2 MATERIALS AND METHODS

2.1 Materials and animals

2.1.1 Technical equipment

ADInstruments PowerLab/4s
BDK laminar flow hood
BioLogic Science Instrument RSC200 rapid solution changer
Bose Model 100 Speaker
Camera Kappa
Dumont fine forceps
Eppendorf micromanipulator 5171
F.S.T. Scissors
Forma Scientific -86°C freezer
Gilson Miniplus 3 peristaltic pump
HEKA EPC-9 amplifier
Ikamag Reo magnetic stirrer
Julabo MP and Medingen waterbaths
Kleindieck Nanotechnik Nanomotor
Leica KL750 Lamp
Leica inverted microscope system DM IRB with fluorescent lamp
Leica Ms5 microscope
Mettler Toledo pH meter
Microplan table
Physiotemp Model BAT-12
Scientific Industries Vortex-Genie 2
Sony Monitor
Sylgard 184 Silicone Elastomer base World Precision Instruments Inc.
Tektronix TDS 210 Two Channel Digital Realtime Oscilloscope
Dounce homogenizer
Schleicher and Schüll Nitrocellulose membranes

Micropipette laserpuller ModelP-2000, Sutter Instruments, Novato, CA

Eppendorf Centrifuge 5415R and 5810R

Heraeus Instruments Megafuge 1.0R

Heidolph promax 1020 shaker

Biorad power PAC 300

Hypercassette Amersham Pharmacia Biotech

AGFA Curix60 developer

Test tube rotator Snijders

Steri-cult 200 Incubator

Leitz DM IL microscope

2.1.2 Chemicals and reagents

REAGENT AND CHEMICAL	COMPANY
Lipofectamine	Gibco
Paraformaldehyde	Sigma-Aldrich
Triton X-100	Sigma-Aldrich
Tween-20	Pierce Chemical, P/N 28320
Protein molecular mass Standard	BioRad
Protease inhibitor cocktail	Sigma-Aldrich
ECL	Amersham Biosciences

2.1.3 Buffers and Solutions

BUFFER AND SOLUTION	COMPOSITION
10X TBS	0.5M Tris/HCl, pH 7.9 1.5 NaCl
4% paraformaldehyde	4g paraformaldehyde in 100ml PBS pH 7.4
PBS	Instamed PBS Dulbecco w/o Ca^{2+}, Mg^{2+}
SDS PAGE Running buffer	25mM Tris/HCl, pH 8.3 190mM Glycine 0.1% SDS

SIF (synthetic interstitial fluid)	2mM $CaCl_2$ 5.5 mM glucose 10mM Hepes 3.5mM KCl 0.7 $MgSO_4$ 123mM NaCl 1.5mM NaH_2PO_4 7.4mM saccharose Set to pH 8.4 with 10N; carbogene used for oxygenation during the experiment will set pH 7.4
Solution I (resuspension buffer for bacterial culture)	100mM Tris HCl, pH 7.5 10mM EDTA 400µg/ml RNAse I
Solution II (Alkaline lysis of bacterial culture)	1M NaOH 5.3% (w/v) SDS
Solution III (neutralisation buffer)	3M potassium-acetate pH 5.5
RIPA lysis buffer	50mM Tris-HCl, pH 7.4, 150mM NaCl, 1mM EDTA, 0.5% DOC, 0.5% Trition X-100, 0.1% SDS, 1mM DTT, 1:500 Protease inhibitor cocktail
Intracellular solution (patch)	110mM Methanesulfonic acid, 30mM KCl (BDH), 1mM $MgCl_2$, 10mM HEPES, pH 7.35 (with KOH)
Extracellular solution (patch)	154mM NaCl, 10mM HEPES, 8mM glucose, 5.6mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$, pH 7.40 (with NaOH)

2.1.4 Culture media

HEK293 medium: 10% FCS (Biochrom)
2mM glutamine (Gibco)
100u penicillin/100µg/ml streptomycin (Gibco)
in DMEM/F12 nut mix (Gibco)
Pharmaceuticals only where mentioned

2.1.5 Kits

KIT	COMPANY
Qiagen Plasmid Midi kit	Qiagen

2.1.6 Plasmids

PLASMID	COMPANY
pcDNA 6B/Myc-His	Invitrogen
pcDNA 3.0	Invitrogen

2.1.7 Antibodies

ANTIBODY	COMPANY
M2 (anti-Flag)	Sigma-Aldrich
Secondary Antibodies	Dianova, Hamburg
Agarose beads	Sigma-Aldrich
Anti-myc (9E10)	Roche

2.1.8 Consumables

PRODUCT	COMPANY
Eppendorf tubes	Eppendorf
Cell Culture dishes	Falcon
15ml and 50ml tubes	Falcon, Greiner
X-ray films	Amersham Biosciences
Borosilicate glass electrodes with 1.5mm filament	Hilgenberg Malsfeld

2.1.9 Animals and genotyping

C57Bl/6 mice were obtained from Bomholtgard and kept in the animal house of the Max-Delbrueck Centrum fuer Molekular Medizin until they were used for experiments. The *ASIC3* -/- mutant mice were obtained from Michael Welsh, Howard Hughes Medical Institute, Iowa, USA. The *stomatin* -/- mutant mice were obtained from Narla Mohandas, Lawrence Berkeley Laboratory, San Fransisco, USA. All mutant mice were bred in the Charite Campus Benjamin Franklin, Einrichtung fuer Experimentelle Medizin, and in the animal house of Max-Delbrueck Centrum fuer Molekular Medizin, Berlin, Germany.

ASIC3 -/- s*tomatin* -/- (A3-/-S-/-) double mutant mice were generated by crossing *ASIC3* -/- with *stomatin* -/- mice at the Max-Delbruek Centrum, and at the Charite Campus Benjamin Franklin, Einrichtung fuer Experimentelle Medizin, Berlin. The A3-/-S-/- double mutant mice were apparently healthy and viable.

Animals were kept in cages under constant temperatures (23 ˚C) with free access to food and water. All animal work was done in accordance with animal care bylaws of the Max-Delbrueck Centrum animal house. Genotypes were determined by PCR amplification of tail DNA using the following forward and reverse primers for ASIC3: (5'-GAACCTGGAAAACAGAGGCAGGAAGGAT-3' and 5'-CAGGGAGTAAG ATCTTATGTAGCCTGGC-3'), and for stomatin (5'-GTGGATAATACAAACTTCACGAGG-3' and 5'-AATGGAGGAGAAGACACGC-3'). Reaction conditions were: 1 cycle of 94˚C for 7 min, 60˚C for 30 sec, 72˚C for 2 min, then 40 cycles of 94˚C for 30 sec and 60˚C for 35 sec

followed by 72˚C for 2 min. A3-/-S-/- mutant mice were kept on a 129SV/C57Bl/6 mixed background.

2.2 Methods

2.2.1 Biochemistry: Co-immunoprecipitation Experiment of ASIC3 and stomatin-like proteins

2.2.1.1 Maintenance of HEK293 cell line

Standard methods were used to culture HEK293 cells. Cells were grown in a Steri-Cult 200 incubator at 37°C, 92% humidity and 5% CO_2 concentration in HEK293 medium. To passage the cells, they were first washed with PBS and dissociated from the culture dish, then pipetted up and down to separate them into single cells. The cells were centrifuged at 1500rpm for 2min, resuspended in fresh medium and counted in a Thoma chamber so that an appropriate number of cells were plated in a new dish.

2.2.1.2 Transfection

HEK293 cells were transfected using the Lipofectamine system as described in the manufacturer's instructions. Cells were cultured in Ø10cm culture dishes overnight until they reached 70-90% confluency. The DNA was prepared prior to transfection. Serum-free medium containing 0.5µg pDNA/µl medium was combined with 10% Lipofectamine/serum-free medium solution and incubated for 30min at RT. Cells were rinsed twice in the culture dish with serum-free medium. Transfection medium was diluted 1:10 in serum-free medium prior to applying it to the cells and incubated for 5 hours under normal cell culturing conditions. Transfection medium was carefully removed, covered with normal, serum-containing medium and allowed to grow undisturbed for 3 days. Cells were passaged, diluting their number 1:10 before replating. They were plated in a new dish in selection medium.

2.2.1.3 Protein isolation

HEK293 cells were collected and washed 3 times in PBS with Protease inhibitor 1:500 with centrifugation at 1500 rpm, 3 min, 4ºC. Samples could be stored at -80ºC.

2.2.1.4 SDS-polyacrylamide-gel electrophoresis

Utilizing a SDS-polyacrylamide-gel electrophoresis (SDS-PAGE) in a miniapparatus, the miniprotean II from BioRAD, proteins were separated according to their molecular mass under reducing conditions (2% β-mercaptoethanol) at 110-130V (Lämmli, 1970). The size of the protein bands was determined by comparing them to molecular mass standard (BioRad).

2.2.1.5 Western Blotting

Proteins were transferred from the SDS gel to a nitrocellulose membrane for 1h at 100V at around 4ºC in the Mini Transblot Apparatus from BioRad. Membranes were quickly rinsed with distilled H_2O and protein bands detected with a 3% ponceau-red solution. After washing 3 times with TBS-T the membranes were transferred to a WB blocking buffer for 1 h at RT. Primary antibodies, Anti-Flag and Anti-myc were diluted to 1:500 in 5 ml of blocking buffer and incubated overnight at 4ºC. Membranes were washed several times and the appropriate secondary antibodies (goat-anti-mouse fro monoclonal and goat-anti-rabbit for polyclonal) conjugated with horse radish peroxidase (HRP) diluted to 1:10,000 in WB blocking buffer were applied for 1 h at RT. Protein bands were detected using chemiluminescent substrate ECL™ Western Blotting analysis system from Amersham Biosciences. The bands were visualized using Hyperfilm™ ECL high performance chemiluminescent film from Amersham Biosciences developed on an AGFA Curix60 machine.

2.2.1.6 Co-immunoprecipitation

The cell extracts (ASIC3, ASIC3/Stomatin, ASIC3/SLP3, SLP3, Stomatin, and Vector) from a 10cm culture plate were lysed in 750µl RIPA lysis buffer and homogenized with a Dounce homogenizer. The homogenate was cleared in a centrifugation step at 10,000g for 20 min at 4ºC. The supernatant was removed, volume divided by 2 (375µl) and additional RIPA solution added to a final volume of 750µl. Thus we had 2 samples of each cell line lysate. Each sample of a cell

line lysate was precipitated with 3µg of antibodies (either anti-Flag or anti-myc) in a test-tube rotator for 2-4h at 4ºC. Protein agarose beads was prepared by washing it 3X with RIPA solution and then added (30µl/tube) to the precipitate overnight at in a test-tube rotator for 2-4h at 4ºC. This was then washed and centrifuged (300rpm, 3min, 4ºC) 3X with RIPA buffer. The protein agarose microbeads were separated from the RIPA buffer using a 27G needle. 30µl of 4X Western blot loading buffer was added to the beads and together cooked at 95ºC for 5 min and then centrifuged for 3min at 10,000g. The mixture was loaded on an SDS gel and run according to the western blot protocol. The anti-Flag-precipitate and anti-Myc-precipitate blots were then treated with anti-Myc and anti-Flag primary antibodies respectively. Detection of the bands was done according to usual western blot protocol.

2.2.2 Electrophysiology: *in vitro* Skin nerve preparation

The mouse *in vitro* skin nerve preparation was described in detail by Koltzenburg and Lewin (Koltzenburg et al., 1997). Some minor modifications were introduced in the mechanical stimulation technique. This method was originally adapted from a rat preparation (Reeh et al., 1986, kress et al., 1992) in which the properties of sensory afferents *in vivo* did not differ from the *in vitro* setting.

2.2.2.1 Mouse Preparation

Adult mice from a C57Bl/6 genetic background, a hybrid strain derived from and sv129 together with their mutant counterparts were used in the study. The experiments were done blind to genotype. The mice were of either sex and weighed between 20 and 40 g. Mice were killed by CO_2 inhalation and the hindlimb hair was removed with an electric razor.

The saphenous nerve was dissected free from its point of insertion into skin tissue up to the lumbosacral plexus and excised together with the innervated hindlimb skin extending from the toes up to the knee. The hindlimb skin was fixed with insect needles “inside up” in an organ bath and superfused with 32ºC warm oxygen-saturated synthetic interstitial fluid (SIF) at a flow rate of 15ml/ml. In this way tissue oxygenation through the corium side and electrical viability could be maintained for up to 8 hours. The saphenous nerve was pulled through a gap to the recording chamber and laid on top of a small mirror-based platform that served as a dissection plate. To

provide electrical isolation, the aqueous solution in the recording chamber was overlaid with mineral oil in such a way that the interface of the two phases was located just below the surface of the mirror.

2.2.2.2 Recording technique

For single-unit recording, the distal cut end of the saphenous nerve was first freed by removing the perineurium. The nerve bundle was then teased under a dissecting microscope using sharp Dumont's 55 forceps until a fine nerve filament was isolated. Recordings were made from the filament placed on a silver electrode installed in the chamber's wall with the reference electrode contacting the bath phase. Filaments containing less than 10 single sensory neurons were ideal for extracellular recordings.

Single unit action potentials could be identified using either an electrical search stimulus, or a mechanical search stimulus. In the former, the nerve was electrically stimulated with constant current pulses of ~1 Hz frequency delivered by a Neurolog Digitimer via an electrode placed on the nerve path across the skin. In the latter, manual probing of the skin with a glass rod identified the receptive fields of sensory afferents. It is known that manual probing alone activates ~90% of cutaneous afferents in rats (Kress et al., 1992), whereas the electrical search stimulus has the advantage of identifying primary afferents that are insensitive to manual probing.

Nerve activity was amplified using the Neurolog^{TM} system from Digitimer Ltd., which included a low-noise differential amplifier, (AC-coupled amplifier). Only fibers with a signal-to-noise ration greater than 3 were used for further analysis. The amplified signals were visualized with a Tektronix TDS 220 two-channel digital real time oscilloscope and connected to a speaker. Simultaneously, the signals were also acquired on a PC with powerLab/4s converter from AdInstruments. Off-line analysis using Chart v5 software and spike Histogram extension of software from ADInstruments enabled the discrimination of action potentials from individual units according to their width, (in μsec) and height (in points), and construction of peristimulus time histograms.

In this way, single-unit activity from Aβ-fibers and Aδ-fibers could be recorded from the finely divided filaments. For unit recordings from C-fibers, a fine nerve filament usually contained the activity of more than one unit, and the activity of the unit with the largest amplitude action

potential was chosen for study. Recordings from single units were confirmed on the basis of the amplitude, width and shape of the action potential displayed on the oscilloscope and whether electrical stimulation of the skin region excited only a single unit. After ensuring that the recording was from a single unit, the sensory properties of the units were examined.

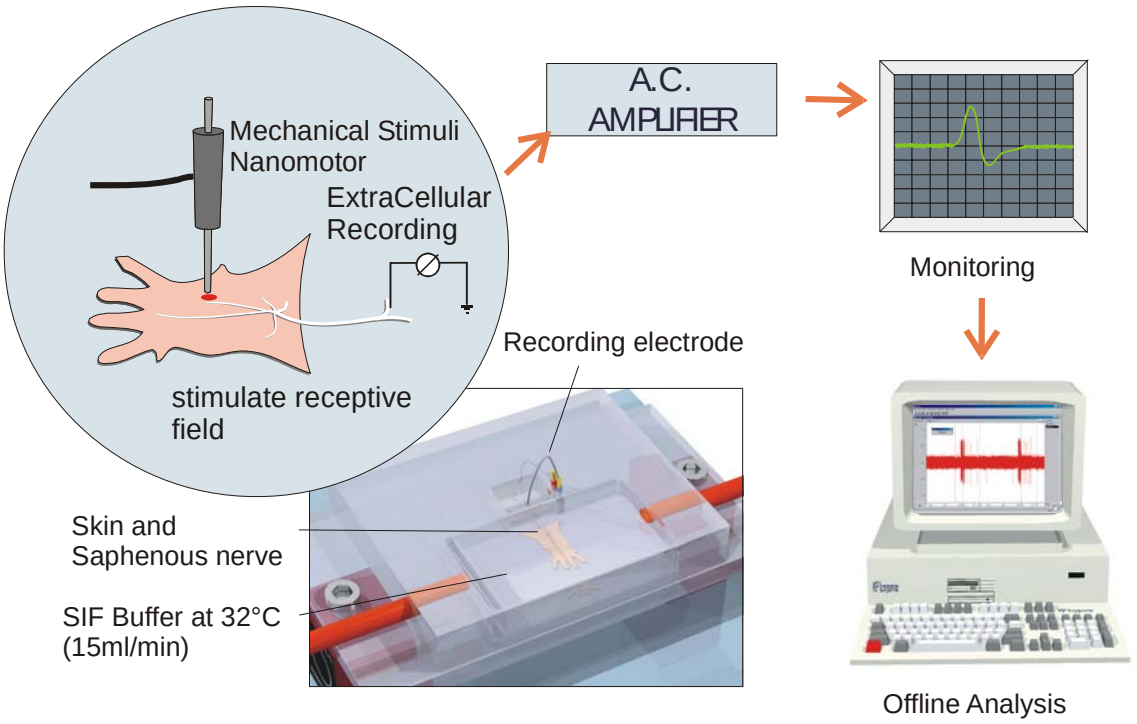

Figure 6: Scheme of the in vitro skin nerve setup

The conduction velocity was determined by electrically stimulating the most sensitive spot of the receptive field with constant current in square wave pulses at 1Hz using a Teflon-coated silver electrode with an uninsulated tip (Ø<0.5mm). The stimulus intensity was set at approximately two-times the threshold with a pulse duration of 50-500µsec. Conduction velocity of axons was calculated by dividing the conduction distance by the latency following electrical stimulation. In agreement with other studies three major classes of mechanosensitive units could be distinguished based on conduction velocity. Units conducting faster than 10m/s were considered as thickly myelinated Aβ-fibers, those conducting between 1-10m/sec as thinly myelinated Aδ-fibers, and those conducting at less than 1 m/sec as unmyelinated C-fibers.

2.2.2.3 Identification of cutaneous mechanoreceptor units

In order to distinguish between the different cutaneous mechanoreceptors encountered, several physiological variables were considered: 1) size and shape of receptive field; 2) mechanical threshold; 3) thermal sensitivity; 4) stimulus-response curve; 5) rate of adaptation to stimulus; and 6) mechanical latencies.

Receptive field size was described as either large or small; shape as spotlike, or punctate, i.e., presence of several sensitive spots. Thermal sensitivity of nociceptors was tested by isolating the receptive fields with a small self-sealing metal ring (6 mm diameter) and the 32 degrees bath solution was exchanged with a hot SIF solution. The temperature of the hot solution was monitored using a temperature tip (from physiotemp) placed within the metal ring. Thus temperatures > 55 degrees were always achieved and the evoked impulses were recorded. This method did not determine the heat threshold, it only identifies nociceptive units activated by heat and thus C-mechanonociceptors could be separated from C-mechanoheat receptors.

2.2.2.3.1 Mechanical thresholds

To determine the mechanical activation threshold, calibrated von Frey monofilaments were applied on the most sensitive spot on the receptive field. The von Frey hairs were of various thicknesses, which accordingly produced bending forces from 0.4mN to 32mN. The mechanical threshold was expressed as the minimum force necessary to evoke an impulse.

2.2.2.3.2 Stimulus-response curve

To determine the mechanical sensitivity of the sensory neurons of the saphenous nerve, a probe fixed to a linear stepping motor under computer control (Nanomotor® Kleindiek Nanotechnik) was used to deliver standardized displacement stimuli applied to the receptive field at regular intervals. The signal driving the movement of the linear motor and raw electrophysiological data were collected with a Powerlab 4.0 system (AD instruments).

The nanomotor consists of a stator (piezo crystal) and a slider that are attached to each other by friction. The piezo crystal expands and contracts to applied voltage, thus voltage pulses change the friction between slider and stator and allows an accurate, stable movement relative to stator.

Using PC software, the nanomotor was operated and controlled to give mechanical stimuli of different displacements as well as different velocities. The nanomotor was calibrated regularly to know the corresponding probe displacement to the voltage steps. The tip of the nanomotor was placed over the most sensitive spot of a receptive field. Since it was not possible to start the mechanical stimulation at the mechanical activation threshold, which is a parameter determined by force of stimulation, the probe was lowered in a decreasing stepwise manner so that the reference point of the recording was when an action potential was fired to a 9.6 µm indentation. From then on various stimulation protocols were used to produce stimulus response functions of individual units based on the type of stimulus to which the fiber responds best.

2.2.2.3.3 Mechanical stimulation varying displacement

In this stimulation protocol, the nanomotor delivered an ascending series of mechanical displacements (9.6, 19.2, 38.4, 76.8, 153.6, 307.2, 614.4 µm/sec) of 10-second duration, in which the ramp phase had a constant velocity of 1100 µm/sec.

2.2.2.3.4 Mechanical stimulation varying velocity

In this protocol the mechanical displacements are constant (50, 100, 150 µm) but with increasing velocities from 1.5 to 3000 µm/sec.

2.2.2.3.5 Mechanical latencies

The mechanical latency defines the time between the start of the mechanical stimulus and the beginning of an action potential. This was calculated for all mechanically activated receptors.

2.2.2.3.6 Rate of adaptation

D-hairs and rapidly adapting fibers normally respond within the ramp phase of the mechanical stimulus whereas slowly adapting fibers fire spikes during the hold phase. According to this criterion low-threshold Aβ fibers were identified.

2.2.2.4 Data analysis and statistics

For construction of the stimulus-response functions for mechanical stimulation, all action potentials were counted in a time period of 10 s after the onset of the force stimulus. Mean discharge frequencies were calculated and plotted against stimulus force for all tested fibers to generate the stimulus response function. Appropriate statistical tests (unpaired 2-sided Student's *t*-test, Mann-Whitney *U* test, and x2 test, ANOVA) were used after fulfillment of the necessary prerequisites with the use of the Statistica software package of Statsoft.

2.2.3 Electron microscopy

Mice were perfused with freshly prepared 4 % formaldehyde in 0.1 M phosphate buffer. Saphenous nerves were dissected and postfixed in 4 % formaldehyde/2.5 % glutaraldehyde in 0.1 M phosphate buffer for 3 days. Following treatment with 1% OsO_4 for 2h, they were dehydrated in a graded ethanol series and propylene oxide and embedded in Poly/Bed^R 812 (Polysciences, Inc., Eppelheim, Germany). Semithin sections were stained with toluidine blue. Ultrathin sections (70 nm) were contrasted with uranyl acetate and lead citrate and examined with a Zeiss 910 electron microscope.

Digital images were taken with a 1kx1k high-speed slow scan CCD camera (Proscan) at an original magnification of 1600x. Two ultrathin sections per nerve and genotype were analysed. On each ultrathin section, four images were taken representing an area of 18.25 x 18.27 μm. Myelinated and non-myelinated axons were counted on these areas using the analySIS 3.2 software (Soft Imaging System, Münster, Germany), and normalized to the whole nerve. The imaging was done by Bettina Erdmann at the Max-Delbrueck Centrum fuer Molekular Medizin, Berlin, Germany.