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Phenotyping sensory nerve endings *in vitro* in the mouse

Katharina Zimmermann^{1,2}, Alexander Hein¹, Ulrich Hager¹, Jan Stefan Kaczmarek², Brian P Turnquist³, David E Clapham², and Peter W Reeh¹

¹Department of Physiology and Pathophysiology, Friedrich-Alexander University of Erlangen-Nuremberg, Universitätsstraße 17, 91054 Erlangen, Germany

²Howard Hughes Medical Institute, Department of Cardiology, Children's Hospital Boston, Department of Neurobiology, Harvard Medical School, 320 Longwood Ave., Boston, Massachusetts 02115, USA

³Department of Mathematics and Computer Science, Bethel University, 3900 Bethel Drive, St. Paul, Minnesota 55112, USA

Abstract

This protocol details methods to identify and record from cutaneous primary afferent axons in an isolated mammalian skin–saphenous nerve preparation. The method is based on extracellular recordings of propagated action potentials from single-fiber receptive fields. Cutaneous nerve endings show graded sensitivities to various stimulus modalities that are quantified by adequate and controlled stimulation of the superfused skin with heat, cold, touch, constant punctate pressure or chemicals. Responses recorded from single-fibers are comparable with those obtained in previous *in vivo* experiments on the same species. We describe the components and the setting-up of the basic equipment of a skin–nerve recording station (few days), the preparation of the skin and the adherent saphenous nerve in the mouse (15–45 min) and the isolation and recording of neurons (approximately 1–3 h per recording). In addition, stimulation techniques, protocols to achieve single-fiber recordings, issues of data acquisition and action potential discrimination are discussed in detail.

INTRODUCTION

The skin is the largest sensory organ of the body and is densely equipped with sensory nerve endings. The peripheral nerve endings in the skin provide us with the senses of light touch, mechanical pressure, temperature and pain. The ability to detect these stimuli is critical for survival. Despite this prominent sensory role, our knowledge of the key transducing elements that underlie the perception of these senses is limited. With the increasing access to knockout animals and selective inhibitors, it is now possible to start to identify the molecular components contributing to each sensory pathway. This protocol describes how the rodent

skin–nerve preparation works and how it can be used to identify the molecules underlying the specific sensations experienced through the skin in wild-type and knockout animals.

Nociceptors are damage-sensing neurons that have their cell bodies in the dorsal root ganglion (DRG) and extend long processes to the skin where their terminals end, partly embedded between the keratinocytes of the epidermis. Assessment of specific ion channels in mechanical, heat, cold transduction and action potential generation is mostly based on experiments using heterologous expression systems and cultured DRG neurons. The native peripheral nerve endings are inaccessible to patch-clamping. Spinal ganglia, first from chick embryos with publications dating back to as early as 1884 (see refs. 1,2), have been a favorite for studies on neurons, but increasing availability of genetically altered mice is changing preferred model systems. Dissociation and culturing of DRG neurons^{3–8} has advantages as well as disadvantages. Isolated neurons in primary culture are accessible to intracellular patch-clamp recordings and measurements of Ca^{2+} or other ion activities using fluorescent dyes. Dissociation of DRGs, however, removes axons and satellite cells; the remaining neurons differentiate, or dedifferentiate, in the presence of various culture conditions. As a consequence, the composition of functional membrane proteins changes⁹, increasing differences between isolated cells and native nerve endings. For example, cultured DRG neurons show hardly any heat-activated currents when isolated from TRPV1-null mutant mice (lacking the heat and proton-sensing ion channel¹⁰). In contrast, polymodal nociceptors from the skin of whole animals lacking TRPV1 respond almost normal to noxious heat^{11,12}. In recent studies, cultured DRG neurons from $\text{NaV}1.8^{-/-}$ mice^{13,14} became quiescent when cooled to 10 °C, whereas the excitability of cutaneous nerve endings appeared similar to wild-type animals¹⁵. However, this cold resistance of the sensory terminals was abolished by TTX, suggesting ‘compensatory’ upregulation or modification of TTX-sensitive Na channels in the $\text{NaV}1.8$ knockouts^{14,16}. Thus, radically different conclusions could be drawn from intact animals or skin–nerve preparations on one side and cultured neurons on the other.

Ideally, one would like to selectively inactivate a protein under study, but few pharmacological tools are sufficiently specific. Disabling a specific protein through gene disruption still allows for uncertainty in assigning a protein’s normal function, as compensatory changes occur during animal development. Even complete mRNA disruption by siRNA (rarely possible in mammalian cells) is sufficiently slow to allow for compensatory changes to occur. Isolation and culturing neurons from the treated mice adds an additional layer of complexity.

Terminals of sensory neurons

The response characteristics of mammalian native sensory afferent terminals to stimulation have been studied extensively since the 1950s using *in vivo* preparations of cutaneous sensory nerves from cats, dogs and other species. Nociceptors were studied for the first time in 1969 by Burgess and Pearl¹⁷. The most commonly studied are the lingual, infraorbital, posterior femoral cutaneous, saphenous, sural, and plantar nerves^{17–24}. In intact experimental animals, however, it is difficult to achieve complete control over all environmental variables affecting the skin. For example, there is no method to control the

effective concentration of exogenous chemicals at the receptive site in nociceptive chemosensitivity studies. Furthermore, some routes of administration (injection, pricking and blister induction) result in skin damage that may alter nociceptor sensitivity. Even arterial injection of agents close to the site depends on circulatory regulation, and many substances are highly vasoactive (bradykinin, histamine and acetylcholine) and thus influence their own distribution.

The first *in vitro* preparations of sensory nerves and their attached skin appeared in the late 1950s and were developed in frog, toad, salamander and leech for the study of mechanoreceptors^{25–29}. Mammalian models are more difficult. For studies of visceral nociception, rabbit pleura–phrenic nerve³⁰, dog testis–spermatic nerve³¹, rodent muscle–nerve^{32–34} and rat and guinea pig esophagus–vagal nerve^{35,36}, models have been developed *in vitro*. The last model combines the single-fiber recording technique, with the distinct distribution pattern of nerve endings in the rat esophagus and provided functional insight into signal processing within mucosal sensory terminals of the vagus nerve. In the only cutaneous model, the isolated perfused rabbit ear–auricular nerve preparation, receptor responsiveness appears to degenerate 4 h after isolation^{37,38}. The more robust *in vitro* cornea–ciliary nerve preparation, which has provided insight into impulse generation in nerve terminals, especially in response to cold stimulation, is restricted to guinea pig^{39–43}. A recently described *ex vivo* somatosensory system from Koerber *et al.*^{11,44,45} allows the combination of functional and morphological studies of the primary sensory neuron. In this model, intracellular recordings in response to natural stimulation of the skin can be associated with morphological information, such as neuropeptide content and laminar projection of the respective neuron in the spinal cord; whereas thermal and mechanical stimulation is performed precisely to the intact epidermal side of the respective cutaneous receptive fields, chemical stimulation is limited by the transepidermal diffusion barrier.

A versatile model for studying the primary sensory neuron responses in the mouse skin is the saphenous skin–nerve preparation⁴⁶. It enables extracellular recording of propagated action potentials from the receptive fields of single sensory nerve endings in the skin. Responses are comparable with those obtained *in vivo* in the same species of rodent^{47,48}. One particular advantage is that the preparation of the skin is fast to isolate and can be kept for many hours under superfusion, and a considerable number of recordings (up to ten or more, depending on the experience of the experimenter) can be obtained from one mouse (i.e., two preparations). In addition, compounds are applied directly to the corium at a known concentration, which allows tight control of the concentration of chemicals and avoids diffusion barriers. In general, even very large water-soluble molecules, such as the peptide CGRP (3,790 g mol^{−1}), and also the smaller alkaloid TTX (319 g mol^{−1}) show effect in the terminal nerve endings in less than 5 min in the rat and less than 2 min in the mouse (with much thinner skin)^{15,49}. The hydrophobic molecules, mustard oil (allyl isothiocyanate, 99 g mol^{−1}) and capsaicin (306 g mol^{−1}) and also menthol (156 g mol^{−1}), often show immediate excitatory effects or require less than 2 min to reach their target. Therefore, this preparation has been used for pharmacological studies of a wide variety of proalgesic and analgesic compounds on nociceptive nerve endings^{49–58}. In addition, the saphenous skin–nerve preparation is particularly valuable for larger sampling studies and, in

combination with intracellular recordings from DRGs, it can provide valuable information about the function of distinct ion channels in their physiological context. It takes advantage of the uniform genetic background of inbred mice and genetically altered mice to evaluate the influence of single proteins or genes on the sensory transduction process, and on action potential electrogenesis and propagation^{15,49,59}. The basic setup can also be used to investigate the effects of inflammatory conditions⁶⁰ and primary afferent aspects of neuropathic pain⁶¹. A major limitation of the preparation, however, is that a lot of patience and time is required to find and record from single units. Each fiber has to be identified as a single fiber, whereas with patch clamp recordings, cells are usually plated or cultured as single cells and can be recorded immediately. Therefore, the time frame for beginners to learn the technique may well be several months.

Organization of the protocol

This protocol focuses on the establishment of an *in vitro* extracellular recording station to be used for split-fiber preparations. It shows the anatomical context of the saphenous nerve innervation territory and describes how the saphenous skin–nerve preparation is performed in adult mice. Major importance is attached to procedures for discriminating and recording single unmyelinated C-fibers in multifiber strands (C-fibers are grouped in Remak bundles that cannot be separated by further splitting). Several techniques are described that are used for the identification of a single unmyelinated C-fiber and its receptive area in the skin within a thin multifiber strand that is placed on the recording electrode. The marking technique uses the analysis of post-excitatory changes of the conduction velocity. By combining electrical and natural (mechanical, chemical, heat, cold) stimulation, interference of action potentials from other receptive fields and C-fibers that are within the same multifiber strand can be recognized. This technique uses the activity-dependent slowing of action potential propagation, which appears as increased latency between electrical stimulation and arrival of the action potential at the recording electrode, to make sure that the recording is gained from one single fiber. Other techniques, such as analysis of the interspike interval and the spike shape, are pointed out and shown in ANTICIPATED RESULTS. In addition, issues of data acquisition and other aspects that are relevant for extracellular electrophysiological recordings are discussed in Box 1. Typical characteristics of responses of single fibers to natural (temperature) and electrical stimulation are pointed out in ANTICIPATED RESULTS.

BOX 1

RECORDING EXTRACELLULARLY AND DATA ACQUISITION SOLUTIONS

Extracellular recordings Extracellular recordings of single nerve fiber activity are the least invasive electrophysiological methods. With a pair of gold wire electrodes, a recording electrode and a signal reference, the potential difference between one point along the length of the nerve (which is a bundle of several axons) and the grounded extracellular milieu are measured. Extracellular potentials are in the μV range, requiring an overall gain of $\sim 10^5$. In practice, this requires two stage of amplification: an AC-coupled differential preamplifier to eliminate offsets is connected in series with a second single-ended 10^4 -gain amplifier band pass filtered between 100 and 1,000 Hz. As the

signal is variable in the range of μV , the actual gain for this sort of extracellular recording varies from 50 to 500 K. For myelinated A-fibers, gains are 10–100 K, whereas 100–500 K gains are needed for C-fiber activity. The signal is monitored on an amplified loudspeaker and an oscilloscope. Figure 1 shows the circuit. The output signal of the amplifier is filtered and recorded using commercially available software systems.

Data acquisition

A large range of digital acquisition cards are commercially available. For nerve extracellular recordings, sampling rates are $<20\text{ kHz}$. Spike 2 (Cambridge Electronic Design) and DAPSYS (John Hopkins University) are two systems that differ principally in their method of data acquisition. Although both systems allow open-channel recording for a period limited only by available disc space memory, the DAPSYS system can collect variable-length, threshold-triggered events. The Spike 2 system offers both template-matching and Principal Component Analysis as methods for discriminating spikes, whereas DAPSYS has implemented digital versions of the analog window discriminator.

(A) DAPSYS data acquisition system (<http://www.dapsys.net>; Brian Turnquist, turnquist@bethel.edu). Using the Dapsys data acquisition system, the output of the amplifier is sampled at 33 KHz and filtered. This system uses a standard PC-running Windows XP for the user-interface and a Microstar DAP5200 data acquisition processing board installed into a PCI slot. The DAP board uses an AMD K6-III+ processor and runs a real-time operating system and includes custom software for data acquisition, filtering and stimulator control. Analog inputs and analog control outputs are $\pm 10\text{ V}$. Threshold-triggering and window discrimination of the analog signal provide an initial classification of action potentials. Most conveniently, each action potential is assigned an ID and a timestamp. In addition, selected clusters of action potentials can be copied in subfilters and filtered manually. Correlation with digital triggers is also possible along with waterfall displays and automated latency measurement (Fig. 2). The data is analyzed offline using the Dapsys software package that provides a template-matching procedure for automatic spike discrimination^{80,81}. All data can be exported into Microsoft Excel and further analyzed.

(B) Spike 2 from Cambridge Electronic Design (<http://www.ced.co.uk>). In conjunction with the CED micro1401 AD card, the CED Spike2 system allows control of multiple I/O analog and digital channels. The working input range of analog input channels is $\pm 5\text{ Volts}$. Systems can be cascaded and linked through a master clock offering large-scale acquisition possibilities. The most powerful aspect of this system is the C-like scripting capabilities that allow custom features to be realized with basic coding skills. These include possibilities for feedback control through board, serial or parallel ports, fuzzy-logic control as well as online decision-making. For nerve activity, both real-time and post hoc analysis are available. Similar to the DAPSYS system, initial spike detection is based on threshold crossing and subsequent template matching over a defined time domain. Figure 3 illustrates a typical offline analysis in Spike 2.

MATERIALS

REAGENTS

Composition of synthetic interstitial fluid

- Sodium chloride (NaCl; Sigma, cat. no. S9888)
- Potassium chloride (KCl; Sigma, cat. no. P9333)
- Magnesium sulphate, heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; Sigma, cat. no. M1880)
- Sodium bicarbonate (NaHCO_3 ; Sigma, cat. no. S5761)
- Sodium phosphate monobasic, dihydrate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$; Sigma, cat. no. 71500)
- Gluconic acid, sodium salt (Sigma, cat. no. G9005)
- Glucose (Sigma, cat. no. G7021)
- Sucrose (Sigma, cat. no. S7903)
- Ca^{2+} chloride, dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; Sigma, cat. no. C5080)
- Adult mice weighing 26–32 g ! **CAUTION** All experiments involving live rodents must conform to appropriate local and national regulations. **▲ CRITICAL** The larger the mice, the easier the isolation of single units (there is less overlap of receptive fields).

EQUIPMENT

- Cylinder with compressed carbogen gas (5% CO_2 , 95% O_2) including pressure-reducing and flow-adjusting valves
- Container for storing extracellular fluid during the experiment (6–10 liters of volume, e.g., Carboy, Fisher Scientific)
- Column: for gassing the extracellular fluid, custom designed (from transparent inert plastic material, height ~120 cm, diameter ~4 cm, wall ~3 mm), connected to the container with extracellular fluid. The column is needed to build up hydrostatic pressure for the superfusion of the preparation
- Filtering candle: Winzer, Laboratory Glassware, 97866 Wertheim, Germany (cat. no. 23-076-14, product specification “Porosity 4”), to be connected to the carbogen gassed through tubing and immersed in the extracellular fluid in the column
- Flow regulation: Fluid flow is best regulated through a drop chamber of an infusion system (i.v. extension set; dial-a-flo, Abbott)
- Fiberoptic light source (e.g., Leica, Fiber-Lite)
- Tubings, connectors: Tygon Tubing (Tygon R-3603 Laboratory Tubing) of different diameters and connectors of different sizes (e.g., Fisher Scientific) are required for perfusion of the recording chamber. Suggested tubing sizes are as follows:
- ID 4.8 mm (3/16 inch) and OD 7.9 mm (5/16 inch);

- ID 6.4 mm (1/4 inch) and OD 9.5 mm (3/8 inch);
- ID 7.9 mm (5/16 inch) and OD 11 mm (7/16 inch);
- in all sizes, the wall should be 1.6 mm (1/16 inch).
- Heat exchanger from glass: to prewarm solution before it enters the organ bath (e.g., Radnoti Glass Technology)
- Heated circulator: controls the temperature of the heat exchanger (e.g., VWR, Pharmacia Biotech)
- Refrigerated circulator: optional, required for cold stimulation (e.g., VWR, Pharmacia Biotech)
- Amplifier: the ISO-80 is an insulated low-noise AC-coupled differential amplifier with high- and low-pass filters (World Precision Instruments) A disadvantage is the long cable connection (1 m) between the preparation and the signal input; an alternative is the DP-301 (Warner instruments) ▲ **CRITICAL** A second-stage amplifier should be equipped with a Vernier adjustment, a Noise Cut (anti-noise filter that cuts out inevitable resistive noise) and a Notch filter (a band-pass filter centered on 60 Hz that eliminates 60-Hz cycles).
- A set of custom-designed amplifiers: frequency range 100–2 kHz; voltage gain: pre-amplifier 100, main amplifier 0–5,000, signal line outlet and Noise Cut audio outlet; integrated impulse generator including trigger outlet with adjustable stimulus width and repetition frequency to control an external stimulus isolator. The preamplifier is provided by an ultra low-noise isolation amplifier (intronics IA297, Edwardsville, KS, USA) with a peak-to-peak noise of <2 μ V. A custom-designed set of amplifiers is available from *Labortechnik Franken* through Katharina Zimmermann ▲ **CRITICAL** Using the differential recording mode for preamplification minimizes interferences and allows experiments to be done without a Faraday cage.
- Oscilloscope: an inexpensive two-channel digital storage oscilloscope is adequate
- Amplified loudspeaker
- Digital thermometer: with miniature thermocouple and ‘analog out’ option
- Standard PC running Windows XP including appropriate hardware and a data acquisition system for extracellular recordings (see Box 1 and Figs. 1–3)
- Electrostimulator (e.g., SD9 Square Pulse Stimulator, Grass Telefactor; A395 Linear Stimulus Isolator, World Precision Instruments)
- Metal microelectrodes: can be custom made or purchased, e.g., from Frederick Haer. According to the requirements of the experiment they have high (e.g., 9–12 M Ω , cat. no. UEWSHGSELN2M) or low (e.g., 50–100 k Ω cat. no. UEXMHGSEAN2M) impedance. Materials are epoxy-insulated steel or tungsten ▲ **CRITICAL** High-impedance electrodes are suitable for excitability measurements and require precise positioning, whereas low-impedance microelectrodes are used

when current or voltage is used as the search stimulus (instead of a mechanical stimulus) and needs to spread widely.

- Custom-designed rings: aluminum, stainless steel, Delrin, glass
- Petroleum jelly (vaselina alba): used to line the bottom edge of the ring to make a watertight connection between the ring and the skin
- Set of von Frey hairs: either as calibrated monofilaments (e.g., <http://www.touch-test.com/home.htm>) or as gravity-driven von Frey hairs (both sets can be acquired from the workshop of the Department of Physiology, University of Erlangen-Nuremberg, through Peter Reeh) ▲ **CRITICAL** The tips of commercial von Frey hairs should be of uniform diameter (e.g., 0.8 mm) and blunted using nail varnish to avoid falsely low thresholds produced by the sharp edges of the hairs. Our sets consist of 18 hairs that are calibrated from 1 to 362 mN in a geometric series

$$[x_i - x_{i-1} \times \sqrt{2}].$$

Optional instrumentation for thermal and mechanostimulation: a computer-controlled stimulation system ('Physio Girl III'), including a radiant heat module, a peltier-based contact cold stimulator and a mechanostimulator, is available from Hofmann Elektronik Erlangen (Alexander Hofmann, alx.hofmann@web.de).

Radiant heat devices feedback-control the power of the radiant heat lamp by using the corium-side temperature. The difference between epidermal and corium temperature during radiant heat stimulation is only about 1 °C in mouse skin, up to 5 °C in rat skin (higher in the epidermis).

Surgical dissection tools (e.g., Fine Science Tools): 1 pair of scissors, 1 pair of spring-leaded scissors, 1 surgical forceps, 1 pair of sharpened forceps, 1–2 needle holders, 1 scalpel

10-ml syringe

Electric razor

Vacuum cleaner

Lint-free cloth (e.g., Kimwipes)

Surgical silk: silk fiber, 4/0 USP, 1.5 metric

Q-tips

Parafilm

Wedge, e.g., from plastic 5 cm × 5 cm, 1–0.3 cm height: to immobilize the animals' foot during the surgical procedure using glue or double sided tape

Forceps: several pairs of watchmaker forceps are required to separate fibers (Fine Science Tools, Dumont no. 5, 11 cm, straight tip 'Biologie,' no. 11252-20) ▲ **CRITICAL** The tips require further sharpening (sharpening stone for Dumont forceps, no. 29008-22, FST and sandpaper grade 800–1,000). Another pair of forceps (FST, standard tip, no. 11251-20) is useful for other tasks.

Silicon rubber glue (Dow Corning, cat. no. 3140)

Stainless Steel Minutien Pins (insect pins; 0.2 mm diameter; Fine Science Tools, cat. no. 26002-20)

REAGENT SETUP

Synthetic interstitial fluid—Sodium chloride (107.8 mM), potassium chloride (3.5 mM), magnesium sulphate, heptahydrate (0.69 mM), sodium bicarbonate (26.2 mM), sodium phosphate monobasic, dihydrate (1.67 mM), gluconic acid, sodium salt (9.64 mM), glucose (5.55 mM), sucrose (7.6 mM) and calcium chloride dihydrate (1.53 mM). Before adding Ca^{2+} , dissolve all components in ultrapure distilled water and gas with carbogen (5% CO_2 , 95% O_2) for at least 5 min. The pH will then drop from ~7.8 to ~7.4 and CaCl_2 will dissolve well (ref. 62) (see TROUBLESHOOTING). ▲ **CRITICAL** SIF should be prepared fresh at the beginning of each experiment. Solution stored over 24 h or longer at 4 °C potentially induces ongoing activity in the preparation.

EQUIPMENT SETUP

Recording chamber—Chamber consisting of two compartments, an organ bath for superfusion and oxygenation of the skin flap, and an adjacent recording chamber where single-fiber activity is recorded from nerve filaments. The recording chamber is equipped with one pair of recording gold wire electrodes and one grounding electrode. The electrodes are connected through shielded cable connections to the differential amplifier. The pair of recording electrodes is best installed in closest proximity to the recording chamber. The recording chamber contains a small socket of 5-mm height on top of which a mirror glass is glued. The main chamber is 4 mm deeper than the recording chamber to leave space for a 4-mm-deep transparent silicon rubber bottom (Sylgard). Both compartments are separated by a 1-mm-thick acrylic sheet with two holes at the bottom (of 1.5–2 mm diameter). The holes are required to allow for exchange of aqueous fluid between both compartments. One hole is located close to the mirror and is needed to thread the nerve through. The distance between the mirror and the acrylic sheet is usually 1.5–2 mm. A custom-designed Delrin chamber (adapted for use with either mouse or rat preparations) is commercially available from the workshop of the Department of Physiology, University of Erlangen-Nuremberg, through Peter Reeh. ▲ **CRITICAL** The springy gold wire recording electrodes in the recording chamber are best adjusted for position with a micromanipulator (Little Giant Series, U-1C, Narishige) and a custom-designed holder, e.g., from a thin glass rod (0.3 mm diameter).

Mirror—Cut mirror glass into small pieces (8 mm × 10 mm) and glue on top of the socket in the recording chamber (e.g., using silicon rubber glue). The reflective surface of the mirror is the efficient base for teasing fibers apart. ▲ **CRITICAL** With respect to the limited length of the mouse saphenous nerve, it is convenient to fix the mirror close to the barrier, which separates the recording chamber from the organ bath (ideally approximately 3–4 mm). There the mirror can be fixed in a slightly angled position to easily allow threading the nerve through the hole (e.g., 3–4 mm distance to the barrier for the right edge facing the hole and 2–3 mm for the left edge).

Insect needles—Insect needles are used to pin the skin to the silicon bottom of the chamber. Needles should be stainless steel to avoid corrosion by the physiological buffer (Minutien Pins (insect pins)). The needles are bent to a right angle, and shortened with fine pliers. ▲ **CRITICAL** The sharp end must be no longer than the depth of the Sylgard chamber.

Synthetic gauze material—Synthetic gauze material is placed in the inlet of the chamber to laminarize the fluid flow.

Silicone elastomer—Sylgard 184, a two-component silicone elastomer, forms the bottom of the chamber and should be replaced every 3–6 months due to increasing porosity.

Electrodes—Solder the end of a fine gold wire (diameter 0.15 mm, ~5 cm long) into a 1-mm male gold connector pin (Multi-Contact) and cover the top ends with thermoplastic shrink tubing to reduce the surface for electric contact and to conserve the soldering point where the wire can easily break (Fig. 4a). The chamber contains in-built female 1-mm gold connectors that form the connection to the amplifier and the stimulus isolator. ▲

CRITICAL Although platinum polarizes least, gold wire is superior because it is easier to solder, more convenient to bend and less springy. Silver oxidizes and sulphatizes.

Glass rods—Glass rods with different custom-designed tips (e.g., Moretti glass rods, 0.5 cm diameter, length at least 30 cm). The rod is heated and bent to form a blunt tip, the final length of the shaft should be around 12–15 cm (see Fig. 4b).

Binocular—Mount a binocular microscope above the recording chamber with a stable, custom-designed pivot-mounted arm. The working distance should be at least 8–15 cm with 40× magnification (e.g., Olympus SZ40).

Two 3-way micromanipulators with magnet base—For example, Narishige, FST, WPI; one micromanipulator is required for precise electrostimulation: fasten the microelectrode in a holder and attach it to the micromanipulator, then establish connections to the stimulus isolator. The second micromanipulator is used to hold and move the fluid application system, which is described in Box 2.

BOX 2

ASSEMBLY OF SYSTEM FOR THERMAL AND CHEMICAL STIMULATION OF ISOLATED RECEPTIVE FIELDS (1–2 D)

Special materials

Steel cannula: afferent: OD ~1.1 mm, ID ~0.5 mm, length tail/length tip ~15 cm/~2 cm, efferent OD ~1.5 mm, ID ~0.9 mm, length tail/length tip ~24 cm/~1.5 cm (SWS-Edelstahl GmbH, Emmingen, Germany)

Resistive wire: Isotan with insulation, e.g., polyurethane-based self-fluxing wire enamel. A resistance of $10 \Omega \text{ m}^{-1}$ is optimal for fast heat dissipation (OD = 0.25 mm). Higher resistance values require more voltage (that may exceed the power-supply abilities); lower resistance wires tend to overheat, leading to destruction of the wire with consequent noise interference

Thermal adhesive: e.g., Arctic Silver5 (<http://www.arcticsilver.com/as5.htm>) is used to establish an optimal thermal contact between the resistive wire and the steel cannula

Power Supply: the PeakTech 1885 can be computer-controlled through software that is included

Tubing pump: the Reglo Digital from Ismatec is a compact multi-channel microprocessor-controlled dispensing pump that can be remote-controlled through an RS232 interface. We recommend using tubing with a diameter of 1.85 mm for the afferent channel and 2.29 mm for the efferent channel

Bracket: custom designed, e.g., from brass

Needle: common 18 G bore or larger (e.g., Becton Dickinson)

Tygon tubing: different diameter

Small diameter silicone/polyethylene/teflon tubing: needs to fit tight to the afferent cannula

Refrigerated circulator: e.g., Pharmacia Biotech, Multi TempIII

3-way valves ($n = 4$): ID 4 mm (e.g., VWR or Fisher Scientific)

Tools:
Regular sandpaper or metal file
Diagonal cutting pliers
Thermoplastic shrinking tube

The system allows to superfuse the cutaneous receptive fields, isolated by a steel cylinder, with heated or cooled physiological solution containing, or not, conditioning chemicals. It runs in push–pull mode whereby the longer cannula (Fig. 11a) is afferent and the shorter one is efferent, defining the fluid level inside the ring and connected to slightly greater diameter tubing (of the roller pump). The afferent tubing is jacketed over a stretch of ~1 m, running inside a larger tubing that comes from one of two thermostatic pumps and ends in a V-shaped custom-welded metal pipe (Fig. 11b), the other leg returning to the thermostat. One of the thermostats is adjusted to a prewarming temperature of 30 °C for heat stimulation, the other one to ice-cold 0 °C for cold stimulation; manually controlled valves allow one to switch between the thermostats that perfuse the jacketing tubing and pipe. The afferent core tubing connects to a steel cannula that passes the V pipe and extends toward the outlet being wrapped with a resistive heating wire for adjustment of the final superfusion temperature.

Altering the tips of the application system (Fig. 11a)

Cut the two steel cannulas to the required length. File away the kinked ends. Bend the two cannulas at one side to a right angle to form the application tip required for the perfusion of the metal ring around the receptive field. The cannula must not be kinked (use a curved metal pipe to achieve a smooth bend). At the bent top end, the afferent cannula should be slightly longer than the efferent cannula (i.e., 3–5 mm, Fig. 11a).

Installing the resistive wire for heating

Line the afferent cannula with Arctic Silver, a two-component thermal adhesive (allow to dry). Coil the resistive wire around the afferent cannula over a length of ~14 cm, starting from the top end behind the bend (280–300 winds). Solder the ends of the wire to cables and insulate the connections with thermoplastic shrinking tubes. Connect the cable ends to banana plugs for the power supply. Attach both cannulas side by side using tape or shrink tubing. Connect the afferent and efferent cannulas through silicon tubing to the tubing pump. Applying current to the resistive wire heats the solution pumped through the cannula. Computer control of the power supply allows design of ramp- or step-shaped heat stimuli (Fig. 11c). The stimulus temperature can be recorded by attaching a thermocouple to the tip of the afferent cannula (see also Fig. 11). The system can also be used effectively for Ca^{2+} imaging⁸⁴.

Fluid circulation system—A sketch of the experimental setup is provided in Figure 5. The recording chamber for skin–nerve superfusion and recording should be perfused by extracellular solution saturated with carbogen (95% O_2 , 5% CO_2)⁶². Prewarm the fluid with a pump-driven heat-exchanger to maintain a temperature of 30–32 °C. Use Tygon tubing to realize the fluid circulation. Integrate the heat exchanger (connected to the thermostat) and a column that is required to saturate the extracellular fluid in the circuit with gas. To gas the synthetic interstitial fluid (SIF), use a filtering candle and connect through an intermediary pressure valve to the gas cylinder containing carbogen.

System for thermal and chemical stimulation of isolated receptive fields—See Box 2.

PROCEDURE

Surgical procedure ● TIMING 20–25 min for each preparation

- 1| Asphyxiate the mouse in CO_2 ; remove the fur from both hindpaws up to the inguinal region using an electric razor. Remove excess hair from the mouse with vacuum suction, or with a tissue cloth soaked in water.

▲ **CRITICAL STEP** Remove as much hair as possible; this makes immersion of the skin preparation into the organ bath easier.

- 2| Use double-sided sticky tape to fix the plantar side of the paw with the tape to the wedge. Stretch the skin, and then make a circular incision at approximately 1–2 mm above the knee joint. The skin distal to the incision will form the skin part of the skin–nerve preparation (see Fig. 6).
 - 3| To expose the saphenous nerve, make two parallel incisions, laterally and medially from the knee toward the inguinal ligament. Remove the skin to make the saphenous nerve visible on its entire length up to the inguinal ligament (Fig. 7a). Rinse the nerve and the exposed muscle with SIF from a syringe frequently.
- ▲ **CRITICAL STEP** Keep the exposed part of the nerve wet with physiological saline at all times during the preparation.

- 4| Separate the saphenous nerve from the connective tissue by using a pair of sharpened forceps. Detach the nerve from the saphenous vessels running side by side with the nerve (as shown in Fig. 7a). Remove the connective tissue from the nerve carefully millimeter by millimeter from distal to proximal until you reach the inguinal ligament (Fig. 7b).

▲ CRITICAL STEP Never pinch the nerve but the epi-/perineurium. A pinched segment is transparent and usually does not recover.

? TROUBLESHOOTING

- 5| Above the inguinal ligament, search the stem of the femoral nerve that is embedded in the superficial muscle layer. This is a thick bundle, and it contains the fibers of the saphenous nerve. Lift the whole bundle (place the tip of the forceps underneath) to pull a short piece of surgical silk underneath it. Make a knot around the nerve with the surgical silk. Cut the whole nerve proximal to the knot, using the pair of spring scissors (Fig. 7b).

▲ CRITICAL STEP By including the stem of the femoral nerve above the inguinal ligament (the saphenous nerve fibers run within the femoral nerve), extra length can be gained that will be required later during the recording (especially in mouse). The saphenous nerve is included in the femoral nerve as a thinner separate bundle. Fibers from the femoral nerve can be removed easily while setting up the experiment (see Step 17).

- 6| Cut the inguinal ligament and lift the end of the nerve with the forceps. Cut the side branches of the nerve that innervate the muscles (Fig. 7b). Remove all remaining adhesions with the pair of spring scissors and/or the forceps until the nerve is free of any adhesions to the accompanying vessels and muscle.

▲ CRITICAL STEP Do not stretch the nerve, as it leads to tearing of the axons. Exposure to air should be as short as possible.

- 7| Leave the nerve loosely convoluted and covered with a piece of lint-free absorbent (e.g., KimWipes) soaked with SIF (Fig. 7c).

- 8| *Skin flap preparation of the forefoot and the calf:* using the scalpel, make a circular cut at the border of hairy and glabrous skin around the forefoot (Fig. 7d). This cut reaches from the insertion of the Achilles tendon at the calcaneus to the lateral edge of the foot, goes over the middle phalanges of the toes and reaches around the medial edge of the foot back to the heel where it started from. This incision should confine a skin flap including the whole hairy skin of the back of the forefoot and excluding glabrous skin. Bisect the skin on the back of the calf with a straight incision, starting from the insertion of the Achilles tendon up to the popliteal fossa (Fig. 7e). The saphenous nerve should be located about equidistant to each side of this incision. As a result, the nerve will be running in the middle of the preparation (see inset in Fig. 6). Then, separate the hairy skin subcutaneously from the tendons starting from the toes backward

to the ankle by lifting the free margin with a forceps and pulling it slowly backward; use the second forceps or scissors to remove adhesions (Fig. 7f).

▲ CRITICAL STEP Verify that the branches of the saphenous nerve are present and intact as pointed out in Figure 7f. No tendons adhere to the skin flap.

- 9| Continuing with the free medial margin of the skin, separate it from the underlying muscle toward the saphenous nerve. On its descent to the ankle, the saphenous nerve closely follows the medial tibial border (compare Fig. 7g with Fig. 6).

▲ CRITICAL STEP The saphenous nerve runs in a slightly curved route on the medial malleolus. It is easy to accidentally injure the nerve, denervating all receptive fields in the entire distal skin flap.

- 10| Continue with the lateral free margin of the skin.

▲ CRITICAL STEP Make sure to remove muscle eventually attached to the nerve. Thick chunks of muscle cannot survive under superfusion and release potassium, which leads to a depolarizing conduction block.

- 11| Place the whole skin flap with the saphenous nerve in a pre-gassed SIF-filled beaker. Close the beaker with parafilm and keep it cold.

▲ CRITICAL STEP The whole dermis, including the nerve, must be in contact with fluid.

PAUSE POINT The preparation can be kept for up to 8 h at 4 °C.

Setting up the experiment ● TIMING 15–20 min

- 12| Place a small amount of synthetic wool in the inlet funnel of the chamber and let the chamber fill with gassed prewarmed SIF. Adjust the flow at a rate of 180 ml h⁻¹.

- 13| Mount the preparation in the organ bath chamber by tightly piercing with insect needles through the very edges of the skin into the silicon rubber. Slightly stretch the skin. The epidermis faces the bottom of the chamber, exposing the corium (dermis) to the extracellular solution and allowing access to receptive fields (Fig. 8a).

- 14| Thread the end of the nerve (free length 25–35 mm for rats; 15–20 mm for mice) through the (~2 mm) hole into the adjacent recording chamber and place it on the surface of the mirror.

▲ CRITICAL STEP Do not touch or strain the nerve with the forceps, rather use the end of the surgical silk knotted around the cut end to move the nerve.

? TROUBLESHOOTING

- 15| Apply paraffin oil of low viscosity on top of the aqueous solution in the recording chamber.

Establishing stable recording conditions ● TIMING 1–10 min

- 16| Adjust the interface of the two liquids to just at the mirror so that the mirror is mostly free of aqueous fluid (a thin layer is inevitable, but there should be no floating of the nerve; compare schematic in Figs. 5 and 8b). If necessary, immerse a pair of forceps in the liquid to visualize the height of the liquid interface.

▲ CRITICAL STEP Stable recording conditions are established when the recording electrode is positioned in the oil above the mirror and the reference electrode is dipped in the aqueous solution below the mirror. The aqueous solution is grounded through a separate electrode. It is crucial to ensure that the aqueous solution on the surface of the mirror is minimized. A small amount of aqueous solution surrounds the nerve and facilitates separation of the fibers. Importantly, the action potentials are bigger, the closer to the nerve stem the teased filament is contacting the electrode. Care has to be taken that there is no short-circuiting through an eventual drop of aqueous fluid in the angle between filament and nerve or mirror surface.

? TROUBLESHOOTING

- 17| Using the spring scissors, cut off the silk knot from the nerve stem and, with the pair of forceps, remove the bundle of axons that belong to the dead end of the femoral nerve trunk. If necessary, cut the saphenous nerve trunk again with the spring scissor. Pull off the thin sheath (epi-/perineurium) of the saphenous nerve trunk (like a stocking; arrow tip in Fig. 8c) over 2 mm and place the whole, desheathed end of the nerve on the recording electrode.

? TROUBLESHOOTING

- 18| Switch on the amplifier and test the innervation of the skin and the signal-to-noise ratio of the recording. Apply light pressure with a blunt glass rod to the skin, going from proximal to distal. Masses of A β - or A δ -fiber discharge (myelinated nerve fibers in somatic nerves, conducting nerve impulses at a rate of 1.6–12 m s⁻¹ (A δ) and >16 m s⁻¹ (A β)) should be visible and audible at a gain of less than 500,000.

? TROUBLESHOOTING

Obtaining a single-fiber recording ● TIMING Limited by luck and patience: 5 min to several hours

- 19| To investigate the response properties of a single-fiber terminal, a single-fiber receptive field must be identified: begin with teasing a strand of about one-fifth of the whole nerve out of its desheathed end. Subdivide it further into two or three smaller filaments of which one is placed on the electrode (Fig. 8c).

▲ CRITICAL STEP Several pairs of sharpened watchmaker forceps are helpful. Forceps must be kept sharp (using grindstone and sandpaper) as blunt

forceps hinder precise filament teasing, and the mirror must be changed occasionally (sharp forceps scratch the smooth surface of the mirror).

▲ CRITICAL STEP Always keep the recording electrode clean, for example, by wiping it with a Q-tip dipped in alcohol. Dried nerve material coating the electrode makes it too sticky and tears the filaments when they are removed from the electrode.

▲ CRITICAL STEP Precise insertion of a microelectrode (stem electrode) in the trunk of the nerve where it enters the recording compartment can also be undertaken at this stage and constitutes an accessory tool, especially for beginners. The ‘stem electrode’ allows, during the whole nerve recording, the identification of the electrical threshold of the C-fiber (unmyelinated nerve fibers, conducting nerve impulses at a velocity of $0.2\text{--}1.2\text{ m s}^{-1}$) compound action potential and can ease the identification of a single fiber later on during the experiment because it allows the determination of the number of fibers present in a teased filament. This technique is particularly helpful for recordings in the rat where the whole nerve is thicker and less fragile than in the mouse.

- 20| Subdivide filaments until the discharge evoked by stimulating the skin with a glass rod results from only a few separate or overlapping mechanosensitive receptive areas.

▲ CRITICAL STEP Subdivision of filaments should lead to increased signal-to-noise ratio and a reduction in the required gain.

- 21| At this point, there are several different techniques that can be used or combined to reduce the multifiber recording to a functional single-fiber recording: by manipulating the gain (option A), by action potential shape discrimination (option B), by distance (option C) or by further subdivision (option D).

(A) Obtaining a single-fiber recording by manipulating the gain

- i. Subtle regulation of the amplifier gain allows one to dispose of smaller spikes that vanish in the background noise; taller spikes from a single fiber can be followed, especially when recording not only from large amplitude A β - or A δ -fibers, but also from C-fibers. This principle is shown in Figure 9.

(B) Obtaining a single-fiber recording by action potential shape discrimination

- i. If two fibers differ markedly in their action potential shape and their receptive fields are in close proximity, then the window discrimination software DAPSYS can provide a preliminary sorting of the two action potentials or filter out the unwanted second signal.

▲ CRITICAL STEP Identification of a C-fiber by spike shape alone is prone to error because the shape of the action potential can change in an activity-dependent manner and also over time (rundown of the signal). More common and sometimes unavoidable is the contamination of the extracellularly recorded signal by a second axon with a similar spike shape, and filtering programs may provide help only in

the case of action potential shapes being visibly different (see also ANTICIPATED RESULTS).

(C) Obtaining a single-fiber recording by distance

- i. A single fiber can be recorded if the receptive fields of other fibers are located distant enough from the fiber of interest, and lack spontaneous activity.

(D) Obtaining a single-fiber recording by further subdivision

- i. If nothing else works, the filament must be further subdivided. Patience and dexterity are required.

▲ CRITICAL STEP In any case, filaments should always be subdivided into filaments as thin as possible.

Marking protocol for verification of single C-fibers within a multifiber strand ● TIMING <5 min for each test

- 22| Insert a microelectrode in the receptive field (at the spot of greatest mechanosensitivity). The electrode is positioned using a micromanipulator. Using a stimulus isolator, determine the latency of the C-fiber (for mouse nerves, the latency usually ranges between 30 and 100 ms). Then, track the latency of the propagated action potential with repetitive pulses of at least twice the electrical threshold and at a constant interval of 2 s or more (Fig. 10, traces 1–2). The action potential should appear at the same latency in subsequent traces.
- 23| Using a blunt glass rod, evoke a burst of action potentials by stimulating the mechanosensitivity of the receptive field in proximity to the microelectrode without touching the microelectrode. A typical result is shown in Figure 10, trace 3–5, where green waveforms represent mechanically evoked action potentials. In response to mechanical stimulation, the latency of the electrically evoked action potentials is increased (red waveforms in traces 3–5 of Fig. 10).
- 24| Observe that the electrically evoked action potential travels back to its initial latency value (compare the dotted line in Fig. 10 and traces 6–8, see examples in ANTICIPATED RESULTS).

Characterization of identified single receptive fields by classification of fiber type ● TIMING 15–20 min

- 25| Determine the latency of the identified action potential so that conduction velocity can be calculated ($cv = \text{distance of receptive field from recording electrode in mm} / \text{latency in ms}$): Electrically stimulate the mechanically identified single C- or A-fibers inside their receptive fields, then remove the microelectrode.
- 26| Test the threshold to mechanical stimulation with a set of von Frey monofilaments.

- 27| Before starting further recording (data capture), adjust the gain: the whole action potential waveform should be well resolved on the oscilloscope or computer screen.

▲ CRITICAL STEP There should be no clipping. This is necessary to recognize possible contamination of responses by a second fiber (see ANTICIPATED RESULTS). In long-lasting recordings, readjustment of the gain may be necessary throughout the experiment because of rundown of the action potential amplitude.

- 28| Test all identified single receptive fields by the following three routine procedures to subclassify the fibers according to their thermosensitive properties: option A for heat stimulation through superperfusion at the corium side, option B for heat stimulation through epidermal radiant heat and option C for cold stimulation.

(A) Heat stimulation: through superperfusion at the corium side

- i. To assess C-fiber heat sensitivity, isolate the receptive field from the surrounding extracellular bath solution by using a metal ring. Line the bottom wall of the ring with petroleum jelly to make the isolation watertight and place it on the receptive field.
- ii. Evacuate the fluid with a pipette (compare Fig. 8a).
- iii. Use the superperfusion temperature control system, as described in Box 2 and shown in Figure 11, to apply heat.

(B) Heat stimulation: through epidermal radiant heat, feedback controlled

- i. To assess C-fiber heat sensitivity, isolate the receptive field from the surrounding extracellular bath solution by using a metal ring. Line the bottom wall of the ring with petroleum jelly to make the isolation watertight and place it on the receptive field.
- ii. Evacuate the fluid with a pipette and place a miniature thermocouple in the receptive field touching the corium (be careful not to evoke mechanostimulation of the receptive field with the tip of the thermocouple).
- iii. Direct a parabolic halogen lamp's light (in focus distance) through the translucent bottom of the organ bath.

▲ CRITICAL STEP To avoid overheating of the epidermal side, the fluid within the ring must be evacuated before heat stimulation!

(C) Cold stimulation

- i. To assess sensitivity to cold, superfuse the corium side of the skin with cold extracellular solution. Box 2 shows how this can be realized.
- 29| Classify the fiber according to the obtained responses from Steps 25, 26 and 28; the classification is listed in Table 1.

Testing the effects of chemicals on single receptive fields of nerve endings ● TIMING

Variable

30| As soon as you have successfully classified the single fiber, you can start the individual protocol, e.g., the application of certain chemicals to the receptive fields of the nerve endings. Depending on the drug, it is usually sufficient to use similar or 10 times higher concentrations in the skin than in patch clamp or calcium imaging conditions. Drugs are usually administered following stable control responses (e.g., superfusion of the skin with extracellular solution and two heat or cold responses of comparable magnitude; the effects of chemicals on mechanosensitivity can be tested using a computer controlled mechanostimulator as demonstrated in ref. 63).

▲ **CRITICAL STEP** The most convenient way is to use an application system that allows control of temperature and rate of the applied solution (Box 2 and Fig. 11). This bears the advantage that potential sensitizing effects of compounds can be tested simultaneously (e.g., heat-sensitizing effect of capsaicin or cold-sensitizing effect of menthol).

▲ **CRITICAL STEP** The solvent DMSO should be used with care. Concentrations of 1% and lower excite A-fibers. Ethanol (<0.2%) is a better alternative, although it is known to exert agonist effects on TRPV1 and blocking effects on TRPM8 (see ref. 64).

▲ **CRITICAL STEP** In hind-paw skin of rodents, the loose corium does not provide a diffusion barrier to chemicals, in contrast to back skin that contains a muscle layer. In particular cases, e.g., when the receptive field is located below a nerve or blood vessel, diffusion may take longer. To our experience, a superfusion of the skin with a compound for 4–5 min is enough to expect acute, heat- or cold-sensitizing effects. Evans Blue can be mixed with the chemical to stain the receptive field allowing treated skin areas to be distinguished from untreated receptive fields (Fig 12).

● TIMING

EQUIPMENT SETUP (including establishment of superfusion temperature control (Box 2)): setting up a skin–nerve recording station from scratch may require a few days

REAGENT SETUP: 15 min before each experiment

Steps 1–11, preparation of the skin–nerve flap: 15–45 min for one preparation (longer for inexperienced people)

Steps 12–15, setting up the skin for the recording: 15–20 min

Steps 16–27, getting one neuron ready for recording, i.e., isolating one neuron including marking test and determination of conduction velocity, von Frey threshold and thermosensitivity: minutes to hours

Steps 28–30, recording and capturing data: will vary depending on the individual protocol, usually 20–80 min (is also limited by ‘survival’ of the signal-to-noise ratio)

? TROUBLESHOOTING

Electrical noise

Reduction of electrical noise, usually 50/60 cycles per second ('hum') or multiples, is crucial to measuring μV signals. If the two electrode lines entering the differential headstage or preamp were exactly the same, any external noise would be subject to the common mode rejection of the differential amplifier and be eliminated. However, the reference electrode dips into the buffer solution with hardly any contact resistance, whereas the recording electrode contacts a fine single-fiber filament with a considerable resistance. Fortunately, 50/60 cycles noise voltages finally refer to ground and can thus be diverted into the internal ground connection provided by the headstage. Well grounded, the aqueous fluid in the recording and storage chambers of the organ bath turns into a shield against electrical noise. The cables from the electrodes to the headstage should be as short as convenient, shielded and grounded at the headstage. No other ground line must enter this internal ground, and the case of the headstage should not be in metallic contact with anything than the connecting cable to the main amplifier. All electrical devices near the organ bath, in particular those with electromotors (pumps, thermostats, lamps with fan), should be well grounded in a star-like manner to one common ground connector. The same applies to all metallic parts in close vicinity that could act as a hum aerial (binocular, mantled light guides, micromanipulators, table top, radiant heat lamp and so on). Absolutely avoid double grounding ('ground loops'). Thermocouple probes in the organ bath can be a rich source of noise if not well insulated. The experimenter him- or herself should contact a metallic grounded surface when manipulating in the organ bath. If these general rules for μV recording are complied with, remaining, hopefully small, noise can be left to the notch filter ('hum bug') in the main amplifier.

When there is ongoing activity in the preparation

The bathing solution contains bicarbonate as the buffer, and must be gassed with carbogen. Alkalinization of the pH leads to precipitation of Ca^{2+} and increased excitability. Solutions should be prepared fresh daily.

When there is no signal

Signal transmission issues

- The signal reference is not correctly placed: check if the signal reference is in good contact with the grounded aqueous solution. If not, use the chamber gate and the flow control to regulate the height of the oil–water interface. Adjust the interface of both solutions to ~1 mm below the mirror.
- The recording reference electrodes may appear intact but have a high resistance discontinuity where soldered to the plug. Set an Ohmmeter to the lowest resistance scale and check if well conductive. Check also if there is no short-circuit to ground or shield.

- Check all cable connections involved in signal transmission. Pull ground electrode out of the fluid, and touch recording and ground electrode or their plugs with a forceps. In all cases, maximal noise should occur.

Experimental preparation issues

- Inspect the course of the nerve stem with the binocular and remove any surplus tissue (muscle may be transparent because the myoglobin is rapidly washed out under constant superfusion). Damaged and attached muscle fibers leak potassium, which depolarizes the nerve and blocks conduction.
- Check extracellular solutions. High or sustained low potassium leads to a depolarization block of the nerve. Missing or precipitated calcium increases general excitability, whereas excess calcium decreases general excitability. Check solution osmolality to detect missing or surplus electrolytes.
- If the nerve has been desheathed and covered by oil for several hours, it undergoes ‘run down’; and should be shortened by the free end and newly desheathed. This procedure often recovers the whole experimental session as if an entirely new preparation was set up.
- If the nerve appears to be nonconductive, it may have been pinched or damaged during the surgical procedure. Stimulate the nerve stem with the needle electrode (distal to proximal) to locate the conduction block; C- and A-compound action potentials occur at and proximal to the site of rupture (axontmesis).

Problems with filtering algorithms

Commercially available filtering algorithms need to be applied with caution; in reality, they may provide help only in gross sorting if action potential shapes of contaminating axons are clearly different. Indeed, the most frequent problem in teased-fiber preparations is the contamination of the extracellular recording by a second axon that carries a similar shape. In general, filtering algorithms are often not sensitive enough and discrimination by spike shape alone is prone to error. This problem can be approached only by electrostimulation and the marking test (see ANTICIPATED RESULTS, point 2), by logical analysis of the response (see ANTICIPATED RESULTS, point 3) and sometimes by further splitting of a multifiber strand.

ANTICIPATED RESULTS

Point 1: The action potential waveform as differentiator

The action potential waveform is an important characteristic of a fiber. As it is used as the differentiator in extracellular recordings, a couple of factors that alter the shape of the waveform should be kept in mind. Determined by the setting of the band-pass filter (in general, 100 Hz to 1–2 kHz), C-fibers appear more triphasic than A-fibers (flattened third phase). A-fibers exhibit faster upstrokes and the amplitude of their narrow waveform is limited by the setting of the low pass filter, whereas the high pass filter (100–500 Hz) mainly affects the C-fibers, the amplitude and predominantly downward deflection of their

action potentials (Fig. 13a). More crucial, however, are changes to the waveform that cannot be influenced by the experimenter and occur during the time course of the experiment.

Figure 13b shows that the waveform of the action potential undergoes activity-dependent changes. At high discharge rate, the amplitude of the waveform decreases. The waveform reverts to its initial amplitude as soon as the activity drops. The important fact is that, in the given example, a common filtering algorithm recognizes two different waveform groups (black and red in Fig. 13b) when the tolerance band multiplier for the creation of the templates is set to 1 (tolerating a ± 1 V deviation of each data point within the waveform). Above an instantaneous discharge rate of 36 spikes per second, the amplitude of the waveform is significantly altered, i.e., more than 50% of the data points differ by more than ± 1 V and are sorted in a different template.

A similar reduction in spike amplitude occurs also over time, especially in C-fibers (due to rundown), and sometimes requires adjustment of the gain during the experiment.

Even at high discharge rates, extracellularly recorded single-fiber action potentials maintain a triphasic waveform with individual propositions of upward and downward deflections. This makes the waveform an important distinctive feature to recognize the presence of a second axon in a recording. Even if a second unit in a filament is indistinguishable by waveform from a first one, it may become conspicuous by occasional superimposition or merger of action potentials. Owing to the refractory period (RF), spikes from one and the same fiber could never merge. The interspike interval for a single C-fiber is at least 9–14 ms, whereas it can be as short as 2.5–3 ms in A-fibers. Figure 13c shows this for an A δ -fiber and Figure 13d for a menthol-sensitized cold receptor.

Point 2: Marking tests and latency analysis

Punctate electrostimulation of the skin provides—by means of current spread—insight into the distribution of other axons in the area of the receptive field of one axon from which recordings are to be taken. The time delay (latency) between the electrical stimulus and the arrival of the action potential at the site of the recording electrode are primarily determined by the properties of the individual fiber (presence and thickness of myelin sheath). Injected current forms a concentric gradient around the microelectrode, and the intensity of the decaying current determines where (at what distance to the recording electrode) an axon is stimulated and whether a second (or third) axon is reached by suprathreshold current. This concept is best shown by a target provided in Figure 14a.

Hence, electrostimulation serves not only to detect a mechanically identified single-fiber electrically (and to determine its conduction velocity), but also to detect potential branches of other fibers in the vicinity of the receptive field of interest that may interfere with the recording. In case more than one fiber is excited by the electrical stimulus (Fig. 14b,c), electrostimulation applied to the receptive field at regular intervals in combination with orthodromic activation of the ending, e.g., by mechanostimulation, allows one to track the time course of post-excitatory latency changes. This is called the marking test. An axon can carry repetitive impulses at intervals greater than the absolute RF. If the interval is greater than the relative RF, the waveform of a consecutive action potential is fully restored and

identical to the foregoing one. However, the conduction velocity is reduced and takes several seconds to recover (Fig. 14b). This ‘marking phenomenon’ is used to identify the particular electrically evoked spike within a train of others or within a compound waveform (consisting of two or more spikes of the same latency) that can also be evoked by natural stimulation of the receptive field under investigation. If the electrically evoked impulse is not slowed by a naturally evoked burst of action potentials, the spikes must result from two different C-fibers with overlapping receptive fields. In this case, the strength of the electrical stimulus may not suffice to excite both receptive fields; increasing the current strength or changing the position of the electrode may show action potentials from two receptive fields. Taken together only the fiber that—in response to mechanical stimulation—shows increased latency of the electrically evoked action potential and subsequent restitution to the original latency is the activated C-fiber under investigation.

Figure 14b,c show two situations. In Figure 14b, a second axon is present (at 12 ms), but it does not respond to mechanical stimulation. Nonetheless, a single-fiber recording (of the unit at 28 ms marked red) can be tried; however, any response of the fiber to other than mechanical stimuli has to be analyzed with caution because the second unit (marked blue) could contribute and thereby lead to a false magnitude estimate of the response of the originally isolated fiber. More complicated is the setting encountered in Figure 14c. Here the action potentials of two fibers appear at the same latency and are entirely superimposed, i.e., the voltage signals of both action potentials add up and appear as one waveform. The marking test then shows changes in latency in response to natural stimulation in both fibers. Trace 6 shows that the action potentials of both fibers show a similar shape. A recording in this case would not be meaningful, because both fibers are responsive to natural stimulation at the same site. The multifiber strand must be further subdivided or discarded. In this case the recording was from two CC-fibers and the natural stimulus was cold. Both CC-fibers were in the same Remak bundle and separation was impossible.

Point 3: Recognizing an assumed single-fiber recording as multifiber recording

The following example describes a recording where a single-fiber was successfully isolated using the marking test. The response to cold stimulation of this fiber appeared unusually vigorous and non-adapting, conspicuous of at least a second fiber contributing. Therefore more troubleshooting was required to acquire a single-unit response.

Figure 15a shows a recording from a mechanosensitive C-fiber in response to cold stimulation. This response pattern is suspicious for a contamination by a second unit for several reasons: (1) Mouse CMC-fibers respond to cold stimulation with peak firing rates below 20 Hz (in contrast to CC-fiber responses to cold or CMH-fiber responses to heat, compare points 4 and 5). (2) Thermal responses (to cold or heat) usually show more or less rapid adaptation at constant or slowly changing temperature (compare point 5). Such a response pattern is best observed in a logarithmic plot of the instantaneous discharge rate (reciprocal value of the interspike interval). (3) In general there are no bursts in mouse CMC-fiber cold responses (in contrast to CC-fibers exposed to prolonged cooling).

Figure 15a shows the cold response of the fiber(s) in strip chart representation and as instantaneous discharge rate plot. A close look at the strip chart shows one bar that exceeds

the upper and lower 10-V limit of the voltage window, and the corresponding action potential waveform shows clipping. Here, the waveforms of two action potentials are superimposed (the voltages add up in the window). The dot plot shows a burst-like firing pattern with a peak rate up to 256 s^{-1} . The corresponding waveform shows that two very similar action potentials crossed the trigger level with less than 1-ms time lag, which leads to near-merger of both waveforms. Merger of two waveforms is always due to contamination with a second axon (compare Fig. 13). Similarly, a burst-like firing pattern at such a discharge rate is suspicious for at least one second axon. Therefore, the next step in this recording attempt should be to remove the metal ring and repeat electrostimulation.

Figure 15b shows that the application of a higher voltage to the same receptive field(s) shows a second axon at shorter latency with a virtually identical waveform. Next, one would try to divide the filament into two strands and then to see whether if the two units run in separate bundles. Indeed, in this case, one of the C-fibers could be found in one of the strands, whereas the second one got lost during the teasing procedure. Figure 15c shows the cold response of the remaining unit. Note that now the firing rate does not exceed 8 s^{-1} , which means that the shortest interspike interval is $\sim 120\text{ ms}$. This recording is consistent with a single fiber recording.

Point 4: C-fibers with ongoing activity at 30–32 °C

Ongoing activity at normal bath temperature rarely occurs in ‘uninjured’ skin–nerve preparations but frequently under pathological conditions, e.g., if the preparation was taken from an inflamed hind paw or after partial nerve injury^{65,66} (see also TROUBLESHOOTING).

Ongoing activity in an apparently uninjured preparation may be related to damaging the nerve during dissection or to cutting through receptive fields at the edge of the skin. In addition, ongoing activity can be induced following electrostimulation with a not perfectly balanced isolation unit releasing miniature but steady DC. This leads to electrolytic damage of the nerve endings.

A frequent cause of ongoing activity at 30–32 °C is (successful) noxious heat stimulation of mechano-heat-sensitive C-fibers (polymodal nociceptors, CMH), up to half of which develop sustained low-frequency irregular discharge after a silent period of 1–2 min following heat stimulation (typically 32–48 °C in 20 s). The mechanism of this activity is unclear but has not to do with major sensitization to heat, as a subsequent heat stimulus after 5 min usually evokes a slightly desensitized response (in mice not rats). This is in contrast to the after-effects of traumatic heat stimulation (60 °C) or to superfusion of receptive fields with bradykinin or low pH (6.1), under which conditions the nociceptive heat threshold drops to or below ambient temperature (22–25 °C), which readily explains ongoing discharge at bath or body temperature^{55,67,68}. To show such dramatic sensitizing effects, the receptive field first needs to be cooled to 10–12 °C to stop the ongoing activity; a subsequent heating ramp (e.g., 12–48 °C in 45 s) will then discover the lowered heat threshold and a log-linear increase in firing rate throughout the temperature-encoding (‘working’ or ‘dynamic’) range of the particular CMH-fiber⁵⁵.

Mechanosensitive cold-fibers (cold nociceptors, CMC-fibers)—In rare cases, mechano-cold-sensitive fibers (CMC) may present with ongoing activity at bath temperature and increasing firing rate upon cooling. Figure 16a shows the case of a CMC-fiber with ongoing activity at bath temperature that reduces its activity when heated or rewarmed after cooling and increases its firing rate when cooled. Application of menthol increases the ongoing activity at 32 °C and strongly sensitizes to cooling. However, CMC-fibers without ongoing activity at bath temperature are much more common (compare point 5) and have their thresholds to cold activation at lower temperatures than the cold receptors (see below).

Mechanoinsensitive cold sensitive fibers (cold receptors, CC-fibers)—These have been described in the *in vitro* guinea pig cornea where their major characteristic is ongoing activity at ambient temperature with burst-like discharge^{39,41,43}. They have been investigated more extensively in the cat *in vivo*^{69–71}. Similar fibers also exist in mouse skin, where the thresholds of the majority of units are below bath temperature (25–35 °C). Upon further cooling, they are activated with log-linearly increasing discharge rates. In contrast to CMC-fibers, they show high peak firing rates, often between 30 and 80 Hz. Figure 16b shows a representative sample recording of a mouse CC-fiber that was also excited and sensitized by menthol, similar to cold receptors in other species¹⁸. Many of these fibers show ‘paradoxical’ heat-induced discharge at high temperatures (in bursts and not temperature-encoding, see Fig. 16b, inset).

Mechanosensitive warm-fibers (CMW-fibers)—Warm-sensitive fibers rarely exist in the mouse skin. They are sometimes mechanosensitive in addition and show ongoing activity at 32 °C. They increase their firing rate progressively and reach peak between 37 and 46 °C; after reaching the peak, they inactivate abruptly. Cooling silences them and rewarming brings the activity back, often with an initial overshoot indicating adaptation (Fig. 16c). Remarkably, the response to warming/heating in this particular fiber was reduced to half by application of menthol, and in the absence of menthol, the warm response recovered to its previous size (not shown). Although these fibers appear polymodal, the fiber subtype probably corresponds to the function of warm fibers in the trigeminal-innervated skin of cat²² and other species⁷²; mechano-insensitive warm-sensitive fibers are very rare in the saphenous nerve of rodents; however, they show the same discharge pattern in response to thermal stimulation as fibers recorded *in vivo* in cats and other species⁷³.

Point 5: Quantifying thermal responses of polymodal nociceptors and cold nociceptors in mice

Classical physiology has provided information about thermosensory specialization among subclasses of peripheral sensory neurons^{18–23}, and in recent years, we have gained insight into the molecular mechanisms by which these cells transduce thermal stimuli and trigger action potentials^{10,59,74–77}. Considering that major physiological aspects of sensory transduction are still poorly understood, the skin–nerve preparation can serve as a read-out for the function of distinct ion channels or receptors in the sensory transduction process. The way is to conduct large sampling studies in transgenic mouse strains and to characterize the function of a particular gene product within the system of thermo- and mechanotransduction or action potential electrogenesis⁵⁹.

Well-controlled cold and heat stimuli are sufficient to characterize the thermal responsiveness of sensory nerve fibers. If one wishes to functionally characterize certain subsets of ion channels in the sensory terminals, certain chemicals may be of further help. However, in intact C-fiber endings, chemical compounds are rarely well defined and as specific in action as they appear in heterologous expression systems. An exception surely is TTX, a blocker of TTXs sodium channel subtypes that reliably leaves Nav1.8 as a sole action potential generator in nociceptive terminals. Certain TRP channels that function as transducer channels in the nerve endings, such as TRPM8, can be tested for by using menthol that reliably sensitizes TRPM8-expressing nerve endings to cold. However, many other TRP-channel agonists may have different effects in native nociceptors than in heterologous expression systems. An example is capsaicin, an agonist of TRPV1. Its desensitizing actions predominate, even at threshold concentration, in the skin–nerve preparation over excitatory and heat-sensitizing actions in heterologous expression systems. In contrast, capsazepine, which is a blocker of TRPV1 in heterologous expression systems, has heat-sensitizing and excitatory actions on C-fiber terminals⁷⁸.

As for mechanotransduction, the application of noxious mechanical stimuli can be particularly helpful. Even if threshold excitability (von Frey sensitivity) is not altered in a transgenic mouse strain, a channel of interest could still be relevant for the encoding and transmission of suprathreshold mechanical stimuli (e.g., in the cold)¹⁵.

Figures 17 and 18 show the profile of cold and heat responses across a population of polymodal fibers recorded from adult C57BL6/J mice. Both heat and cold responses have a wide range of temperature thresholds as well as firing patterns. Polymodal nociceptors are typically silent at bath temperature in the absence of stimulation⁷⁹ (see Figs. 17c and 18c), but roughly half of nociceptors develop low-frequent ongoing activity after heat stimulation ($n = 16/33$; Fig. 17c). Similarly, both heat- and cold-induced discharges slowly adapt during plateau phases of temperature and when the rate of temperature change is not high enough (Figs. 17a and 18c). When analyzing the firing pattern of heat responses across a population, the majority ($n = 22/33$) of the fibers fire less than ten spikes per heat stimulus of 20 s duration, and have thresholds above 40 °C (Fig. 17b,d,e). When challenged with a step stimulus (45 °C) to test for static discharge, these fibers show more or less complete adaptation (not shown). A relatively small percentage of fibers (<10%), however, have heat thresholds closer to bath temperature, and responses reach high peak firing rates. These fibers typically respond to heat ramps with a steeper log-linear increase of the firing rate, and present with static discharge and slow biphasic adaptation when challenged with a step stimulus (Fig. 17a). Figure 17g shows that the magnitude of the response correlates inversely with the threshold temperature. Similarly, the peak discharge rate and the magnitude of the response show a functional relationship (Fig. 17f). However, the histograms (Fig. 17d and e) are skew but continuous and do not allow for demarcation of subpopulations.

The peak firing rate of cold nociceptors is much lower than that of heat polymodals, typically around 2 s⁻¹ (Fig. 18f). When analyzing the responses across a population, the majority ($n = 20/30$) of the fibers discharged less than ten spikes per cold stimulus of 60 s duration (Fig. 18d). As with the heat responses, the peak firing rates were correlated with the

magnitude of the response (Fig. 18g). The cold thresholds of cold nociceptors appear to form a bimodal distribution (Fig. 18e). About half of the fibers have thresholds between room and bath temperature and show dynamic sensitivity with slow but complete adaptation (Fig. 18a,e). The other half presents with thresholds in the noxious range, and these units show much less or no adaptation (Fig. 18b,e).

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References

1. Nakai J. Dissociated dorsal root ganglia in tissue culture. *Am J Anat.* 1956; 99:81–129. [PubMed: 13362126]
2. Murray, MR.; Kopech, G. A Bibliography of the Research in Tissue Culture. Academic Press; New York: 1953.
3. Drew LJ, Wood JN, Cesare P. Distinct mechanosensitive properties of capsaicin-sensitive and -insensitive sensory neurons. *J Neurosci.* 2002; 22:RC228. [PubMed: 12045233]
4. Cesare P, McNaughton P. A novel heat-activated current in nociceptive neurons and its sensitization by bradykinin. *Proc Natl Acad Sci USA.* 1996; 93:15435–15439. [PubMed: 8986829]
5. Reid G, Flonta ML. Physiology. Cold current in thermoreceptive neurons. *Nature.* 2001; 413:480. [PubMed: 11586349]
6. Renganathan M, Cummins TR, Waxman SG. Contribution of Na(v)1.8 sodium channels to action potential electrogenesis in DRG neurons. *J Neurophysiol.* 2001; 86:629–640. [PubMed: 11495938]
7. Wood JN, et al. Capsaicin-induced ion fluxes in dorsal root ganglion cells in culture. *J Neurosci.* 1988; 8:3208–3220. [PubMed: 3171675]
8. Wood JN, Boorman JP, Okuse K, Baker MD. Voltage-gated sodium channels and pain pathways. *J Neurobiol.* 2004; 61:55–71. [PubMed: 15362153]
9. Bhawe G, Gereau RW. Posttranslational mechanisms of peripheral sensitization. *J Neurobiol.* 2004; 61:88–106. [PubMed: 15362155]
10. Caterina MJ, et al. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature.* 1997; 389:816–824. [PubMed: 9349813]
11. Woodbury CJ, et al. Nociceptors lacking TRPV1 and TRPV2 have normal heat responses. *J Neurosci.* 2004; 24:6410–6415. [PubMed: 15254097]
12. Zimmermann K, et al. The TRPV1/2/3 activator 2-aminoethoxydiphenyl borate sensitizes native nociceptive neurons to heat in wildtype but not TRPV1 deficient mice. *Neuroscience.* 2005; 135:1277–1284. [PubMed: 16165301]
13. Akopian AN, Sivilotti L, Wood JN. A tetrodotoxin-resistant voltage-gated sodium channel expressed by sensory neurons. *Nature.* 1996; 379:257–262. [PubMed: 8538791]
14. Akopian AN, et al. The tetrodotoxin-resistant sodium channel SNS has a specialized function in pain pathways. *Nat Neurosci.* 1999; 2:541–548. [PubMed: 10448219]
15. Zimmermann K, et al. Sensory neuron sodium channel Nav1.8 is essential for pain at low temperatures. *Nature.* 2007; 447:856–859.
16. Matsutomi T, Nakamoto C, Zheng T, Kakimura J, Ogata N. Multiple types of Na(+) currents mediate action potential electrogenesis in small neurons of mouse dorsal root ganglia. *Pflügers Arch.* 2006; 453:83–96. [PubMed: 16838161]
17. Bessou P, Perl ER. Response of cutaneous sensory units with unmyelinated fibers to noxious stimuli. *J Neurophysiol.* 1969; 32:1025–1043. [PubMed: 5347705]
18. Hensel H, Zotterman Y. The effect of menthol on the thermoreceptors. *Acta Physiol Scand.* 1951; 24:27–34. [PubMed: 14877581]

19. Hensel H, Zotterman Y. The response of mechanoreceptors to thermal stimulation. *J Physiol.* 1951; 115:16–24. [PubMed: 14889427]
20. Hensel H, Zotterman Y. The response of the cold receptors to constant cooling. *Acta Physiol Scand.* 1951; 22:96–105. [PubMed: 14933132]
21. Zotterman Y. Touch, pain and tickling: an electro-physiological investigation on cutaneous sensory nerves. *J Physiol.* 1939; 95:1–28. [PubMed: 16995068]
22. Hensel H, Kenshalo DR. Warm receptors in the nasal region of cats. *J Physiol.* 1969; 204:99–112. [PubMed: 5352076]
23. Hensel H, Huopaniemi T. Static and dynamic properties of warm fibres in the infraorbital nerve. *Pflügers Arch.* 1969; 309:1–10. [PubMed: 5815315]
24. Bessou P, Burgess PR, Perl ER, Taylor CB. Dynamic properties of mechanoreceptors with unmyelinated (C) fibers. *J Neurophysiol.* 1971; 34:116–131. [PubMed: 5540574]
25. Catton WT. Some properties of frog skin mechanoreceptors. *J Physiol.* 1958; 141:305–322. [PubMed: 13539840]
26. Catton WT. Responses of frog skin to local mechanical stimulation. *J Physiol.* 1957; 137:81–2P. [PubMed: 13463789]
27. Gonzalez C, Sanchez J, Concha J. Changes in potential difference and short-circuit current produced by electrical stimulation in a nerve–skin preparation of the toad. *Biochim Biophys Acta.* 1966; 120:186–188. [PubMed: 5962276]
28. Diamond J, Holmes M, Nurse CA. Are Merkel cell–neurite reciprocal synapses involved in the initiation of tactile responses in salamander skin? *J Physiol.* 1986; 376:101–120. [PubMed: 3795072]
29. Weston KM, Foster RW, Weston AH. The application of irritant chemicals selectively to the skin of the leech ganglion/body wall preparation. *J Pharmacol Methods.* 1984; 12:285–297. [PubMed: 6536832]
30. Wedekind C. Receptive properties of primary afferent fibres from rabbit pleura, *in vitro*. *Somatosens Mot Res.* 1997; 14:229–236. [PubMed: 9443365]
31. Kumazawa T, Mizumura K, Sato J. Response properties of polymodal receptors studied using *in vitro* testis superior spermatic nerve preparations of dogs. *J Neurophysiol.* 1987; 57:702–711. [PubMed: 3559698]
32. Wenk HN, McCleskey EW. A novel mouse skeletal muscle–nerve preparation and *in vitro* model of ischemia. *J Neurosci Methods.* 2007; 159:244–251. [PubMed: 16959326]
33. Taguchi T, Sato J, Mizumura K. Augmented mechanical response of muscle thin-fiber sensory receptors recorded from rat muscle–nerve preparations *in vitro* after eccentric contraction. *J Neurophysiol.* 2005; 94:2822–2831. [PubMed: 16160095]
34. Taguchi T, Matsuda T, Tamura R, Sato J, Mizumura K. Muscular mechanical hyperalgesia revealed by behavioural pain test and c-Fos expression in the spinal dorsal horn after eccentric contraction in rats. *J Physiol.* 2005; 564:259–268. [PubMed: 15677691]
35. Lennerz JK, et al. Electrophysiological characterisation of vagal afferents relevant to mucosal nociception in the rat upper oesophagus. *J Physiol.* 2007; 582:229–242. [PubMed: 17478536]
36. Yu S, Udem BJ, Kollarik M. Vagal afferent nerves with nociceptive properties in guinea-pig oesophagus. *J Physiol.* 2005; 563:831–842. [PubMed: 15649987]
37. Juan H, Lembeck F. Inhibition of the action of bradykinin and acetylcholine on paravascular pain receptors by tetrodotoxin and procaine. *Naunyn Schmiedebergs Arch Pharmacol.* 1975; 290:389–395. [PubMed: 1196404]
38. Juan H, Lembeck F. Action of peptides and other algesic agents on paravascular pain receptors of the isolated perfused rabbit ear. *Naunyn Schmiedebergs Arch Pharmacol.* 1974; 283:151–164. [PubMed: 4369745]
39. Brock JA, McLachlan EM, Belmonte C. Tetrodotoxin-resistant impulses in single nociceptor nerve terminals in guinea-pig cornea. *J Physiol.* 1998; 512:211–217. [PubMed: 9729630]
40. Carr RW, Pianova S, Brock JA. The effects of polarizing current on nerve terminal impulses recorded from polymodal and cold receptors in the guinea-pig cornea. *J Gen Physiol.* 2002; 120:395–405. [PubMed: 12198093]

41. Carr RW, et al. Effects of heating and cooling on nerve terminal impulses recorded from cold-sensitive receptors in the guinea-pig cornea. *J Gen Physiol.* 2003; 121:427–439. [PubMed: 12695483]
42. Carr RW, Brock JA. Electrophysiology of corneal cold receptor nerve terminals. *Adv Exp Med Biol.* 2002; 508:19–23. [PubMed: 12171110]
43. Madrid R, et al. Contribution of TRPM8 channels to cold transduction in primary sensory neurons and peripheral nerve terminals. *J Neurosci.* 2006; 26:12512–12525. [PubMed: 17135413]
44. Elitt CM, et al. Artemin overexpression in skin enhances expression of TRPV1 and TRPA1 in cutaneous sensory neurons and leads to behavioral sensitivity to heat and cold. *J Neurosci.* 2006; 26:8578–8587. [PubMed: 16914684]
45. Koerber HR, Woodbury CJ. Comprehensive phenotyping of sensory neurons using an *ex vivo* somatosensory system. *Physiol Behav.* 2002; 77:589–594. [PubMed: 12527004]
46. Reeh PW. Sensory receptors in mammalian skin in an *in vitro* preparation. *Neurosci Lett.* 1986; 66:141–146. [PubMed: 3725179]
47. Cain DM, Khasabov SG, Simone DA. Response properties of mechanoreceptors and nociceptors in mouse glabrous skin: an *in vivo* study. *J Neurophysiol.* 2001; 85:1561–1574. [PubMed: 11287480]
48. Reeh PW. Sensory receptors in a mammalian skin–nerve *in vitro* preparation. *Prog Brain Res.* 1988; 74:271–276. [PubMed: 3187037]
49. Mogil JS, et al. Variable sensitivity to noxious heat is mediated by differential expression of the CGRP gene. *Proc Natl Acad Sci USA.* 2005; 102:12938–12943. [PubMed: 16118273]
50. Bernardini N, et al. Muscarinic M2 receptors on peripheral nerve endings: a molecular target of antinociception. *J Neurosci.* 2002; 22:RC229. [PubMed: 12045234]
51. Kress M, Petersen M, Reeh PW. Methylene blue induces ongoing activity in rat cutaneous primary afferents and depolarization of DRG neurons via a photosensitive mechanism. *Naunyn Schmiedeberg Arch Pharmacol.* 1997; 356:619–625. [PubMed: 9402042]
52. Kress M, Riedl B, Reeh PW. Effects of oxygen radicals on nociceptive afferents in the rat skin *in vitro*. *Pain.* 1995; 62:87–94. [PubMed: 7478712]
53. Kress, M.; Reeh, PW. Chemical excitation and sensitization in nociceptors. In: Belmonte, C.; Cervero, F., editors. *Neurobiology of Nociceptors*. Oxford University; Oxford: 1996. p. 258–297.
54. Petho G, Derow A, Reeh PW. Bradykinin-induced nociceptor sensitization to heat is mediated by cyclooxygenase products in isolated rat skin. *Eur J Neurosci.* 2001; 14:210–218. [PubMed: 11553274]
55. Reeh PW, Petho G. Nociceptor excitation by thermal sensitization—a hypothesis. *Prog Brain Res.* 2000; 129:39–50. [PubMed: 11098680]
56. Ringkamp M, et al. Activated human platelets in plasma excite nociceptors in rat skin, *in vitro*. *Neurosci Lett.* 1994; 170:103–106. [PubMed: 8041482]
57. Steen KH, Wegner H, Reeh PW. The pH response of rat cutaneous nociceptors correlates with extracellular [Na⁺] and is increased under amiloride. *Eur J Neurosci.* 1999; 11:2783–2792. [PubMed: 10457175]
58. Steen KH, Steen AE, Reeh PW. A dominant role of acid pH in inflammatory excitation and sensitization of nociceptors in rat skin, *in vitro*. *J Neurosci.* 1995; 15:3982–3989. [PubMed: 7751959]
59. Alloui A, et al. TREK-1, a K⁺ channel involved in polymodal pain perception. *EMBO J.* 2006; 25:2368–2376. [PubMed: 16675954]
60. Kirchhoff C, Jung S, Reeh PW, Handwerker HO. Carrageenan inflammation increases bradykinin sensitivity of rat cutaneous nociceptors. *Neurosci Lett.* 1990; 111:206–210. [PubMed: 2336185]
61. Koltzenburg M, Kress M, Reeh PW. The nociceptor sensitization by bradykinin does not depend on sympathetic neurons. *Neuroscience.* 1992; 46:465–473. [PubMed: 1542419]
62. Bretag AH. Synthetic interstitial fluid for isolated mammalian tissue. *Life Sci.* 1969; 8:319–329. [PubMed: 5781321]
63. Schlegel T, Sauer SK, Handwerker HO, Reeh PW. Responsiveness of C-fiber nociceptors to punctate force-controlled stimuli in isolated rat skin: lack of modulation by inflammatory mediators and flurbiprofen. *Neurosci Lett.* 2004; 361:163–167. [PubMed: 15135919]

64. Benedikt J, Teisinger J, Vyklicky L, Vlachova V. Ethanol inhibits cold-menthol receptor TRPM8 by modulating its interaction with membrane phosphatidylinositol 4,5-bisphosphate. *J Neurochem.* 2007; 100:211–224. [PubMed: 17074062]
65. Handwerker HO, Anton F, Kocher L, Reeh PW. Nociceptor functions in intact skin and in neurogenic or non-neurogenic inflammation. *Acta Physiol Hung.* 1987; 69:333–342. [PubMed: 3661217]
66. Kocher L, Anton F, Reeh PW, Handwerker HO. The effect of carrageenan-induced inflammation on the sensitivity of unmyelinated skin nociceptors in the rat. *Pain.* 1987; 29:363–373. [PubMed: 3614971]
67. Lyfenko A, et al. The effects of excessive heat on heat-activated membrane currents in cultured dorsal root ganglia neurons from neonatal rat. *Pain.* 2002; 95:207–214. [PubMed: 11839420]
68. Liang YF, Haake B, Reeh PW. Sustained sensitization and recruitment of rat cutaneous nociceptors by bradykinin and a novel theory of its excitatory action. *J Physiol.* 2001; 532:229–239. [PubMed: 11283237]
69. Bade H, Braun HA, Hensel H. Parameters of the static burst discharge of lingual cold receptors in the cat. *Pflügers Arch.* 1979; 382:1–5. [PubMed: 118427]
70. Duclaux R, Schafer K, Hensel H. Response of cold receptors to low skin temperatures in nose of the cat. *J Neurophysiol.* 1980; 43:1571–1577. [PubMed: 7411177]
71. Hensel H, Schafer K. Static and dynamic activity of cold receptors in cats after long-term exposure to various temperatures. *Pflügers Arch.* 1982; 392:291–294. [PubMed: 7070961]
72. Hensel H. Static and dynamic activity of warm receptors in *Boa constrictor*. *Pflügers Arch.* 1975; 353:191–199. [PubMed: 1168336]
73. Kress M, Koltzenburg M, Reeh PW, Handwerker HO. Responsiveness and functional attributes of electrically localized terminals of cutaneous C-fibers *in vivo* and *in vitro*. *J Neurophysiol.* 1992; 68:581–595. [PubMed: 1527577]
74. McKemy DD, Neuhauser WM, Julius D. Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature.* 2002; 416:52–58. [PubMed: 11882888]
75. Colburn RW, et al. Attenuated cold sensitivity in TRPM8 null mice. *Neuron.* 2007; 54:379–386. [PubMed: 17481392]
76. Dhaka A, et al. TRPM8 is required for cold sensation in mice. *Neuron.* 2007; 54:371–378. [PubMed: 17481391]
77. Kang D, Choe C, Kim D. Thermosensitivity of the two-pore domain K⁺ channels TREK-2 and TRAAK. *J Physiol.* 2005; 564:103–116. [PubMed: 15677687]
78. Pethö G, Izydorczyk I, Reeh PW. Effects of TRPV1 receptor antagonists on stimulated iCGRP release from isolated skin of rats and TRPV1 mutant mice. *Pain.* 2004; 109:284–290. [PubMed: 15157689]
79. Koltzenburg M, Stucky CL, Lewin GR. Receptive properties of mouse sensory neurons innervating hairy skin. *J Neurophysiol.* 1997; 78:1841–1850. [PubMed: 9325353]
80. Forster C, Handwerker HO. Automatic classification and analysis of microneurographic spike data using a PC/AT. *J Neurosci Methods.* 1990; 31:109–118. [PubMed: 2319811]
81. Turnquist B, Leverenz M, Swanson E. Neural spike classification using parallel selection of all algorithm parameters. *J Neurosci Methods.* 2004; 137:291–298. [PubMed: 15262073]
82. Cook, MJ. *The Anatomy of the Laboratory Mouse.* Academic Press Inc; New York: 1965.
83. Mogil JS, Adhikari SM. Hot and cold nociception are genetically correlated. *J Neurosci.* 1999; 19:RC25. [PubMed: 10479718]
84. Munns C, AlQatari M, Koltzenburg M. Many cold sensitive peripheral neurons of the mouse do not express TRPM8 or TRPA1. *Cell Calcium.* 2007; 41:331–342. [PubMed: 16949152]

Figure 1.

The experimental setup: schematic representation of data capture. A pair of recording electrodes feed the signal into an AC-coupled differential preamplifier that is connected in series with a second single-ended 10^4 -gain amplifier and a band-pass filter (between 100 and 1,000 Hz). The signal is fed to audio speakers and oscilloscope and processed through a DAP board, where the action potentials above set amplitudes are discriminated and fed into the computer. Electrical stimulation can be administered to receptive fields in the skin through a set of stimulation electrodes connected to an electrical stimulator.

Figure 2.

Typical view of a recording in Dapsys. **(a)** The main window shows all stimulator, discriminator and digital trigger windows where data are acquired during the recording. **(b)** A discriminator window; the input signal is acquired by crossing a trigger level (blue crosshair), in this case, a negative one. Discriminator windows show an unlimited duration of action potential history as well as optional waterfall display and cursors for measurements. Each signal exceeding the threshold is assigned an ID and a timestamp. Each discriminator window can send out trigger impulses and enables classification of action potentials through three separate secondary filters. **(c)** Contingent secondary filter acquiring only the C-fiber action potential of interest from the main signal discriminator. Action potential separation is achieved in real time by accepting all signals from the discriminator that exceed three additional trigger levels that can be freely positioned irrespective of the primary trigger level (pairs of vertical dots). **(d)** Discriminator window triggered by a digital trigger, in this case from a constant voltage stimulus isolator. Sweeps of variable duration are displayed. Cursors can be placed to allow exact latency measurement. A waterfall display allows browsing through the history of sweeps. **(e)** A latency measurement box measures the latency between different digital triggers, e.g., between an electrical stimulus and an incoming action potential in the secondary filter. **(f)** Stream browser windows plot acquired data over time, e.g., action potentials as waveforms (strip chart), frequency plot or histogram. Data streams of different inputs can be overlaid in one window or opened in separate windows. The waveforms of action potentials shown in the stream browser window can be opened in a fly-out window, which allows the user to magnify the corresponding waveform of each data point in the stream browser, either as single waveforms or in groups of several waveforms (not shown). A cursor allows to browse through the waveforms and to find waveforms easily (not shown, but similar to Fig. 13b). **(g)** A manual event dialog box saves comments. Data are further analyzed offline using a waveform discrimination algorithm^{80,81} (not shown) and Microsoft Excel.

Figure 3.

Typical view of an offline analysis in Spike2. **(a)** Left “Main window”. The main window displays the current recording or file in several data channels. From the bottom to the top, channel one holds the input signal (μV) recorded at a sampling rate of 20 kHz. Channel 2 holds events displayed as frequency plot (Hz); in this case, the action potentials of a rat C-fiber in response to a cold stimulus. To discriminate the action potential of interest from other waveforms of similar size, the input signal has been subjected to a waveform discrimination procedure **(b)** within the time frame of two limiting vertical cursors, shown as vertical dotted lines carrying the numbers 1 and 2. Channel 3 holds the temperature time course ($^{\circ}\text{C}$). Channel 30 displays marked events triggered while a script for repetitive electrostimulation was operated (see right window in **(a)** “Sweep View”). Channel 31 holds keyboard markers and Channel 32 digital trigger events activated by a constant current stimulus isolator controlled by a running script and the CED. The top trace displays the superimposed waveforms extracted from the input signal using the WaveMark filter feature **(b)**. This spike-sorting feature can also be used in real time; however, it allows only limited influence on and insight into the template matching procedure (version 5). The right “Sweep view” window in **(a)** represents a waterfall display window recorded and analyzed with a separately written script of repetitive electrostimulation. Sweeps can be displayed at variable length and listed in variable time frames; in this case, the time frame between the two vertical cursors positioned in the main window **(a)** was chosen. Sweeps recorded at 2-s intervals are shown, demonstrating cold-induced slowing of the latency of the C-fiber subjected to the cold stimulation displayed in the main window in **(a)**. **(b)** WaveMark showing offline template editing. This feature allows shape-dependent template formation and reclassification of data in a WaveMark channel (compare top trace in window **(a)**). Spike 2 conveniently allows traces to be printed or directly exported into an image-editing program.

Figure 4.

Custom-made gold wire electrodes and glassrods. **(a)** Wire electrodes. One 1-mm male gold-plated pin (left inset) is soldered to the end of a fine gold wire (0.15 mm, right inset). The top end is covered with thermoplastic shrink tubing. The pins correspond to in-built female 1-mm gold-plated connectors in the chamber and connect to the differential amplifier and the stimulus isolator. **(b)** Glass rods. Glass rods with different custom-designed tips are useful for mechanostimulation of receptive fields in the skin and can be custom made by heating and bending the glass rod. All tips must be blunt.

Figure 5.

The experimental set-up: schematic representation of organ bath and fluid circulation. A custom-designed skin-nerve chamber for superfusion and recording is integrated in a one-way hydrostatic perfusion system supplying gassed extracellular solution. The temperature of the solution is thermostatically controlled. Superfusion of isolated receptive fields is realized with metal rings of different sizes and an application system connected to a roller pump. Electrical stimulation is performed through metal microelectrodes. Optimal recording conditions are maintained by tight control of the level of the oil-water interface in the recording chamber by height adjustment of the weir (double-headed arrow). Labels: (1) metal ring used to isolate receptive fields; (2) schematic representation of an application system; (3) pair of stimulation electrodes; (4) pair of wire electrodes used for recording a differential signal.

Figure 6.

Anatomical context of the saphenous nerve (lower leg, adapted from ref. 82). The saphenous skin-nerve preparation includes the skin of the lower hind limb of the mouse with the saphenous nerve in continuity. The saphenous nerve is a terminal sensory branch of the femoral nerve, arising from the third lumbar root. At the level of the thigh (not shown), the saphenous nerve separates from the femoral nerve in the femoral triangle and descends on the lateral side of the femoral vessels to enter the adductor canal. It crosses the vessels obliquely to lie on their medial side, passes down in front of the lower end of the adductor magnus muscle and on the medial side of the leg. Lower leg: the saphenous nerve supplies the skin of the medial side and front of the knee. It is accompanied by the saphenous vessels and closely follows the medial tibial border to the level of the ankle innervating large parts of the lower leg. At the ankle, it passes anterior to the medial malleolus to innervate skin on the medial and dorsal aspects of the foot. Note that in some cases the saphenous nerve splits into two branches at the knee. Color code: blue: venous vessels, red: arterial vessels, yellow: saphenous nerve. Inset: The drawing shows a sketch of a preparation from the right leg and the innervation territory supplied by the saphenous nerve (yellow) and the superficial

peroneal nerve (grey). The territory innervated by the saphenous nerve includes the medial side of the leg and the medial side of the foot.

Figure 7.

The surgical procedure in a left leg saphenous nerve preparation. **(a)** The femoral vessels and the saphenous nerve (color code: red: arterial; blue: venous; yellow: nerve) run in the middle of the medial aspect of the thigh. With a pair of sharp forceps, the connective tissue is removed from the saphenous nerve. **(b)** Opening the field of view toward the groin with a lateral and a medial cut gives view on the inguinal ligament (white shadow). In the region of the groin, the saphenous nerve runs within the femoral nerve and intersects the inguinal ligament. Distal to the ligament, the femoral nerve splits into a thick lateral nerve trunk (containing branches to the rectus femoris and vastus femoris muscles) and the much thinner saphenous nerve, which runs medially. A knot of surgical silk is made around the whole femoral nerve trunk above the inguinal ligament. **(c)** After detaching the saphenous nerve from all adhering connective tissue, the saphenous nerve is placed in the middle of the thigh and covered with a piece of lint-free cloth. The cloth is soaked with plenty of physiological solution to keep the nerve wet. **(d)** Medial view of the thigh, lower leg and forefoot: a

circular cut is made around the forefoot at the border of hairy and glabrous skin. (e) Lateral view of the lower leg: a straight cut from the popliteal fossa to the heel separates the skin flap at the back. (f) The cutaneous branches of the saphenous nerve (highlighted in yellow) are embedded in the subcutaneous connective tissue of the forefoot skin flap. Inset: overview. (g) Medial view of the lower leg: the saphenous nerve closely follows the medial (inner) border of the tibia. Connective tissue is carefully removed with forceps.

Figure 8.

Views of organ bath and recording chamber. **(a)** Top view of the skin–nerve chamber. The chamber consists of an organ bath where the skin is exposed, corium (dermis) side up, and superfused with SIF. The receptive field of a sensory nerve fiber is isolated by use of a metal ring. A microelectrode is positioned within the receptive field for electrical stimulation. An application system's tip is positioned within the ring to superfuse the receptive field with solution and to apply temperature stimulation. Dotted black box: magnified in **b**. **(b)** Detailed view of the adjacent recording chamber for extracellular recordings from isolated filaments of the saphenous nerve. The desheathed and filamented saphenous nerve is covered with paraffin oil. The pair of recording electrodes is on the top left, and a grounding electrode for the organ baths' aqueous solution is on the right. Dotted white circle: magnified in **c**. **(c)** Highly magnified view through the binocular lens showing the nerve and several teased filaments; one filament is placed on the recording electrode. White arrow tip: the sheath of the nerve is pulled distally like a stocking.

Figure 9.

Sweep showing several C-fiber action potential waveforms. Original recording in response to electrical stimulation to the receptive field showing several action potential waveforms; arrow: A-fiber volley merged with the stimulation artifact; *x* axis: latency in ms; *y* axis: amplitude of the voltage signal (window size ± 10 V). With electrical stimulation (of sufficient intensity), the number and waveform of potentially contaminating excitable nerve fibers (afferent and efferent) can be determined at a spot where a receptive field of an identified single-fiber had been located. At large, each waveform in the sweep corresponds to one axon close to the site of stimulation, and only one, usually the tallest, is of interest. In this case, mere reduction of the gain would suffice to achieve a functional single-fiber recording, even in the presence of the other fibers. Smaller waveforms would disappear in the background noise yielding a functional single-fiber recording on the basis of the largest C-fiber waveform.

Figure 10.

The marking test. Eight consecutive recordings from a C-fiber in response to electrical stimulation at 2-s intervals. Sweep 1–2: the waveform of the C-fiber appears repeatedly at a stable latency of 52 ms (dotted vertical line). Sweep 3–5: application of a mechanical stimulus to the receptive field triggers a burst of action potentials (green waveforms), which delay the electrically evoked action potentials (red waveforms). Sweep 6–8: the latency of the electrically evoked action potential gradually returns to its initial value. The *x* axis: latency in ms; *y* axis: amplitude of the voltage signal (window size ± 10 V); green: action potential in response to mechanical stimulation; red: action potentials elicited by electrical stimulation.

Figure 11.

The parts of an application system for thermostatic control. **(a)** Two steel tubes are mounted side by side and serve for push–pull superfusion. Both are bent to a right angle to form the nozzle. Note that at the end, the afferent tube is slightly longer than the efferent one. The afferent tube is wrapped with a resistive wire. To heat the solution flowing through the tube, current is applied through the resistive wire. The longer of the tubes is connected to the outlet of the mantle pipe and bracket (shown in **b**), whereas the shorter one serves to evacuate the fluid from the cylinder. Both are connected to the roller pump. Inset: shows a receptive field, which is isolated from the surrounding fluid by a cylinder (15 mm OD). The top end of the application system is positioned within the cylinder and the receptive field is superfused with solution (stained with Evans Blue). A thermocouple is attached to the tip of the application system and a needle electrode is positioned within the receptive field. **(b)** A custom-designed bracket serves to fasten the application tip to a micromanipulator and enables a water-tight connection to tubing. For details, see Box 2. **(c)** Examples for heat and cold stimuli. Upper panel: average of 30 cold stimuli. Lower panel: average of 33 heat stimuli. Error bars represent $\pm$ standard deviation.

Figure 12.

Views of organ bath and isolation of a receptive field with a hollow steel cylinder. **(a)** Detail showing a superfused receptive field (solution dyed with Evans Blue) encompassed by a metal cylinder. Inside the cylinder, the tips of the application system can be seen with the thermocouple (red tip) attached side by side. A needle electrode, attached to a holder, is inserted in the receptive field for electrical stimulation. The custom-designed cylinder restricts the application of the solution to the surface of the receptive field. **(b)** Blue-stained receptive field after an experiment (where the ring had been in **a**).

Figure 13.

Factors that influence the shape of the action potential waveform. **(a)** Bandwidth of the band-pass filter. The diagram shows the triphasic action potential waveform of an A-fiber acquired at two different low-frequency cutoffs of the band-pass, 100 Hz to 1 kHz (black waveform) and 500 Hz to 1 kHz (red waveform). Closing the pass band reduces the amplitude of the A-fiber and renders the waveform four phase. The speed of the rising and falling edges is reduced accordingly. C-fibers are much slower in rise and fall of their action potential and would therefore suffer more from the too high cutoff frequency. **(b)** Activity-induced changes of the waveform shown in a CMH-fiber. The diagram shows the log-linear increase in (instantaneous) discharge rate in response to ramp-shaped heat stimulation and shows how the action potential waveform changes at high firing rate. Top panel: temperature–time course of the heat stimulus; middle panel: instantaneous frequency plot of the heat response, each dot represents one action potential; bottom panel: contains all action potentials waveforms of the response separated in two templates using a tolerance band multiplier filter set to 1. The black waveforms correspond to the black circles in the frequency plot, whereas the red waveforms correspond to the red circles. At the peak of the heat response when the firing rate exceeds 36 s^{-1} , the reduction in amplitude of the action potential waveform reaches significance and subsequent waveforms are sorted into a different template on the basis of a standard deviation of $\pm 1 \text{ V}$. With the activity rate dropping, the waveform progressively reverts to its initial shape and action potentials fit into the initial (black) template. **(c–d)** Interspike interval. The diagram shows that at peak discharge rates, both A- and C-fibers reduce amplitudes but retain the triphasic waveform. **(c)** Mechanostimulation of an A δ -downhair receptor leads to discharge of action potentials at a rate of up to 350 s^{-1} , which determines the minimum interspike interval for these fibers to $\sim 2.8 \text{ ms}$. Left chart: bursts in response to mechanostimulation (black bars) of an A δ -fiber (cv 5.4 m s^{-1} , 1 mN). Right chart: magnification of the time frame at peak discharge (red box in left chart); upper trace: instantaneous firing frequency; lower trace: corresponding waveforms. **(d)** Left chart: cold stimulation of a menthol ($500 \mu\text{M}$)-sensitized cold receptor (CC-fiber; cv 0.9 m s^{-1}) causes discharge of action potentials at a maximum rate of 108 s^{-1} . The minimum interspike interval of these highest-frequency C-fibers comes to $\sim 9 \text{ ms}$. Blue line: temperature–time course integrated in the log scale. Note the dynamic characteristic of the response of CC-fibers; minute temperature changes lead to huge increases in the firing rate (see also Fig. 16b). Right chart: magnification of the time frame at peak discharge (red

box in left chart); upper trace: instantaneous firing frequency; lower trace: corresponding waveforms.

Figure 14.

Electrostimulation, marking test and latency analysis. **(a)** Schematic target showing electrostimulation and current spread in cutaneous tissue. Black cross: site of current injection (position of the microelectrode); colored concentric circles: suggested area in which a given amount of injected voltage/current suffices for the depolarization (to action potential threshold) of all axons located within this area. A higher current/voltage covers a larger area and allows detecting more/other potentially contaminating axons from adjacent receptive fields, e.g., 20 V of injected voltage (0.8–1 μ A using 12-M Ω tip electrode impedance) would spread within the red area and suffice to detect axon a while axon b would only be detected by injecting a higher voltage (compare Fig. 15). Dotted lines confine suggestive areas of two receptive fields (red and blue) with terminal axon branching. **(b,c)** Marking test examples with two fibers involved. **(b)** Eight consecutive traces from a C-fiber in response to electrical stimulation. Sweep 1–2: the waveforms of two C-fibers appear repeatedly at a stable latency of 13 and 30 ms, respectively. Sweep 3–5: mechanical stimulation is delivered to the area of one receptive field and triggers a burst of action potentials (green waveforms). Subsequently, the latency of the electrically evoked action potential of one of the C-fibers (red waveforms) is delayed. Sweep 6–8: the delayed latency gradually returns to its initial value (compare red dotted vertical line). The *x* axis: latency in ms; *y* axis: amplitude of the voltage signal (window size ± 10 V); green: action potentials in response to mechanical stimulation; red: action potentials elicited by electrical stimulation; blue: electrically elicited action potentials of a second C-fiber not mechanically stimulated. **(c)** Nine consecutive traces from a C-fiber in response to electrical stimulation. Sweep 1–2: one waveform appears repeatedly at a latency of 50 ms. Sweep 3–5: mechanical stimulation is delivered to the area of the receptive field and triggers a burst of action potentials with smaller amplitudes. Subsequently, the latency of the electrically evoked waveform slows and falls apart in two spikes. Sweep 6: two very similar waveforms are visible and appear at different delayed latencies. Sweep 7–9: both waveforms gradually return to their initial position and become superimposed (compare dotted vertical line). The *x* axis: latency in ms; *y* axis: amplitude of the voltage signal (window size ± 10 V).

Figure 15.

Troubleshooting a single-fiber recording. **(a)** Cold response of CMC-fiber. Upper panel: strip chart of the cold response shows the amplitude of action potential waveforms; middle panel: instantaneous frequency plot of the cold response in a logarithmic scale; lower panel: time course of the cold stimulus. The frequency plot shows a dense firing pattern in response to cold stimulation that is overlaid by burst firing up to 300 Hz. The strip chart shows amplitudes in a relatively uniform voltage range ($n = 227$ waveforms, s.d. = 0.73), except for one waveform that exceeds the upper and lower 10-V limit of the voltage window (red strip). The action potential waveform that corresponds to this event shows clipping (red waveform in the inset). Comparison of the peak values in the frequency plot to the corresponding waveforms shows one waveform with an interspike interval of less than 1 ms, which is impossible in one and the same fiber (blue waveforms in inset; compare Fig. 13). **(b)** Electrostimulation was repeated and, with application of higher voltages, revealed the presence of a second axon with identical waveform shape. Subsequently, the multifiber strand was removed from the electrode and separated. **(c)** Cold response of one of the CMC-fibers. Upper panel: strip chart of the cold response shows the amplitude of action potential waveforms within the 10-V window frame; middle panel: instantaneous frequency plot of the cold response in a logarithmic scale; lower panel: time course of the cold stimulus. The strip chart shows larger amplitudes with less variation than before; inset: $n = 112$

waveforms, s.d. = 0.47. Peak firing frequency is now 8.4 s^{-1} , corresponding to a minimum interspike interval of ~120 ms.

Figure 16.

Thermosensitive C-fibers with ongoing activity at 32 °C. **(a)** Case of a CMC-fiber with ongoing activity at 32 °C. Heating the receptive field reduces the activity (light-gray arrow tip) and rewarming after a cooling stimulus inhibits the fiber (dark gray arrow head). Menthol enhances ongoing activity at 32 °C and sensitizes to cooling. Only a minor fraction of CMC-fibers responds to menthol. *Note:* in comparison to CC-fibers, the peak firing rates of CMC-fibers are much lower and do not exceed $10\text{--}16 \text{ s}^{-1}$ even in the presence of high concentrations of menthol. **(b)** Sample recordings from a single cold-sensitive (CC) fiber. At a bath temperature of 32 °C, this CC-fiber shows no ongoing activity but when cooled it responds with high firing rate and a large number of spikes. The threshold is close below

bath temperature and at lower slowly falling stimulus temperatures the fiber shows some adaptation. There is a strong dynamic component in the response when the fiber reaches very high peak firing rates (40 s^{-1}). Menthol induces ongoing activity at bath temperature that silenced above 34°C (not shown) but, in this case, did not further increase the firing rate or the magnitude of the response; the threshold, however, was lowered by 4.4°C under menthol. Inset: this CC-fiber shows paradoxical heat-induced discharge at high temperatures (48°C), which does not encode the temperature change by firing rate. (c) Rare case of a CMW-fiber with ongoing activity at 32°C . Warming the fiber rapidly increases firing rate up to 44°C where the fiber reaches its peak firing frequency and rapidly inactivates (red column and inset). Cooling the fiber inhibits the ongoing activity, and rewarming acts like a warming stimulus. Upper traces: temperature–time course; lower traces: instantaneous firing frequency.

Figure 17.

Heat responses of polymodal nociceptors (CMH). The properties of the heat response of polymodal nociceptors were characterized in detail on the basis of 33 single fibers recorded from skin-nerve preparations of adult C57BL/6 mice. The stimulus was a 20-s ramp from 30 to 49–50 $^\circ\text{C}$. C-fiber action potential amplitudes varied within a standard deviation of $\pm 0.47 \text{ V}$. **(a,b)** Sample recordings from two different types of CMH-fibers; **(a)** displays a CMH-fiber with low temperature threshold, high peak firing frequency and large responses. When subjected to a 30-s step stimulus to 45 $^\circ\text{C}$ (right panels), the fiber briskly responded followed by slow adaptation and static discharge at lower frequency. **(b)** A CMH-fiber with a high temperature threshold, low peak firing frequency and a small response. This fiber showed no discharge when subjected to a moderate step stimulus. Upper panel: time course of the heat stimulus; threshold temperature (arrow). Middle panel: instantaneous frequency plot of the heat response. Lower panel: strip chart showing the amplitude of action potentials. **(c)** Averaged histogram of 33 heat responses. Upper panel: mean of heat responses in bins of 2 s, error bars represent the s.e.m. Lower panel: average temperature–time course of the 33 heat stimuli. Note that polymodal nociceptors show no ongoing activity at 30 $^\circ\text{C}$. **(d)** Distribution of the magnitude of the heat responses, given as number of action potentials discharged in 20 s + 2 s of heat stimulation. The majority of the polymodal fibers show small responses and fire less than ten spikes per stimulus. **(e)** The threshold temperature distribution of polymodal fibers is approximately Gaussian, with a maximum between 41 and 43 $^\circ\text{C}$. **(f)** Regression of the peak firing frequency on the magnitude of the

heat response ($R = 0.84$, s.d. = 8.7, $P = 0.0001$). (g) Regression of the heat response magnitude on the heat threshold ($R = -0.67$, s.d. = 0.28, $P = 0.0001$, $n = 33$).

Figure 18.

Cold responses of cold nociceptors (CMC). The properties of the cold response of cold nociceptors were characterized in detail on the basis of 30 single fibers recorded from skin-nerve preparations of adult C57BL/6 mice. The stimulus lasted for 60 s from 30 to 10 $^{\circ}$ C. C-fibers action potential amplitudes varied within a standard deviation of ± 0.41 V. **(a,b)** Sample recordings from two types of CMC-fibers: **(a)** dynamic discharge and a low temperature threshold, **(b)** static discharge and a high temperature threshold. Upper panels: time course of the cold stimulus. The value of the threshold temperature of the response (arrow). Middle panels: instantaneous frequency plot of the cold response. Peak firing frequency is shown. Lower panels: strip chart showing the amplitude of the action potentials. Inset: the average of all action potentials constituting the response. **(c)** Averaged histogram of 30 cold responses. Upper panel: mean of cold responses in bins of 2 s. Lower panel: average temperature-time course of the 30 cold stimuli. Note that cold nociceptors show no ongoing activity at 30 $^{\circ}$ C. **(d)** Distribution of the magnitude of cold responses, given as number of action potentials discharged per 60 s of cold stimulation. The majority of the CMC-fibers have small cold responses and discharge less than ten spikes per stimulus. **(e)** The temperature threshold distribution of CMC-fibers appears bimodal with two maxima between 27–25 and 19–17 $^{\circ}$ C. **(f)** ‘Box and Whiskers’ plot showing the distribution of the peak firing frequencies of CMC-fibers. Error bars $\pm$ s.e.m. The boxes are 25th and 75th centiles; the horizontal lines inside the boxes show the median, and the ‘whiskers’ (enhanced by asterisks) indicate minima and maxima. **(g)** Regression of the peak firing frequency on the magnitude of the cold response ($R = 0.84$, s.d. = 0.86, $P = 0.0001$, $n = 30$).

TABLE 1

Fiber classification.

Class	Type	Abbreviation	Adaptation	Conduction velocity (m/s)	Von Frey threshold (mN)	Temp. threshold (°C) ^d	Notes
C	Mechano-Heat 'Polymodal Nociceptor'	CMH	Slow	<1	<1–128	~40 ^b	Classical 'polymodal' nociceptor ¹⁷ ; chemosensitive
C	High-Threshold Mechano	C(HT)M	Slow	<1	5.7–128	— ^{b,c}	
C	Low-Threshold Mechano	C(LT)M	Slow	<1	<1–5.7	—	
C	Mechano-Cold 'Cold Nociceptor'	CMC	Slow	<1	<1–128	Bimodal ^d	Low peak firing frequencies, bimodal temperature threshold distribution (~27–29° and ~17–22 °C), chemosensitive (e.g., menthol, capsaicin)
C	Mechano-Cold-Heat 'Multimodal Cold Nociceptor'	CMCH	Slow	<1	<1–128	Like CMC; >45	Infrequent but more frequently encountered in mouse than rat, heat responses often small and high threshold, cold response properties like CMC
C	Cold 'Cold Receptor'	CC	Slow	<1	—	Peak 28 ^d	Relatively rare, often spontaneous activity at 30–32 °C, high peak firing frequencies, usually menthol sensitive
C	Warm	CW	Slow	<1	—	~32–34	Peak firing around 38 °C blocked above 44 °C; extremely rare
C	Heat	CH	Slow	<1	—	~40	Rare in mouse and rat, more abundant in cat ^c
Aδ	Mechano-Heat	AMH	Slow	1.6–12	<1–128	~40 ^b	Often excited by bradykinin, pH <6.9, serotonin, histamine; sensitized (to heat) by PGE ₂
Aδ	High-Threshold Mechano	A(HT)M SA	Slow ^e	1.6–12	5.7–128	— ^{b,c}	
Aδ	Low-Threshold Mechano	A(LT)M SA	Slow	1.6–12	<1–5.7	—	
Aδ	Low-Threshold Mechano	A(LT)M RA	Rapid ^f	1.6–12	<1–5.7	—	D-hair receptor (Down hair follicle receptor), firing frequency up to 350 Hz
Aδ	Mechano-Cold	AMC	Slow	1.6–12	<1–128	<28 ^{d,j}	Often activated and sensitized to cold by menthol in rat, less frequent in mouse
Aβ	Rapidly adapting	RA	Rapid ^f	>16	<1–5.7	—	Meissner corpuscle (~50 Hz vibration), G-hair receptor (Guard hair follicle receptor), Pacinian corpuscle (200–300 Hz) ^g , Krause's end bulbs;

Class	Type	Abbreviation	Adaptation	Conduction velocity (m/s)	Von Frey threshold (mN)	Temp. threshold (°C) ^d	Notes
Aβ	Slowly adapting type I	SA I	Slow ⁱ	>16	<1–5.7		Merkel's disc; no directional sensitivity to stretch; up to 200 Hz, irregular discharge
Aβ	Slowly adapting type II	SA II	Slow	>16	<1–20	— ^j	Ruffini's end organ; directional sensitivity to stretch, very regular discharge

According to their sensitivities, mouse C- and A-fibers are categorized as described below.

^aIntracutaneous temperature thresholds and thermal responses depend on the genetic background and are variable between different inbred mouse strains^{49,83}.

^bSome exhibit rewarming discharge after deep cooling.

^cMay develop heat response after sensitization with compounds, such as bradykinin; may develop cold response after sensitization with compounds, such as menthol or icilin.

^dMay show bursting discharge after adaptation; some of the fibers show a 'paradox heat' discharge at a narrow temperature range between 45 and 50 °C.

^eOngoing discharge following the end of strong stimulation is often seen.

^fUsually show an 'off response' at the end of a stimulus.

^gPacini receptive fields are large; they are extremely sensitive (even tapping the experimental platform activates them), ultrarapid adaptation, vibration sensitive. Occasionally show an 'off response' at the end of a stimulus.

ⁱMay show 'spurious' thermal responses.

^jThreshold temperatures sometimes below 10 °C.