Woodlands, TX, USA). The probes are 3′ end labeled (^{35}S) in the presence of terminal deoxynucleotidyl transferase (TdT) (Thermo Fisher Scientific, Waltham, MA, USA) and purified using micro BioSpin-30 columns (Bio-Rad) at 1000 × g. To prepare sections for in situ hybridization, the sections are fixed in freshly prepared 4% paraformaldehyde (in 0.1M PBS, pH7.4), acetylated for 10 min at pH 8.0 with 0.25% acetic anhydride in 0.1 M triethanolamine and 0.9% sodium chloride, dehydrated using an ethanol series, and delipidated with 100% chloroform. The sections are incubated with the hybridization mixture, which consists of the labeled probe, 50% formamide, 4× sodium chloride-sodium citrate, 250 mg/mL yeast tRNA, 250 mg/mL sheared salmon sperm DNA, 10% dextran sulfate, 1× Denhardts, 25 mM sodium phosphate, 1 mM sodium pyrophosphate, and 10 mM dithiothreitol, overnight (17–22 hours) at 42°C in a humidified chamber. Posthybridization steps are performed as follows: slides are rinsed 2× 15 min in 1× SSC with 1 mM dithiothreitol at room temperature, rinsed in the same solution for 1 hour at 55°C, and then transferred through 1× SSC (Saline–Sodium Citrate), 0.1× SSC, 70% ethanol, and 95% ethanol (two dips in each). Tissues are air dried, dipped in Kodak NTB photographic emulsion (Eastman Chemical Co., Kingsport, TN, USA), and exposed in the dark for 3 weeks at 4°C. Slides are then developed at 15°C in D-19 (Eastman Chemical Co.) for 4 min, rinsed with distilled H_2O for 30 s, fixed for 5 min in fixer (Eastman Chemical Co.), and counterstained with thionin. Two different controls are used for specificity controls on adjacent sections. First, competitive blocking of the labeled oligonucleotide is performed using excess concentrations (50-fold) of unlabeled oligonucleotides. Second, sections are incubated with labeled sense oligonucleotides, which should not be hybridized with cellular mRNAs.

Images are captured using a CoolSnap high-resolution digital camera connected to an *Image-Pro4* system under 25× objective.[17] The accumulation of grains over cell bodies is interpreted as hybridization of the probe to its corresponding mRNAs in these neurons, and the number of grains over somata (grains/100 mm^2) is counted. Values are corrected by subtracting average background labeling obtained from five random off-tissue areas on each slide. Ten to 15 cells/section are selected and analyzed in a blinded fashion. Only grains within the perimeter of the cell are counted (number of grains/100 mm^2 of the cell area).

13.3.4. Western Blot

Rats/mice are anesthetized and sacrificed. DRG/TG are removed as described above and placed in a lysis buffer (0.1% SDS, 1% Triton X-100, 1% deoxycholate, protease and phosphatase inhibitor cocktail, 1:100) (Sigma) and are then homogenized and centrifuged. The protein concentration is measured by the bicinchoninic acid (BCA) assay. Protein is separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad). Membranes are probed overnight with rabbit anti-TRP and anti-actin (Sigma) antibodies followed by incubation with horseradish peroxidase-conjugated (HRP) goat anti-rabbit IgG (Santa Cruz Biotechnology) for 1 hour. After incubation with enhanced chemiluminescence reagents (Santa Cruz Biotechnology),

membranes are scanned using the Hitachi genetic systems (Hitachi Software Engineering, Tokyo, Japan), and blots are analyzed using GeneTools Analysis Software (SynGene, Frederick, MD, USA).

13.3.5. Tritiated Agonist and Antagonist Binding

Binding studies with [^{3}H]agonists and antagonists are carried out according to methods described by Szallasi and Blumberg.[18,19] Nonspecific ligand binding is reduced by adding bovine α1-acid glycoprotein (100 μg per tube) after the binding reaction has been terminated. Binding assay mixtures contain [^{3}H]ligand, nonradioactive ligands, and 0.25 mg/mL bovine serum albumin (BSA, Cohn fraction V), along with at least 40 μg of DRG membrane proteins. The final volume is adjusted to 500 μL (competition binding assays) or 1000 μL (saturation binding assays). Nonspecific binding is defined as binding that occurs in the presence of 1-μM nonradioactive ligand. For saturation binding, [^{3}H]ligands are added in increasing concentrations. Competition binding assays are performed in the presence of a fixed concentration of [^{3}H]ligand and various concentrations of competing ligands. The binding reaction is initiated by placing the assay mixtures into a 37°C water bath and is terminated after a 15-min incubation period by cooling the tubes on ice. Membrane-bound ligand is separated from free and the α1-acid glycoprotein-bound ligands by pelleting the membranes in a Beckman 12 bench top centrifuge (15 min, maximal velocity). The radioactivity is determined by using a scintillation counter (Beckman Instruments, Fullerton, CA, USA).

13.3.6. Release of Neuropeptides (CGRP and SP) from Nerve Terminals

DRG neurons are peptidergic or nonpeptidergic. Calcitonin gene-related peptide (CGRP) and substance P (SP) are stored in peptidergic nerve terminals (central and peripheral). To study the changes in peripheral and central nerve terminals, CGRP and SP release can be measured in response to TRP channel activation from paw skin and spinal cord tissues, respectively.[20] The tissues are transferred into a beaker containing 200–300 mL of Kreb's solution that contains (in mM): 119 NaCl, 25 $NaHCO_3$, 1.2 KH_2PO_4, $MgSO_4$, 2.5 $CaCl_2$, 4.7 KCl, and 11 D-glucose. To minimize peptide degradation, 0.1% BSA, 1 mM phosphoramidon, and 1 mM captopril are added to Kreb's solution. The solution is aerated with 5% CO_2 and 95% O_2, brought to 37°C, and left to aerate for 1 hour for stabilization. Each tissue piece is transferred into an Eppendorf tube containing 400 μL Kreb's or experimental solution. After 10 min, the tissue is transferred to tubes containing TRP channel agonists for 10 min. The samples are then dried to remove excess liquid and weighed within 0.1 mg. The test solutions are freeze-dried and stored at –80°C.[21] CGRP release is measured by using the ^{125}I CGRP radio immunoassay kit from Peninsula Labs (San Carlos, CA, USA) following the manufacturer's protocol. Similarly, freeze-dried samples are analyzed for SP concentration using an SP ELISA kit, following the protocol of the manufacturer (Cayman Chemical Company, Ann Arbor, MI, USA).

13.4. FUNCTIONAL STUDIES OF TRP CHANNELS IN SENSORY NEURONS

13.4.1. Function of TRP Channels in Isolated Sensory Neurons

13.4.1.1. Ca^{2+} Fluorescence Imaging

Relative Ca^{2+} Flux: To detect acute increases in intracellular Ca^{2+} levels by TRP channel activation, cultured or dissociated DRG neurons grown on cover slips are used. In order to obtain relative change in fluorescence representing Ca^{2+} influx, the neurons are incubated with 2 μM Fluo-4 AM (Invitrogen) for 20 min at 37°C. Fluo-4 is excited at 488 nm, emitted fluorescence is detected with a 535 ± 25 nm bandpass filter using a Leica microscope (DMIRE2), and data are read into a computer running Scanalytics software (Rockville, MD, USA). Changes in fluorescence are expressed as F/F_0, where F is the fluorescence at time t, and F_0 is the background fluorescence.

Ratiometric Ca^{2+} Imaging: To quantify the increase in intracellular Ca^{2+} concentrations, ratiometric Ca^{2+} imaging is performed with Fura-2 AM dye (Invitrogen) and analyzed using Scanalytics software. DRG/TG neurons are loaded with 3 μM Fura-2 AM and placed into a recording chamber containing (in mM): 140 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 glucose, and 10 HEPES, titrated to pH 7.4 with NaOH. Pairs of images are collected every 2 s at alternating exposures of 340 and 380 nm using a Polychrome V monochromator (Lamda DG4, Sutter Instruments, Novato, CA, USA) and a CCD camera (Retiga EX, Leeds Precision Instruments Inc. Minneapolis, MN, USA). After the subtraction of background fluorescence, the ratio of fluorescence at 340 and 380 nm is calculated following application of TRP channel agonists. By constructing a standard curve of known concentrations of Ca^{2+}, the absolute concentration of intracellular Ca^{2+} can be determined.

Neurons under study are perfused continuously with the control solution from a 300-μm barrel positioned 50–100 μm away from the neuron (complete solution exchange is achieved in <100 ms). Solutions containing agonists and antagonists are applied by activating solenoid valves (ASCO, Florham Park, NJ, USA) that switch between different solutions. Thermal stimuli (heat/cold) are applied through a computer-controlled heating/cooling peltier device (Warner Instrument Corporation, Hamden, CT, USA). The temperature is measured at the mouth of the flow pipes using a thermocouple (Warner Instrument).

Ca^{2+} Permeability: Changes in the reversal potential (ΔE_{rev}) are used as an index of Ca^{2+} permeability relative to those for Cs^+. Change in the reversal potential (ΔE_{rev}) for TRP channel currents is measured after replacing a Cs^+-based reference solution (140 mM CsCl, 10 mM HEPES, titrated to pH 7.2 with CsOH) with a solution in which CsCl is substituted with a different concentration of Ca^{2+} and *N*-methyl-D-glucamine (NMDG) and titrated to pH 7.2 with HCl. The pipette solution consists of (in mM): 140 CsCl, 10 EGTA, and 10 HEPES, titrated to pH 7.2 with CsOH. Because TRP channels exhibit strong rectification, a ramp protocol that changes the voltage from –100 to +100 mV in 1–2 s can be used. By subtracting the base line current, agonist-induced currents are plotted to determine the reverse potential. While perfusing the solutions of different compositions, it is necessary to adjust the junction potential[22] (http://web.med.unsw.edu.au/PHBSoft/). ΔE_{rev} values are converted to P_{Ca}/P_{Cs} using the Lewis equation, as described by Wollmuth and Sakmann.[23]

Fractional Ca^{2+} Currents (P^f): Ca^{2+} permeability estimated by changing the extracellular Ca^{2+} and determining the reversal potential requires the use of unphysiological concentrations of Ca^{2+}. In order to determine the Ca^{2+} flux through TRP channels at physiological concentrations of the ion, the fractional Ca^{2+} current (P_f) can be determined by simultaneous recording of Ca^{2+} fluorescence by imaging and membrane current using the patch-clamp technique. DRG neurons

are patched with an intracellular solution that contains (in mM): 140 KCl, 10 HEPES and 1 K_5·fura-2 (Invitrogen), titrated to pH 7.2 with KOH. The extracellular bath solution contains (in mM): 140 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES, titrated to pH 7.4 with NaOH.[24–26]

Fura-2 fluorescence (380-nm excitation and 510-nm emission) is gathered using a photomultiplier (Photon Technology International, South Brunswick, NJ, USA). In order to standardize the sensitivity of the microscope and the photomultiplier tube, the fura-2 signal is normalized to a "bead unit" (BU). One BU equals the average fluorescence of seven Fluoresbrite carboxy BB 4.6-μm microspheres (Polysciences, Warrington, PA, USA). Fractional Ca^{2+} currents (P_f) are quantified using the following equation:

$$P_f\,(\%) = 100 \times Q_{Ca}/Q_T$$

where Q_T is the total charge and is equal to the integral of the agonist-induced transmembrane current. Q_{Ca} is the part of Q_T carried by Ca^{2+} and is equal to ΔF_{380} divided by the calibration factor F_{max}. F_{max} is calculated in a separate series of experiments under conditions in which Q_T is expected to equal Q_{Ca}. Data are analyzed in Clampfit 8.1 (Molecular Devices, Sunnyvale, CA, USA), and calculations are performed using Microcal Origin (Northampton, MA, USA).

13.4.1.2. Whole-Cell Patch-Clamp Recording (Voltage-clamp and current-clamp)

Voltage-Clamp Recording: Patch-clamp techniques[27,28] are used to record membrane currents from cultured or dissociated DRG/TG neurons. Neurons for the study can be chosen according to their size, which largely represents the type of neuron (small diameter <25 μm are nonmyelinated C-fibers; medium diameter <40 μm are thinly myelinated Aδ fibers; >40 μm are Aβ fibers). The type of TRP channels expressed depends on the type of neuron. Patch pipettes are fabricated from borosilicate glass (World Precision Instruments, Sarasota, FL, USA) using a two-step electrode puller (Narishige, Tokyo, Japan). The electrode is lowered to the neuronal plane using a micromanipulator (EXFO Life Sciences Group, Mississauga, Ontario, Canada), and a gigaseal is obtained by gently pressing the electrode against the neuronal cell body and applying gentle suction. After forming a gigaseal, the amount of suction is gradually increased causing the membrane within the patch pipette to rupture without disrupting the gigaseal, resulting in a whole-cell configuration mode. In the whole-cell mode, currents are recorded in response to application of TRP channel agonists and blockade of agonist responses by antagonists. Currents are recorded at a holding potential of –60 and +60 mV, using voltage steps of 20-mV increments, or using a ramp protocol that changes the voltage from –100 to +100 mV in 1–2 s. Because most of the TRP channels exhibit outward rectification (the current flow into the cell is restricted), it is necessary to record and analyze currents at positive and negative potentials to quantify the extent of rectification. The currents are recorded using a patch-clamp amplifier (Axopatch 200B, Molecular Devices or EPC10, HEKA Elektronik, Lambrecht/Pfalz, Germany), and the data are collected with an open filter in the amplifier, digitized (VR-10B, Instrutech Corp., Great Neck, NY, USA), and stored either on videotapes or directly in the computer. For whole-cell recording, the bath solution contains (in mM): 140 Na-gluconate, 10 NaCl, 1 or 2 $MgCl_2$, 10 HEPES, and 10 EGTA, titrated to pH 7.3 with NaOH, and the pipette solution contains (in mM): 140 Na-gluconate/140 K-gluconate, 10 NaCl, 1 or 2 $MgCl_2$, 10 EGTA/BAPTA, 10 HEPES, 2 K_2ATP, and 0.25 GTP, titrated to pH 7.3 with NaOH/KOH. For whole-cell current analysis, the data are

filtered at 2.5 kHz (–3 dB frequency with an eight-pole low-pass Bessel filter, LP10, Warner Instrument) and digitized at 5 kHz. Data analysis is done by pCLAMP software (Molecular Devices).

Because TRP channels are modulated by second messenger molecules and the phosphorylation state of the receptor, it is essential to keep the interior of the neuron intact without dialysis. Therefore, the perforated patch-clamp technique is used.[28] In this technique, access to the cell interior is achieved by adding nystatin or amphoteracin B (250 μg/mL) to the pipette solution, which forms pores that allow passage of only monovalent cations, thus preventing dialysis of the cell interior.

Current-Clamp Recording: To determine the change in the membrane potential, the current-clamp mode can be used in the patch-clamp technique. The current-clamp mode allows for the study of the resting membrane potential and depolarization caused by activation of various TRP channels. If the depolarization is sufficient to reach the threshold, action potentials are generated. In the current-clamp mode, injection of depolarizing currents elicits action potentials. Using the firing pattern in response to depolarizing pulse, it is possible to categorize the type of neuron from which recordings are made (DRG versus DH neurons). For current-clamp recording, the pipette solution contains (in mM): 130 K-gluconate/$KMeSO_3$, 10 NaCl, 1 $MgCl_2$, 5 or 10 EGTA, 2 K_2ATP, and 10 HEPES, titrated to pH 7.35 with KOH. The bath solution contains (in mM): 140 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES, titrated to pH 7.35 with NaOH.

13.4.1.3. Single-Channel Recordings

Gigaseal patch-clamp techniques[27,28] are used to record single-channel currents. For single-channel recording using the cell-attached mode, NaCl in the bath solution is replaced by KCl in order to neutralize the membrane potential and also to identify the K^+ channels that may interfere with the recording (at 0 mV, there should not be any TRP channel activity with Na-gluconate in the pipette because TRP channels are nonselective cation channels). For cell-attached patches, the bath solution contains (in mM): 140 K-gluconate, 2.5 KCl, 5 HEPES, and 1.5 EGTA, titrated to pH 7.35 with NaOH. The patch pipettes are made from glass capillaries (Drummond, Microcaps, Broomall, PA, USA), coated with sylgard (Dow Corning, Midland, MI, USA) to minimize the pipette capacitance, and are filled with a solution that contains (in mM): 140 Na-gluconate, 10 NaCl, 2 $MgCl_2$, 1.5 EGTA, and 5 HEPES, titrated to pH 7.35 with NaOH. Ca^{2+} free extracellular solutions are used to avoid desensitization and tachyphylaxis. While studying the effects of extracellular Ca^{2+}, EGTA is eliminated, and 2 mM $CaCl_2$ is added to the solution; the concentration of $MgCl_2$ is reduced to 1 mM. For inside-out patches, the bath solution contains (in mM): 140 Na-gluconate, 10 NaCl, 1 or 2 $MgCl_2$, 10 EGTA, 10 HEPES, 2 K_2ATP, and 0.25 GTP, titrated to pH 7.3 with NaOH, and the pipette solution contains the same solution as the cell-attached patch. For outside-out patches, the pipette solution contains (in mM): 140 Na-gluconate, 10 NaCl, 1 or 2 $MgCl_2$, 10 EGTA, 10 HEPES, 2 K_2ATP, and 0.25 GTP, titrated to pH 7.3 with NaOH/KOH, and the bath solution contains (in mM): 140 Na-gluconate, 10 NaCl, 2 $MgCl_2$, 1.5 EGTA, and 5 HEPES, titrated to pH 7.35 with NaOH. Using these solutions, it is possible to eliminate K^+ and Cl^- currents contaminating the recording. For recording currents from a large number of channels in isolated patch configurations, the macropatch technique[29] can be used. Data are collected with an open filter in the amplifier, digitized (VR-10B), and stored on videotapes. For analysis of amplitude and open probability (P_o), the data are filtered at

2.5 kHz and digitized at 5 kHz; for kinetic analysis, the data are filtered at 10/20 kHz and digitized at 50/100 kHz using pCLAMP (Molecular Devices) based hardware and software. Single-channel current amplitude and P_o are estimated from all-point current-amplitude histograms (pCLAMP) and fitted to Gaussian densities (Microcal Origin). For current-voltage relationships, 10–50 single-channel openings are grouped, and the amplitude is determined by fitting a Gaussian curve. P_o is determined using unedited segments of data, which is typically 1–5 min long. For multiple-channel patches, mean P_o is measured as NP_o divided by N (N is the number of channels in the patch). Chord conductance is measured at +60 or –60 mV, and the slope conductance is determined by plotting a current-voltage curve and fitting the inward and outward currents with linear functions.

Patches that apparently have a single TRP channel (assessed by the lack of overlapping events at +60 mV, when P_o is >0.8) are used for dwell-time analysis. However, this criterion is not valid when lower concentrations of agonists are used. The number of channels in the patch can be determined by exposing the patch to higher agonist concentrations at the end of the experiment. Single-channel currents are idealized (10-kHz bandwidth) using a modified Viterbi algorithm (QUB software, www.qub.buffalo.edu). Dwell-time distributions are fitted with mixtures of exponential densities using a method of maximum likelihood. Additional exponential components are incorporated only if the maximum log likelihood increases more than 2 log likelihood units.[30–35] A dead time (τ_d) of 50 μs is imposed retrospectively; events shorter than 50 μs are ignored.

13.4.1.4. Mechanosensitivity

Determining the mechanosensitivity of ion channels has proven to be a daunting task. Mechanosensitive ion channels have been studied extensively, but the mechanosensitivity of TRP channels is still unclear. In order to conclusively determine the type of mechanical stimulus a channel responds to, it is necessary to use several types of mechanical stimuli with multiple controls.

Mechanosensitive channels can be activated by changes in osmolarity, so hypotonic solutions are often used to study mechanosensitivity. The standard extracellular solution for electrophysiological measurements contains (in mM): 150 NaCl, 6 CsCl, 1 $MgCl_2$, 5 $CaCl_2$, 10 glucose, and 10 HEPES, titrated to pH 7.4 with NaOH. The osmolarity of this solution, as measured with a vapor pressure Vapro 5520 osmometer (Wescor, Inc, Logan, Utah, USA), is around 320 milliosmolar. For measuring currents due to a change in osmolarity, an isotonic solution is used that contains (in mM): 105 NaCl, 6 CsCl, 5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 90 D-mannitol, and 10 glucose titrated to pH 7.4 with NaOH (~320 milliosmolar). Hypoosmolar solution is obtained by omitting mannitol from the solution (240 milliosmolar).

Mechanical stress induced by changing osmolarity represents a diffused mechanical stimulus. However, responses to mechanical stimuli can be due to punctate indentation of the membrane. To study this properly, a piezo-controlled probe (EXFO Life Sciences Group)[36,37] that advances in 0.5–2 μm/ms steps can be used. The stimulus is applied for a duration of 200–500 ms. The probe is advanced until a change in membrane conductance is observed.

In order to determine the direct mechanical sensitivity, the pressure-clamp technique developed by McBride and Hamill[38,39] can be used. The positive and negative pressure is applied by

opening a piezo-driven valve. The extent of valve opening regulates the final pressure applied. The pressure is measured using a pressure transducer that has been calibrated. The pressure is changed in a ramp-like fashion from 0 to 100 mmHg over a period of 20 s. The ramp protocol changes the pressure over time and enables recording of the single-channel activity in response to increasing magnitude of pressure. Patch pipettes are pulled on a Narishige vertical puller to have a tip diameter of ~2 μm. Single mechanosensitive channels are analyzed as described above.

13.4.2. Function of TRP Channels in the Central Terminals of Sensory Neurons

13.4.2.1. Synaptic Current Recordings from DH Neurons in DRG-DH Co-Cultures

Spontaneous and Miniature Synaptic Currents: Cover slips with the co-cultured neurons are mounted on the stage of an Olympus IMT-2 microscope for patch-clamp recordings. The DH neurons are distinguished from the DRG neurons based on their morphology and electrophysiological properties. DH neurons are fusiform, pyramidal, or multipolar in shape, in contrast to the rounded pseudounipolar characteristics of the DRG neurons. Moreover, when a DRG neuron is voltage clamped, no miniature excitatory postsynaptic currents (mEPSCs) are observed, and capsaicin application may result in an inward whole-cell current. However, mEPSCs in controls and upon TRP channel agonist application can be recorded when a DH neuron is voltage clamped. The bath solution contains (in mM): 150 Na-gluconate/NaCl, 5 KCl, 2 $MgCl_2$, 0.1 $CaCl_2$, 10 glucose, and 10 HEPES, titrated to pH 7.4 with NaOH. The recording pipettes are made from borosilicate glass, which has a resistance of 3–10 MΩ when filled with a solution that contains (in mM): 140 Cs-Gluconate/$CsMeSO_3$, 5 CsCl, 10 HEPES, 5 $MgCl_2$, 10 EGTA, 5 Mg-ATP, and 1 Li-GTP, titrated to pH 7.4 with CsOH. Experiments are performed at the desired temperature by adjusting the temperature of the flow solution. In order to record EPSCs, DH neurons are voltage clamped (EPC10) at –60 mV (close to E_{Cl}). In order to record inhibitory postsynaptic currents (IPSCs), the currents are recorded at 0 mV. The capacitance and the series resistance are compensated, and the input resistance of the cell is monitored. The data are filtered at 2.5 kHz and digitized at 5 kHz. Adequate voltage clamp is confirmed by recording the mEPSCs at different voltages and determining their reversal potentials. A new cover slip should be used for every experiment to prevent rundown of mEPSCs and the activation of second messenger pathways.

Neurons are perfused with standard bathing solution and lidocaine (10 mM), strychnine (1 μM), and bicuculline (10 μM) to study glutamatergic transmission, and CNQX (20 μM) and APV (50 μM) to study GABAergic and glycinergic transmission. A high concentration of lidocaine is used to block both TTX-sensitive and TTX-resistant Na^+ channels. TRP channel agonists/antagonists are bath-applied for 30 s, and changes in mEPSCs are recorded continuously thereafter. Effects of phosphorylation can be studied using kinase activators such as phorbol 12,13-dibutyrate (1 μM).

Evoked Synaptic Currents: DRG and DH neurons are voltage-clamped simultaneously using a dual-electrode voltage-clamp technique. Monosynaptic EPSCs are recorded in DH neurons in the voltage-clamp mode by generating an action potential in DRG neurons in the current-clamp mode. The bath solution contains (in mM): 150 NaCl, 5 KCl, 2 $MgCl_2$, 0.5 $CaCl_2$, 10 glucose, and 10 HEPES, titrated to pH 7.2 with NaOH. The pipette solution for the DRG neuron contains

(in mM): 140 KCl, 10 HEPES, 5 $MgCl_2$, 10 EGTA, 5 Mg-ATP, and 1 Li-GTP, titrated to pH 7.4 with KOH. The pipette solution for DH neurons contains $CsSO_4$ instead of KCl. Monosynaptic connections are confirmed by the constant latency of the EPSCs. It is necessary to monitor the membrane capacitance (C_m), membrane resistance (R_m), and access resistance (R_a) continuously to avoid erroneous interpretation of the results.

13.4.2.2. Synaptic Current Recording from Spinal Cord Slices

Spontaneous and Miniature Synaptic Currents: Spinal cord or CSTN slices are placed on the stage of an upright near-infrared differential interference contrast microscope, Olympus BX-50wi, for patch-clamp recording.[40,41] In these preparations, only the TG and DRG neuronal terminals are intact without the cell bodies. Pipettes with tips of 2–5 MΩ are pulled from thick-walled borosilicate glass (1B150F-4, World Precision Instruments) with a horizontal pipette puller P-97 (Sutter Instruments). Slices are visualized with an upright microscope and Gibralter stage using a 40× objective and infrared filter (Olympus). The image is sent to a TV monitor. For voltage-clamp studies, the pipette solution contains (in mM): 140 $CsMeSO_3$, 10 EGTA, 10 HEPES, 5 CsCl, 5 $MgCl_2$, 5 MgATP, and 1 LiGTP, titrated to pH 7.4 with CsOH. For current-clamp studies, a pipette solution contains (in mM): 130 K-gluconate, 10 NaCl, 1 $MgCl_2$, 0.2 EGTA, 1 K_2ATP, and 10 HEPES, titrated to pH 7.4 with NaOH. Whole-cell currents are recorded using a patch-clamp amplifier (EPC 10/Axopatch 200B) and acquisition software (Pulse 8.6/pCLAMP). The data are collected with the filter set at 2.5 kHz (–3 dB frequency with an eight-pole low-pass filter, LP10), digitized at 5 kHz (VR-10B), and stored on a hard disk. The recording chamber (PH1, Warner Instrument) is perfused at 4 mL/min with an extracellular solution, artificial cerebrospinal fluid (aCSF) that contains (in mM): 126 NaCl, 2.5 KCl, 1.4 NaH_2PO_4, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 25 $NaHCO_3$, and 10 glucose, titrated to pH 7.4 (290 mOSM), and is aerated with a 95% O_2–5% CO_2 gas mixture. Temperature is held at 28°C using a glass water jacket and circulating water bath (VWR 1130, VWR, Batavia, IL, USA). The tip potential is cancelled before forming a gigaseal. Junction potential can be determined and corrected according to Barry and Lynch.[22] The activities of GABA, glycine, AMPA, NMDA, and voltage-gated Na^+ channels are blocked when necessary with 30 μM bicuculline, 5 μM strychnine, 100 μM CNQX, 20 μM APV, and 500 nM TTX (all dissolved in the extracellular solution), respectively. The lidocaine derivative (QX-314) is included to prevent action potentials in the recording neuron.

Evoked Synaptic Currents: To obtain evoked EPSCs or IPSCs, the neurons are voltage-clamped at –60 or 0 mV. A concentric bipolar stimulating electrode is placed on the spinal trigeminal or Lissauer tract. For horizontal slices of CSTN, the electrode is placed caudal to the recording site for orthograde transmission. A Grass Stimulator (S88) with stimulus isolation unit PSIU 6 (Grass Technologies, West Warwick, RI, USA) triggered by a Master 8 (A.M.P.I., Jerusalem, Israel) is used to stimulate a concentric bipolar electrode (Rhodes Medical Instruments, Tujunga, CA) placed on the sensory fiber tract. Stimulus duration is 100 μs, and half maximal stimulus intensity is used (less than 800 μA, usually 200–800 μA for C-fibers). Spontaneous and evoked EPSCs are low-pass-filtered at 2.5 kHz and digitized at 5 kHz. The digitized signal is stored on a hard drive on a PC-compatible computer. Fast and slow capacitance compensation is performed in Pulse 8.6/ pCLAMP. Input resistance and series resistance are measured every 2–5 min in the voltage-clamp mode with three small (ΔV 10 mV, 150 ms) hyperpolarizing voltage steps. Cells

showing greater than 20% change in series resistance should not be included for further analysis. Offline data analysis is done with pCLAMP software. sEPSCs are analyzed using the Mini Analysis Program (Synaptosoft, Decatur, GA, USA). The threshold for event detection (usually 10 pA) is at least 3 times baseline noise levels.

Blind Patch-Clamp Technique: Using the blind patch-clamp technique, a randomized unbiased sample of neurons in a given preparation can be accomplished without sophisticated equipment. Electrophysiological recordings can be made in spinal cord slices using a standard patch-clamp amplifier, dissecting microscope, and recording chamber. In addition, some preparations require thick slices, and direct visualization is not possible.[42]

Sharp Electrode Technique: In situations where slices from adult animals are used, it is difficult to form gigaseals routinely. Therefore, the sharp electrode technique (single electrode voltage-clamp or current-clamp)[43] is used, in which the neurons are impaled using a manipulator (PCS-5100, EXFO Life Sciences Group), and the potential inside the cell membrane can be recorded with minimal effect on the ionic constitution of the intracellular fluid. The electrodes are like those for patch clamp (pulled from glass capillaries), but the tip size is much smaller (10 to 100 s MΩ). Signals from impaled neurons are amplified with an Axoclamp 2A amplifier (Molecular Devices). Neurons are recorded under current-clamp or voltage-clamp conditions. While using voltage-clamp conditions, it is necessary to make sure that adequate voltage-clamp is achieved because of high-resistance electrodes. The data collection and analysis are similar to those described for patch-clamp techniques.

13.5. EX VIVO PREPARATIONS TO STUDY TRP CHANNEL FUNCTION IN SENSORY NEURONS

13.5.1. Skin-Nerve Preparation

The details of skin-nerve preparation have been described previously.[44–46] Rats/mice are anesthetized with isoflurane and killed by cervical dislocation. The saphenous nerve and its innervated skin are located in the medial-dorsal side of the hind paw and exposed by removing the hair. The nerve and skin are carefully excised. The skin is placed corium side up in the in vitro perfusion chamber and is superfused with a modified Krebs-Hensleit solution containing (in mM): 110.9 NaCl, 4.8 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2SO4, 24.4 NaHCO3, and 20 glucose, which is saturated with 95% O_2–5% CO_2. The temperature of the bath is maintained at 34°C. The saphenous nerve is drawn through a small hole into the recording chamber, which is filled with a layer of paraffin oil. The nerve is placed on a mirror, and individual nerve fibers are separated to record single-unit activity.

Fiber types are categorized by conduction velocity. Units conducting slower than 1.2 m/s are classified as unmyelinated C-fibers; those conducting between 1.2 and 10 m/s are classified as thinly myelinated Aδ-fibers; and units conducting faster than 10 m/s are classified as myelinated Aβ-fibers. Mechanically sensitive Aβ-fibers are further categorized as slowly adapting (SA) if they responded throughout a sustained force of 10-s duration or rapidly adapting (RA) if they responded only at the onset or offset of force. Mechanically sensitive Aδ-fibers are classified as A-mechanoreceptor (AM) fibers if they exhibit slowly adapting responses to sustained force, or as down-hair (D-hair) receptors if they are rapidly adapting.[45,46]

Receptive fields are identified by probing with a blunt glass rod in the corium side of skin.

Conduction velocity of the fiber is determined by monopolar electrical stimulation (variable intensity, 0.2 Hz, and 2–3 ms duration) into the receptive field. The distance between the receptive field and the recording electrode (conduction distance) is divided by the latency of the action potential. The mechanical threshold of units is tested with a set of calibrated von Frey hairs made from nylon filaments (Stoelting, Wood Dale, IL, USA). To test the heat sensitivity of the localized receptors, radiant heat stimulation from a halogen lamp (150 W) is applied to the epidermal surface. A thermocouple is placed at the corium side in the receptive field for feedback control. The temperature is increased linearly from 32° to 47°C in 15 s. For applying chemical solutions, a metal ring is used to isolate the receptive field.

Action potentials are amplified, filtered, and displayed on an oscilloscope and continuously recorded on videotape using an analog–digital converter (VR-10B). The data are analyzed offline using pCLAMP software. The magnitude of the responses of a C-fiber nociceptor is determined by counting the total impulses (action potentials) evoked during the 5 min after onset of superfusion. For counting the total impulses, spontaneous discharges during the 60-s control period are multiplied by 5 and then subtracted from the 5-min count after drug addition.[46]

13.5.2. Skin-Nerve-DRG-Spinal Cord Preparation

Sensory neurons project to specific regions of the spinal DH and are correlated with specific modality sensations. The ontogenic changes and the changes that occur in different disease conditions can be studied using an ex vivo somatosensory preparation that includes the skin, nerve, DRG, and the spinal cord.[47] Adult rat/mice are anesthetized with a mixture of ketamine and xylazine (85 and 5 mg/kg, respectively, I.P.). Following the conformation of anesthesia, animals are perfused transcardially with chilled (14°C) and oxygenated (95% O_2–5% CO_2) modified aCSF, which consists of (in mM): 253.9 sucrose, 1.9 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 2.4 $CaCl_2$, 26.0 $NaHCO_3$, and 10.0 D-glucose. The right hind limb and the spinal cord are excised and placed in a constantly circulating bath. The area of the skin innervated by the saphenous nerve and the corresponding DRGs and spinal cord are dissected. The skin is spread flat on a platform and allowed to dry by adjusting the fluid level of the recording chamber. The cell bodies of L2 and L3 DRG neurons are impaled with sharp quartz microelectrodes (Vector Laboratories). The peripheral response properties of the neurons are assessed using controlled thermal, mechanical, and chemical stimuli as described in the previous section.

13.6. IN VIVO BEHAVIORAL STUDIES TO EVALUATE THE FUNCTION OF DRG/TG NEURONS

13.6.1. Measurement of Thermal Sensitivity

13.6.1.1. Paw Withdrawal Latency

Thermal nociceptive responses are determined using a plantar test instrument (Ugo Basile, Camerio, Italy), as described previously.[48] Rats/mice are habituated to the apparatus (1 hour per day for 5 days). A mobile radiant heat source is located under the table and focused onto the desired paw. Paw withdrawal latencies (PWLs) are recorded three times for each hind paw, and the average is taken as the baseline value. A timer is automatically activated with the light source, and response latency is defined as the time required for the paw to show an abrupt withdrawal. The apparatus can be calibrated to give a PWL of approximately 6–20 s. In order to

prevent tissue damage, a cutoff at 20 s must be set. Rats are accustomed to the test conditions 1 hour per day for 5 days. At least 4 days should elapse between two consecutive tests.

13.6.1.2. Tail-Flick Test for Thermal Hyperalgesia

Tail flick latencies to radiant heat are assessed using methods described.[49,50] Tail flick latencies are determined every 5 min four times before and after systemic or intrathecal drug administration.

13.6.1.3. Carrageenan-Induced Thermal Hyperalgesia

After obtaining baseline values of PWL to radiant heat, the animals receive an intraplantar injection of carrageenan (1%, 100 μL) into the left hind paw.[51] After the induction of inflammation (assessed by thickness and volume), PWL to thermal stimuli is determined after 24 to 48 hours.

13.6.1.4. Complete Freund's Adjuvant (CFA) Induced Inflammatory Thermal Hyperalgesia

Chronic peripheral inflammation is induced by injecting 100 μL CFA (1 mg mycobacterium per 0.85 mL paraffin oil and 0.15 mL of mannide monooleate) (Sigma) in the left hind paw of animals. The inflammation develops slowly over a period of a few days and lasts for a few weeks. Hyperalgesia to noxious thermal stimulation is measured by determining the PWL 3 days after CFA administration as described above. Data are plotted to contrast thermal hyperalgesia between inflamed and control paws of each animal in the two groups.

13.6.2. Measurement of Chemical Sensitivity by Nocifensive Behavior

Chemical-evoked nocifensive behavior in rats is defined as lifting (guarding), licking, and shaking of the injected paw.[52] The number of times the rat exhibits guarding, licking, and shaking is counted, and the total duration of this behavior is measured over 5 min immediately after intraplantar administration of agonists.

13.6.2.1. Formalin-Induced Pain Behavior

Each rat is placed in an observation cage and is allowed to adapt to its environment for 10 minutes. Formalin (50 μL, 5%) is then injected intracutaneously into the hind paw, and the animal is immediately transferred to the observation cage. The number of flinches (rapid withdrawal of the injected hind paw) produced by the animal is counted for 60 min. Animals exhibit two phases of pain behavior (phase 1, first 10 min; phase 2, 10–60 min). Data are plotted to represent the number of flinches produced in each 5-min period following formalin injection.

13.6.3. Measurement of Mechanosensitivity

13.6.3.1. von Frey Filament Test

Weekly calibrated nylon monofilaments are used (Stoelting). Filaments 2 to 9 (0.015–1.3 g) are firmly applied to the plantar surface of the mid hind paw (alternating the side of the body being tested) until they bowed for 5 s.[53] Vigorous vertical and/or horizontal movement of the hind paw away from the fiber is considered as a withdrawal response. Fibers are applied in random order; no more than two measurements are made per behavioral state per fiber per rat/mouse.

Mechanical sensitivities are also assessed using a dynamic plantar aesthesiometer instrument using von Frey probe (Ugo Basile, Camerio, Italy).[54] A 0.5-mm-diameter von Frey probe is applied to the plantar surface of the rat hind paw with pressure increasing by 0.05 Newtons/s. The pressure at which a paw withdrawal occurs is recorded, and this is taken as the paw withdrawal threshold (PWT). For each hind paw, the procedure is repeated 3 times, and the average pressure to produce withdrawal is calculated. Successive stimuli are applied to alternating paws at 5-min intervals.

13.6.3.2. Randall-Selitto Paw Pressure Test

The nociceptive flexion reflex is quantified with a Randall–Selitto paw pressure device[55] (Analgesymeter, Stoelting) that applies a linearly increasing mechanical force to the dorsum of the rat's hind paw.[56] The nociceptive threshold is defined as the force in grams at which the rat withdraws its paw. Rats are familiarized in the testing procedure at 5-min intervals for a period of 1 hour/day for 3 days in the week preceding the experiments. Baseline PWT is defined as the mean of six readings.

13.6.4. Chronic Constriction Injury (CCI) Model, Bennett–Xie Model

Male SD rats (250–300 g) are anesthetized with ketamine and xylazine (85 and 5 mg/kg, respectively, I.P.). The skin of the middle thigh is cut, and the muscle is separated to expose the left sciatic nerve. Four ligatures are loosely tied around the nerve by using 4-0 braided silk thread with a 1.5-mm interval. The surgical incision in the skin is closed. Amikacin (10 mg/kg, s.c.) is injected every day for one week to prevent infection. Thermal, mechanical, and chemical sensitivities are tested to determine the changes that occur following nerve injury.

13.6.5. Intrathecal Administration of Drugs by Catheter Implantation

13.6.5.1. Intrathecal Administration by Catheter

In order to target the TRP channels expressed at the central terminals of the sensory neurons, chronic intrathecal catheters are implanted.[57] Male SD rats (225–250 g) are anesthetized with ketamine and xylazine (85 and 5 mg/kg, respectively, I.P.). When they no longer respond to the paw pinch test, the neck area is shaved, and the skin is swabbed with betadine followed by 70% alcohol. A small incision is made in the skin, and the muscles are separated to expose the atlanto-occipital membrane. A small incision is made in the membrane to allow a polyethylene-10 catheter with 0.9% sterile saline to be inserted into the subarachnoid space. The catheter is gently threaded through the space as far as the lumbar enlargement (approximately 7.5 cm). The catheter is then sutured in place with the muscles, and the incision is closed. About 5 cm of catheter is exposed externally for injections. The external port is sealed with Parafilm to prevent flow of cerebrospinal fluid. Rats are allowed to recover for 7 days after surgery. To prevent infection, 10 mg/kg of kanamycin is injected subcutaneously every day for 5 days during recovery. Agents to be tested are administrated in 20-μL volumes by slow infusion followed by 20-μL saline using a Hamilton syringe.[58]

13.6.5.2. Intrathecal Administration of Drugs by Lumbar puncture

All drugs or their appropriate vehicles are injected intrathecally in a volume of 3 μL by lumbar puncture using a Hamilton syringe and 30-gauge needle.[59] The injection is made at the space

between L5 and L6 where the spinal cord ends and the cauda equina begins.[60] A 27-gauge needle is used to puncture the muscle, spinal process, and the dura until a characteristic brief motor response of tail or hind limbs is observed indicating the penetration of the dura.

13.6.6. Behavioral Assays Involving TG Neurons

13.6.6.1. Catheter Implantation

In order to study the changes in peripheral terminals of TG neurons, the nerve innervations of the dura mater can be manipulated.[61] Male SD rats (225–250 g) are anesthetized with ketamine and xylazine (85 and 5 mg/kg, respectively, I.P.). When they no longer respond to the paw pinch test, the hair is removed, and the skin is swabbed with betadine followed by 70% alcohol. The bregma is exposed with a 2-cm incision and retracting the skin. An electric drill is used to bore bilateral (3–4 mm to midsaggital suture) troughs (2-mm diameter and 8–10 mm long). The catheters are constructed using PE-10 tubing (Instech Laboratories, Inc., Plymouth Meeting, PA, USA) and inserted through the troughs about 4–5 mm; they are placed above the occipital lobe. The catheter is then sutured in place with the skin while closing the incision. About 5 cm of the catheter is exposed externally for injections. The external port is sealed with Parafilm to prevent flow of cerebrospinal fluid. Rats are allowed to recover for 7 days after surgery. To prevent infection, 10 mg/kg of kanamycin is injected subcutaneously every day for 5 days during recovery.

In order to sensitize the peripheral terminals of TG neurons, pro-inflammatory agents to be tested, namely, bradykinin (1 µM, histamine (0.1 mM), prostaglandin E2 (2 µM), and serotonin (0.1 mM) (inflammatory soup), are administrated in 5–6 µL volumes by slow infusion followed by 5–6 µL saline.[61]

13.6.6.2. Facial Allodynia

Following the injection of inflammatory soup into the skull, facial allodynia can be assessed using von Frey filaments. All testing is done in a blinded fashion. Areas around the eye, the cheek, the shoulder, and the forearm are tested. There are two methods that can be used: (1) The gram force filament in the middle of the logarithmic range is tested, and then the force is increased or decreased by using different filaments until the animal responds reliably.[62] (2) The gram force filament to which the majority of control rats respond versus the gram force filament to which the majority of allodynic rats respond is determined.[62] Using the method described by Ren,[62] absolute thresholds can be recorded. With the method described by Tawfik et al.,[63] absolute thresholds cannot be determined. Following 5 days of general habituation to handling (~5 min/day), the baseline behavior is assessed. A logarithmic series of 10 calibrated von Frey hairs (Stoelting) are applied as described by Ren.[62] The rat is stimulated 5 times with each filament for 2 s on either side of the face. The filament size is increased if the rat responds 5 times to the filament. These data are then analyzed as previously described.[64]

13.7. STATISTICAL METHODS FOR DATA ANALYSIS

Data are expressed as mean ± S.E.M. When comparing the means of only two groups, Student's *t* test is used. For all tests, a *p* value lower than 0.05 ($p < 0.05$) is considered significant. Statistical procedures are implemented using SPSS 14.0 (SPSS, Chicago, IL, USA).

For in situ hybridization and immunohistochemical data, analysis of variance (ANOVA) is used to determine if differences in background-adjusted mean grain density or staining can be attributed to a change. Tests subsequent to the ANOVA are carried out using Bonferroni corrections to control the overall type I error rate.

For analysis of miniature and spontaneous synaptic events, Kolmogorov–Smirnov (KS) test is used to compare the cumulative probability curves for inter-event intervals and amplitudes.

For conduction velocity, temperature thresholds, and the number of spikes induced by different manipulations, statistical comparisons between two groups are made using an unpaired two-tailed *t*-test. Fisher's exact probability test is used to compare the percentage change of C-fiber responsiveness to various TRP channel agonists. The magnitudes of responses are compared using a nonparametric Mann–Whitney U-test.

For experiments that involve manipulation of one of the legs, data are normalized for each animal as the maximum possible effect (MPE). This value is calculated as follows: MPE = (PDR – IBR)/(CBR – IBR), where PDR is the postdrug response of the ipsilateral paw, IBR is the ipsilateral paw baseline response, and CBR is the contralateral paw baseline response. The nociceptive thresholds are calculated as mean values obtained from both hind paws. The data are subjected to a one-way ANOVA followed, when significant, by a post hoc Dunnett's *t*-test. Data for the touch-evoked agitation test are compared using the Mann–Whitney *U*-test because the data are not continuously distributed. von Frey Filament data are analyzed by performing linear regression applied to percentage withdrawal responses obtained across the entire fiber range and by solving the linear equation for a 50% response. Significance is established by nonoverlapping 95% confidence intervals.

13.8. CONCLUDING REMARKS

In recent years, there have been several discoveries regarding the molecular structures that respond to specific stimuli and their transduction mechanisms in sensory neurons. Several members of the TRP channel family have become important players in sensory transduction. A rich array of techniques is available to study the expression and function of TRP channels in sensory neurons. It is always desirable to use multiple techniques to infer definitive conclusions. While studying the expression of TRP channels, the specificity of antibodies must be confirmed. With lack of change in the message of a particular TRP channel, one must consider the possibility of RNA stabilization and posttranslational modifications before arriving at conclusions. Whole-cell and single-channel recordings can be used to characterize the conductance and kinetic properties and to identify the mechanism of rectification. However, caution should be exercised to prevent activation of channels other than the TRP channel of interest. Agonist and antagonist specificities are important to infer accurate conclusions. TRP channel-mediated responses exhibit Ca^{2+}-dependent desensitization and tachyphylaxis. Therefore, responses obtained with repeated agonist application must be analyzed carefully. While studying evoked synaptic responses, a decrease in the amplitude of evoked responses to application of TRP channel agonists may be due to presynaptic depolarization block.[58] While using in vivo or ex vivo approaches, changes in other channels (e.g., voltage-gated Na^{+} channels) may induce an exaggerated response to the application of TRP channel agonists because activation of TRP channels causes the release of pro-inflammatory agents that can sensitize other channels. Therefore, sensitization of TRP channels as compared to sensitization of

other channels must be taken into consideration. In most TRP channels, both N- and C-termini are intracellular, and generally, agonist binding sites are located at the N-terminus. Therefore, when using membrane impermeable agonists or second messenger molecules, the membrane permeability of the applied compounds must be known. Finally, appropriate statistical methods must be adopted to analyze the data for significance to arrive at conclusions.

ACKNOWLEDGMENTS

I thank Lauren Hughes and Deshou Cao for their help with editing the manuscript. This work was supported by grants from the National Institutes of Health (NS042296 and DK065742 and DA028017) and EAM award from SIUSOM.

REFERENCES

1. Clapham D.E. TRP channels as cellular sensors. Nature. 2003;426:517–524. [PubMed: 14654832]
2. Minke B, Cook B. TRP channel proteins and signal transduction. Physiological Reviews. 2002;82:429–472. [PubMed: 11917094]
3. Nilius B, Owsianik G, Voets T, Peters J.A. Transient receptor potential cation channels in disease. Physiological Reviews. 2007;87:165–217. [PubMed: 17237345]
4. Cortright D.N, Szallasi A. TRP channels and pain. Current Pharmaceutical Design. 2009;15:1736–1749. [PubMed: 19442187]
5. Lewin G.R, Moshourab R. Mechanosensation and pain. Journal of Neurobiology. 2004;61:30–44. [PubMed: 15362151]
6. Lewin G.R, Lu Y, Park T.J. A plethora of painful molecules. Current Opinion in Neurobiology. 2004;15:129. [PubMed: 15321065]
7. Patapoutian A, Tate S, Woolf C.J. Transient receptor potential channels targeting pain at the source. Nature Reviews Drug Discovery. 2009;81:55–68. [PMC free article: PMC2755576] [PubMed: 19116627]
8. Wood J.N. Recent advances in understanding molecular mechanisms of primary afferent activation. Gut. 2007;53 Suppl. 2:9–12. [PMC free article: PMC1867774] [PubMed: 14960551]
9. Gottlieb P, Folgering J, Maroto R, et al. Revisiting TRPC1 and TRPC6 mechanosensitivity. Pflügers Archiv. 2008;455:1097–1103. [PubMed: 17957383]
10. Caterina M.J, Julius D. The vanilloid receptor: a molecular gateway to the pain pathway. Annual Review of Neuroscience. 2001;24:487–517. [PubMed: 11283319]
11. Julius D, Basbaum A.I. Molecular mechanisms of nociception. Nature. 2001;413:203–210. [PubMed: 11557989]
12. Premkumar L.S, Sikand P. TRPV1: a target for next generation analgesics. Current Neuropharmacology. 2008;6:151–163. [PMC free article: PMC2647151] [PubMed: 19305794]
13. Grudt T.J, Williams J.T. mu-Opioid agonists inhibit spinal trigeminal substantia gelatinosa neurons in guinea pig and rat. Journal of Neuroscience. 1994;14:1646–1654. [PubMed: 8126561]
14. Monyer H, Jonas P. Polymerase chain reaction analysis of ion channel expression in single neurons in brain slices. In: Sakmann B, Neher E, editors. Single-Channel Recording. second. New York: Plenum Press; 1995. pp. 357–373. Chapter 16.

15. Ramos-Vara J.A. Technical aspects of immunohistochemistry. Veterinary Pathology. 2005;42:405–426. [PubMed: 16006601]
16. Hofler H. Principles of in situ hybridization. In: Polak J.M, McGee J.O'D, editors. Situ Hybridization: Principle and Practice. Oxford: Oxford University Press; 1990. pp. 15–30.
17. Kus L, Mazzone S.B, Paxinos G, Geraghty D.P. Autoradiographic localisation of substance P (NK1) receptors in human primary visual cortex. Brain Research. 1998;794:309–312. [PubMed: 9622661]
18. Szallasi A, Blumberg P.M. Resiniferatoxin and its analogs provide novel insights into the pharmacology of the vanilloid (capsaicin) receptor. Life Sciences. 1990;47:1399–1408. [PubMed: 2174484]
19. Szallasi A, Blumberg P.M. Specific binding of resiniferatoxin, an ultrapotent capsaicin analog, by dorsal root ganglion membranes. Brain Research. 1990;524:106–111. [PubMed: 2400923]
20. Tognetto M, Amadesi S, Harrison S. et al. Anandamide excites central terminals of dorsal root ganglion neurons via vanilloid receptor-1 activation. Journal of Neuroscience. 2001;21:1104–1109. [PubMed: 11160380]
21. Zygmunt P.M, Peterson J, Anderson D.A, Chuang H, Sorgard M, Marzo V.Di. Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. Nature. 1999;400:452–457. [PubMed: 10440374]
22. Barry P.H, Lynch J.W. Liquid junction potentials and small cell effects in patch clamp analysis. Journal of Membrane Biology. 1991;121:101–117. [PubMed: 1715403]
23. Wollmuth L.P, Sakmann B. Different mechanisms of Ca^{2+} transport in NMDA and Ca^{2+}-permeable AMPA glutamate receptor channels. Journal of General Physiology. 1998;112:623–636. [PMC free article: PMC2229440] [PubMed: 9806970]
24. Schneggenburger R, Zhou Z, Konnerth A, Neher E. Fractional contribution of calcium to the cation current through glutamate receptor channels. Neuron. 1998;11:133–143. [PubMed: 7687849]
25. Frings S, Hackos D.H, Dzeja C, Ohyama T, Hagen V, Kaupp U.B, Korenbrot J.I. Determination of fractional calcium ion current in cyclic nucleotidegated channels. Methods Enzymology. 2000;315:797–817. [PubMed: 10736742]
26. Egan T.M, Khakh B.S. Contribution of calcium ions to P2X channel responses. Journal of Neuroscience. 2004;24:3413–3420. [PubMed: 15056721]
27. Hamill O.P, Marty A, Neher E, Sakmann B, Sigworth F.J. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Archiv. 1981;391:85–100. [PubMed: 6270629]
28. Sakmann B, Neher E, editors. Single-Channel Recording. second. New York: Plenum Press; 1995.
29. Hilgemann D.W. The giant membrane patch. In: Sakmann B, Neher E, editors. Single-Channel Recording. second. New York: Plenum Press; 1995. pp. 307–326. Chapter 13.
30. Chung S.H, Moore J.B, Xia L.G, Premkumar L.S, Gage P.W. Characterization of single channel currents using digital signal processing techniques based on Hidden Markov Models. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences. 1990;329:265–285. [PubMed: 1702543]
31. Qin F, Auerbach A, Sachs F. Maximum likelihood estimation of aggregated Markov processes. Proceedings Biological Sciences. 1997;264:375–383. [PMC free article: PMC1688268] [PubMed: 9107053]

32. Premkumar L.S, Qin F, Auerbach A. Subconductance states of a mutant NMDA receptor channel kinetics, calcium, and voltage dependence. Journal of General Physiology. 1990;109:181–189. [PMC free article: PMC2220056] [PubMed: 9041447]
33. Premkumar L.S, Ahern G.P. Induction of vanilloid receptor channel activity by protein kinase C. Nature. 2000;408:985–990. [PubMed: 11140687]
34. Premkumar L.S, Agarwal S, Steffen D. Single-channel properties of native and cloned rat vanilloid receptors. Journal of Physiology. 2002;545:107–117. [PMC free article: PMC2290669] [PubMed: 12433953]
35. Raisinghani M, Pabbidi R, Premkumar L.S. Activation of TRPV1 by Resiniferatoxin: Implications in chronic pain conditions. Journal of Physiology. 2005;567:771–786. [PMC free article: PMC1474234] [PubMed: 16037081]
36. Drew L.J, Rohrer D.K, Price M.P, Blaver K.E, Cockayne D.A, Cesare P, Wood J.N. Acid-sensing ion channels ASIC2 and ASIC3 do not contribute to mechanically activated currents in mammalian sensory neurons. Journal of Physiology. 2004;556:691–710. [PMC free article: PMC1664992] [PubMed: 14990679]
37. Hu J, Lewin G.R. Mechanosensitive currents in the neurites of cultured mouse sensory neurons. Journal of Physiology. 2006;577:815–828. [PMC free article: PMC1804210] [PubMed: 17038434]
38. McBride D.W Jr, Hamill O.P. Simplified fast pressure-clamp technique for studying mechanically gated channels. Methods Enzymology. 1999;294:482–489. [PubMed: 9916244]
39. McBride D.W Jr, Hamill O.P. A fast pressure-clamp technique for studying mechanogated channels. In: Sakmann B, Neher E, editors. Single-Channel Recording. second. New York: Plenum Press; 1995. pp. 329–339. Chapter 14.
40. Edwards F.A, Konnerth A, Sakmann B, Takahashi T. A thin slice preparation for patch clamp recordings from neurons of the mammalian central nervous system. Pflügers Archiv. 1989;414:600–612. [PubMed: 2780225]
41. Eilers J, Schneggenburger R, Konnerth A. Patch clamp and calcium imaging in brain slices. In: Sakmann B, Neher E, editors. Single-Channel Recording. second. New York: Plenum Press; 1995. pp. 213–227. Chapter 9.
42. Casteñada-Castellanos D.R, Flint A.C, Kriegstein A.R. Blind patch clamp recordings in embryonic and adult mammalian brain slices. Nature Protocols. 2006;1:532–542. [PubMed: 17406279]
43. Sontheimer H. Whole-cell patch clamp recordings. In: Boulton A.A, Baker G.B, Wolfgang W, editors. Patch-Clamp Applications and Protocols, Neuromethods Series. Vol. 26. New York: Springer-Verlag; 1995. pp. 37–73.
44. Reeh P.W. Sensory receptors in mammalian skin in an in vitro preparation. Neuroscience Letters. 1986;66:141–146. [PubMed: 3725179]
45. Kress M, Koltzenburg M, Reeh P.W, Handwerker H.O. Responsiveness and functional attributes of electrically localized terminals of cutaneous C-fibers in vivo and in vitro. Journal of Neurophysiology. 1992;68:581–595. [PubMed: 1527577]
46. Stucky C.L, DeChiara T, Lindsay R.M, Yancopoulos G.D, Koltzenburg M. Neurotrophin 4 is required for the survival of a subclass of hair follicle receptors. Journal of Neuroscience. 1998;18:7040–7046. [PubMed: 9712673]
47. Koerber H.R, Woodbury C.J. Comprehensive phenotyping of sensory neurons using an ex vivo somatosensory system. Physiology and Behavior. 2002;77:589–594. [PubMed:

12527004]
48. Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociciption in cutaneous hyperalgesia. Pain. 1988;32:77–88. [PubMed: 3340425]
49. D'Amour G.E, Smith D.L. A method for determining loss of pain sensation. Journal of Pharmacology and Experimental Therapeutics. 1941;72:74–79.
50. Watkins L.R, Martin D, Ulrich P, Tracey K.J, Maier S.F. Evidence for involvement of spinal cord glia in subcutaneous formalin induced hyperalgesia in the rat. Pain. 1997;71:225–235. [PubMed: 9231865]
51. Winter C.A, Risley E.A, Nuss G.W. Carrageenin-induced edema in hind paw of the rat as an assay for anti-inflammatory drugs. Proceedings of the Society for Experimental Biology and Medicine. 1962;111:544–547. [PubMed: 14001233]
52. Gilchrist H.D, Allard B.L, Simone D.A. Enhanced withdrawal responses to heat and mechanical stimuli following intraplantar injection of capsaicin in rats. Pain. 1996;67:179–188. [PubMed: 8895246]
53. von Frey M. Zur physiologie der juckemfindung. Archives Neerland Physiologies. 1922;7:142–145.
54. Chaplan S.R, Bach F.W, Pogrel J.W, Chung J.M, Yaksh T.L. Quantitative assessment of tactile allodynia in the rat paw. Journal of Neuroscience Methods. 1994;53:55–63. [PubMed: 7990513]
55. Randall L.O, Selitto J.J. A method for measurement of analgesic activity on inflamed tissue. Archives internationals de pharmacodynamie et de thérapie. 1957;111:409–419. [PubMed: 13471093]
56. Taiwo Y.O, Coderre T.J, Levine J.D. The contribution of training to sensitivity in the nociceptive paw-withdrawal test. Brain Research. 1989;487:148–151. [PubMed: 2752281]
57. Yaksh T.L, Rudy T.A. Analgesia mediated by a direct spinal action of narcotics. Science. 1976;192:1357–1358. [PubMed: 1273597]
58. Jeffry J.A, Yu S.Q, Sikand P, Parihar A, Evans M.S, Premkumar L.S. Selective targeting of TRPV1 expressing central terminals of spinal cord for long lasting analgesia. PLoS One. 2009;4:e7021. [PMC free article: PMC2737142] [PubMed: 19753113]
59. Hylden J.L, Wilcox G.L. Intrathecal morphine in mice: a new technique. European Journal of Pharmacology. 1980;67:313–316. [PubMed: 6893963]
60. Sidman R.L, Angevine J.B, Pierce E.T. Atlas of the Mouse Brain and Spinal Cord. Cambridge, MA, USA: Harvard University Press; 1971.
61. Burstein R, Yamamura H, Malick A, Strassman A.M. Chemical stimulation of the intracranial dura induces enhanced responses to facial stimulation in brain stem trigeminal neurons. Journal of Neurophysiology. 1998;79:964–982. [PubMed: 9463456]
62. Ren K. An improved method for assessing mechanical allodynia in the rat. Physiology and Behavior. 1999;67:711–716. [PubMed: 10604842]
63. Tawfik V.L, Nutile-McMenemy N, Lacroix-Fralish M.L, Deleo J.A. Efficacy of propentofylline, a glial modulating agent, on existing mechanical allodynia following peripheral nerve injury. Brain, Behavior, and Immunity. 2007;21:238–246. [PubMed: 16949251]
64. Milligan E.D, O'Connor K.A, Nguyen K.T, et al. Intrathecal HIV-1 envelope glycoprotein gp120 induces enhanced pain states mediated by spinal cord proinflammatory cytokines. Journal of Neuroscience. 2001;21:2808–2819. [PubMed: 11306633]

Copyright © 2011 by Taylor and Francis Group, LLC.

Bookshelf ID: NBK92807 PMID: 22593954