2 Material and Methods

2.1 Material and animals

2.1.1 Technical equipment

ADInstruments PowerLab/4s
Applied Biosystems PRISM 7700 Sequence Detection System
BDK Laminar Flow Hood
Biometra TRIO-Thermoblock PCR machine
BioRad Mini Protean II
BioRad Mini Transblot Apparatus
BioRad PowerPac 300
Cryostat
Digitimer Ltd. NeuroLog Amplifier
Eppendorf Thermomixer Compact and 5436
EquiBio Easyject Electroporation Apparatus
Forma Scientific -80°C Freezer
Forma Scientific Steri-Cult 200 Incubator
Gilson Minipuls 3 Peristaltic Pump
Hamamatsu Digital Camera C4742-95
Harnischmacher Labortechnik DNA Electrophoresis Chambers
Heidolph Duomax 1030 and Promax 1020 Shakers
Heraeus Biofuge 13
Heraeus Megafuge 1.0
Hereaus Biofuge 15R
Herolab E.A.S.Y 429K Digital Camera
Herolab UVT 2035 Transilluminator 302nm
Ikamag Reo Magnetic Stirrer
Julabo MP and Medingen Waterbaths

Kleindieck Nanomotor

Leica DM RBE Upright Fluorescence Light Microscope

Leica KL 750 Fiber Optic Light Source

Leica MS5 dissecting microscope

Mettler Toledo 320 pH Meter

MoTil System (TSE Systems)

Harmacia Biotech Ultrospec 1000 Spectrophotometer

Rotorod Test (TSE Systems)

Scientific Industries Vortex-Genie 2

Stratagene UV Stratalinker 2400

Tektronix TDS 220 Two Channel Digital Real Time Oscilloscope

Uni Equip Unitherm Hybridization oven 6/12

Analytical Software

AnalySIS 3.2 Software (Soft Imaging System, Münster, Germany)

ActiMot Analysis Software (TSE Systems, Germany)

Chart v5.2 for Windows (ADI instruments)

Lasergene Software by DNAStar Inc.

2.1.2 Chemicals and reagents

REAGENT AND CHEMICAL	COMPANY
10 x PCR buffer	Invitrogen Life Technologies
100bp and 1kb ladder	Gibco
20x TaqMan Gene Expression Assay	Applied Biosystems
2x TaqMan Universal Master Mix	Applied Biosystems
5x First-strand buffer	Invitrogen Life Technologies
5x Second-strand buffer	Invitrogen Life Technologies
APES	Sigma-Aldrich
Aqua-Polymount	Polyscience Inc.
Bovine Serum Albumin (BSA)	Invitrogen Life Technologies
dNTPs (10mM each)	Invitrogen Life Technologies

DTT	Invitrogen Life Technologies
ECL	Amersham Bioscience
ExpressHyb solution	Clontech
Gelatine	Sigma-Aldrich
Glygogen	Promega Corporation
Herring sperm DNA	Promega Corporation
Horse serum	Biochrom
Horse radish peroxidase (HRP)	Sigma-Aldrich
Lipofectamine	Gibco
P_{32} dCTPs	Perkin Elmer
Paraformaldehyde	Sigma-Aldrich
Phenol/chloroform/isoamyl alcohol	Roth
poly-L-Ornithin	Sigma
Laminin	Sigma
Protease inhibitor cocktail	Sigma
Protein molecular mass standard	BioRad
Tissue Tek	Miles, Elkhart, Ind. USA
Triton X-100	Sigma-Aldrich
Trizol	Roth
Tween-20	Pierce Chemical
Fetal calf serum	Biochrom

Further chemicals were obtained from Biomol, Merck, Roth and Sigma-Aldrich.

2.1.3 Buffers and solutions

***in situ* hybridisation:**

BUFFER AND SOLUTION	COMPOSITION
20 x SSC	3M NaCl 0.3M Na-citrate pH 7.0
Acetylation buffer	200ml DEPC-H_2O 2.3ml triethanolamine 500µl acetic-anhydride
B1-buffer	0.1M Tris pH 7.5 0.15M NaCl
B2-buffer	1% Blocking Reagent (Roche) in B1
B3-buffer	0.1M Tris 0.1M NaCl 50mM $MgCl_2$
B4-buffer	10ml B3 200µl NBT/BCIP 240µl Levamisole (5 mg / ml)
Hybridisation buffer	50% formamide 4 x SSC 2 x Denhardts 50µg/ml total RNA (clean) 150µg/ml denatured salmon sperm DNA DEPC-H_2O to 50ml
Hydrolysis buffer	60mM Na_2CO_3 40mM $NaHCO_3$ pH 10.2
Neutralisation buffer	200mM Na-acetate 1% acetic acid pH 6.0

Southern blot:

Denaturation solution	0.5M NaOH 1.5M HCl
Depurination solution	0.25M HCL
Neutralising solution	0.5M Tris/HCl pH 7.0 1.5M NaCl
Wash solution I	2X SSC, 0.05% SDS
Wash solution II	0.1X SSC, 0.1% SDS

Others:

BUFFER AND SOLUTION	COMPOSITION
10x TBS	0.5M Tris/HCl pH 7.9 1.5M NaCl
4% PFA	4% paraformaldehyde in PBS pH 7.4
5x Lämmli buffer	60mM Tris/HCl pH 6.9 10% SDS 10% β-mercaptoethanol 50% glycerol 1.5% bromphenolblue
Acetate buffer	Na-acetate 10 mM pH 5
Homogenisation buffer	0.1M PBS
PBS	PBS Dulbecco w/o Ca^{2+}, Mg^{2+}
Phosphate buffer	0.1M KH_2PO_4 0.1M Na_2HPO_4 x $2H_2O$
SDS PAGE running buffer	25mM Tris/HCl pH 8.3 190mM Glycine 0.1% SDS
SIF (Synthetic Interstitial Fluid)	2mM $CaCl_2$ 5.5mM glucose 10mM Hepes 3.5mM KCl

	0.7mM $MgSO_4$ 123mM NaCl 1.5mM NaH_2PO_4 9.5mM Na-gluconate 7.4mM saccharose set to pH 8.4 with 10N NaOH; carbogene used for oxygenation during the experiment will bring it to pH 7.4
Tail lysis buffer	0.05M Tris (pH8) 0.1M EDTA (pH8) 0.1M NaCl 1% SDS
TCA	20% Trichloracetic Acid
TX-100 protein extraction buffer	50mM Tris/HCl pH 7.4 150mM NaCl 1% Triton X100
WB blocking buffer	1 x TBS 0.05% Tween-20 2-5% Milk-powder 0.001% Thimerosal
WB blot buffer	25mM TRIS/HCl pH 8.3 190mM Glycin 20% Methanol

2.1.4 Culture media

HEK293 medium:	10% FCS (Biochrom)
	2mM glutamine (Gibco)
	100u penicillin/100µg/ml streptomycin (Gibco)
	in D-MEM/F12 (Gibco)
OPTI-MEM:	Gibco
DRG medium:	20% HS (Biochrom)
	2mM glutamine (Gibco)
	100u penicillin/100µg/ml streptomycin (Gibco)
	in D-MEM/F12 (Gibco)

2.1.5 Enzymes and molecular weight markers

ENZYME	COMPANY
Collagenase TypeIV	Gibco
DNAse (RNAse-free)	Roche
E. coli DNA polymerase I	Invitrogen Life Technologies
Pfu-DNA polymerase	Stratagene
Phosphatidylinositol phospholipase C	Oxford GlycoScience
Proteinase K	Roth
Restrictionendonucleases	Amersham, Roche
RNA-polymerases (T7, Sp6)	Promega
RNaseA (10mg/ml)	Qiagen
RNaseOUT^{TM}	Invitrogen Life Technologies
SAP (shrimp alkaline phosphatase)	Amersham
Superscript II^{TM} reverse transcriptase	Invitrogen Life Technologies
T4 DNA polymerase,	Invitrogen Life Technologies
T4-DNA ligase	Promega
T4-DNA ligase	Invitrogen Life Technologies

Taq-DNA polymerase	Gibco
Trypsin	Gibco

2.1.6 Kits

KIT	COMPANY
DIG RNA Labeling Kit (SP6/T7)	Roche
GeneClean III Kit	Dianova
QiaEx Gel Extraction Kit	Qiagen
Qiagen Plasmid Midi Kit	Qiagen
Qiagen Plasmid Mini Kit	Qiagen
TA-Cloning Kit (pGEM-T Easy)	Promega
ExSite PCR-based Site-directed Mutagenesis Kit	Stratagene

2.1.7 Plasmids

PLASMID	COMPANY
pGEM-T Easy	Promega
pTracerCMV6	Invitrogen

2.1.8 Constructs

NAME	COMPOSITION
MM1.3	slp3 ORF in pcDNA myc, hisB
PON2B1	pon2 (flag) ORF in pTracerCMV
ZZB	stomatin ORF in pcDNA myc, hisB

2.1.9 Primers

PRIMER NAME	SEQUENCE (5´→3`)
SLP3Ex1up	GATTCACCGGAGAAACTGG
SLP3Ex1low	GGCTTTATCAGCTTGGATG
SLP3Ex4low	TTCGAAGATCTACTTTG
SLP3Ex5low	CAGGAACGTGGCCTGGTGAAC
SLP3Ex4up	GATCTTCGAACGGTTACTTG
GAPDH up	GGCAAATTCAACGGCACAGT
GAPDH low	AGATGGTGATGGGCTTCCC
SLP3 tar up	TCCCAGTCTGAAGACAACAGCG
SLP3 tar low	CTCTCACTTCCCAGGCACCAAC
NEO1	CCTGCGTGCAATCCATCTTGTTC
SLP3 probe up	CAGGGCCTGCAGAAGGAGGC
SLP3 probe low	GCAGTCCTCCTCTGTCTAGTCC
Pon2-cDNA up	CCGCACCATGGGCCGGATG
Pon2-cDNA low	CTAGTGCACTTGCATGCC
Pon1 in situ up	GCCAGCAGTGTCAGAGTTAG
Pon1 in situ low	GGCATCCAACCCAGAGG
Pon2 in situ up	CCGGATGGTGGCTCTG
Pon2 in situ low	GAGCTCGTGTTTAATTGTTTTCAG
Pon3 in situ up	CGCTGCCCTTGCTGGGAGCC
Pon3 in situ low	CACACTCTTGAGAAGTTCATG
Pon3-cDNA up	ATGGGGAAGCTTGTGGCGCTGACCTTG
Pon3-cDNA low	CTAGAGGTCACAGTACAGAGC

Primer and probe sets for quantitative real time PCR:
(all primer and probe sets were obtained from Applied Biosystems: Assay-On-Demands™ Gene Expression Products)

pon1: TaqMan Expression Assay Mm00599936
pon2: TaqMan Expression Assay Mm00447159
pon3: TaqMan Expression Assay Mm00447161
slp3: TaqMan Expression Assay Mm00467671
stomatin: TaqMan Expression Assay Mm00469130
gapdh: TaqMan Expression Assay 99999915_g1

2.1.10 Antibodies

ANTIBODY	SEQUENCE AND COMPANY
Anti-DIG antibody	Roche
BNC1_EC	polyclonal affinity-purified antibody against amino acids 382-396 of ASIC2a, KTS AKY LEK KFN KSE
M2 (anti-Flag) antibody	Eastman Kodak Company
PGP9.5	rabbit polyclonal, UltraClone Ltd, Wellow, UK
Secondary antibodies	Dianova, Hamburg
Stomatin 5.0	polyclonal affinity-purified antibody against amino acids 188-200 of stomatin, KVE RVE IKD VKL P

2.1.11 EST-clones

EST-CLONES	NAME AND COMPANY
PON1	IMAG O064721Q2 (RZPD, Berlin)
PON3	IMAG B128764 (RZPD, Berlin)

2.1.12 Consumables

PRODUCT	COMPANY
15ml and 50ml tubes	Falcon, Greiner
Cell culture dishes	Falcon
Centricon	Millipore
Coverslips	Roth
Dounce homogenize	Roth
Eppendorf tubes	Eppendorf
Hybond-N	Amersham
Micro spin columns	Amersham
MicroAmp Optical 96 well reaction plate	Applied Biosystems
Quartz cuvettes	Roth
Sterile filters	Nalgene, Millipore
Whatman filters	Schleicher & Schuell
X-ray films	Kodak

2.1.13 Animals

C57BL/6N mice were obtained from Charles River Breeding Laboratory, Inc., Wilmington, Massachusetts and kept in the animal house of the MDC until they were used for experiments.

Stomatin$^{-/-}$ mice were obtained from Narla Mohandas, Lawrence Berkeley Laboratory, Berkeley, USA. Animals were also bred in the animal house of the MDC.

SLP3$^{-/-}$ mice were generated in collaboration with Dieter Riethmacher, ZMNH, Hamburg, Germany. Animals were also bred in the animal house of the MDC.

SLP3$^{-/-}$/Stomatin$^{-/-}$ mice were generated by inter-crossing of SLP3$^{-/-}$ and Stomatin$^{-/-}$ mice. Animals were bred in the animal house of the MDC.

2.2 Methods

2.2.1 Molecular biology

Standard methods were performed according to Sambrock et al. (1989) and Asubel et al. (1997).

2.2.1.1 DNA cloning

Gel extraction

The QiaEx or the GeneClean Kit was used according to the manufacturer's instructions to extract DNA fragments from agarose gels.

Ligation

The final ligation volume was 10µl. 50ng of vector were used with the molar ratio of vector to insert being set at 1: 3.

50ng vector
3x insert
1µl 10x buffer
1µl T4-DNA-ligase
ad 10µl with H_2O

3µl of the ligation mixture was used for transformation of competent bacteria (*E.coli* Top10).

Electroporation

Electrocompetent bacteria were thawed on ice for 5–10min. 50 µl of bacterial suspension were mixed with 3µl of the ligation product. Immediately after the electroporation (U = 2.5kV; C = 25µF; R < 200Ω; t_{impuls} = 5 ms), 1ml of SOC-medium was added before transferring the suspension to an Eppendorf tube. Cells were incubated at 37°C for 1h. Subsequently 100µl of the cell suspension were

plated on an agar plate containing the appropriate selection antibiotic for the plasmid and grown at 37°C overnight.

Isolation of plasmid DNA from small amounts of bacteria (mini-prep)

Mini-preps were performed in accordance to the manufacturer's instructions using the Qiagen Plasmid Mini Kit. The final DNA pellet was dissolved in 50 µl H_2O or TE buffer with the concentration set to 1µg/µl and stored at 4°C.

Isolation of plasmid DNA from medium amounts of bacteria (midi-prep)

Midi-preps were performed in accordance to the manufacturer's instructions using the Qiagen Plasmid Midi Kit. The final DNA pellet was dissolved in H_2O or TE buffer with the concentration set to 1µg/µl and stored at –20°C.

Determining nucleic acid concentrations

Using quartz cuvettes to measure the OD_{260}, the nucleic acid concentration was calculated as follows:

DNA	OD_{260} x 50µM/ml
RNA	OD_{260} x 40µM/ml

Sequencing

DNA sequencing was done by InViTek, Berlin-Buch or Seqlab, Göttingen.

PCR (for SLP3 genotyping)

For genotyping $SLP3^{-/-}$ mice the following protocol was used:

1µl genomic tail DNA
2µl 10 x PCR buffer
1µl $MgCl_2$
0.4µl dNTPs (10mM)
0.2µl each 50µM primer (SLP3 tar up, SLP3 tar low, NEO1)
0.2µl Taq polymerase.
ad 20µl H_20

PCR cycling:

Initial denaturation:	94°C	7min
Annealing:	60°C	35sec
Extension:	72°C	2min
Denaturation:	94°C	30sec
Annealing	60°C	35sec
Extension:	72°C	2min
Cycles:	40	
Final extension:	72°C	5min
Storage:	4°C	

PCR reactions using Taq DNA polymerase

DNA was amplified by PCR using a reaction volume of 25µl:

0.5µl template
2.5µl 10 x PCR buffer
1.25µl $MgCl_2$
0.5µl dNTPs (10mM)
0.5µl primer 3´
0.5µl primer 5´
17.5µl H_20
0.25µl Taq DNA polymerase.

PCR cycling:

Initial denaturation:	94°C	2min
Denaturation:	94°C	30–60sec
Annealing	55 – 60°C	30–60sec
Extension:	72°C	30–60sec
Cycles:	25–35	
Final extension	72°C	2min
Storage:	4°C	

PCR products were loaded to an agarose gel, bands of the expected size were gel-extracted and where required cloned into the TA-cloning vector pGEM-T Easy, in accordance to the manufacturer's instructions. PCR fragments were analysed by restriction digest and/or sequencing.

PCR reactions using Pfu DNA polymerase

DNA was amplified by PCR using a reaction volume of 100µl:

0.5µl template (20-200ngDNA template)
10.0µl 10 x PCR buffer
1.25µl $MgCl_2$
0.8µl dNTPs (25mM each NTP)
2.5µl primer 3´ (100ng/µl)
2.5µl primer 5´ (100ng/µl)
2µl Pfu DNA Polymerase
81.2µl H_20

PCR cycling:

Initial denaturation:	94°C	2min
Denaturation:	94°C	45sec
Annealing	primer Tm-5°C	45sec
Extension:	72°C	1-2 min/kb of PCR target
Cycles:	25–35	
Final extension	72°C	10min
Storage:	4°C	

PCR products were loaded to an agarose gel, bands of the expected size were gel-extracted and cloned into mammalian expression vectors.

Computer analysis of DNA and protein sequences

The Lasergene software by DNAStar Inc. was used for the analysis of DNA and protein sequences. The NCBI server was used for online analysis.

2.2.1.2 In situ hybridisation

The riboprobes were prepared as described by Schaeren-Wiemers and Gerfin-Moser (1993).

1µg	linearised DNA template
10µl	5x reaction buffer
5µl	DTT
5µl	DIG RNA labeling mix (10x)
1µl	RNAse inhibitor
3µl	RNA Polymerase (SP6- or T7)

ad 50µl DEPC-H_2O (final reaction volume)

Incubating the reaction mixture 3h at 37°C, labelling reaction was stopped by adding 2µl DNAse for 15min at 37°C.
For better tissue penetration of the probe the reaction mixture was hydrolysed by adding two volumes of carbonate buffer (hydrolysis buffer) for 45min at 60°C. Adding an equal amount of neutralisation buffer stopped the reaction. Ethanol precipitation prior to resuspension of the probe in DEPC-H_2O was performed in order to purify the probe. The probe concentration was estimated by performing dot blot verification as described by Boehringer for non-radioactive *in situ* hybridisation. Probes were stored at –80°C.

Mouse DRGs were dissected and freshly frozen in Tissue-Tek on dry ice. 10 µm thick cryosections were cut and taken up on slides coated with 2% APES (aminopropylethylsilane). Sections were allowed to dry for 30 min and could be stored up to several weeks at –80°C.
Sections were fixed in 4% cold paraformaldehyde for 5min, washed 3x2min in cold PBS and then deacetylated for 10 min by placing the slides into a stirring solution of 2.3ml triethanolamine in 200ml H_2O. Then 500µl acetic anhydride was added dropwise. Afterwards sections were washed 3x2min in PBS at RT.
Prehybridisation was performed using a mixture of 50% formamide and 4xSSC. Slides were covered with hybridisation buffer (without probe) and left for 2-3 hours at RT. For the hybridisation solution, 150ng of DIG-labeled cRNA per ml

hybridisation buffer was added, denatured at 85°C for 5min and then immediately placed on ice. The hybridisation was performed in the humid box at 52°C to 58°C overnight.

Slides were shortly placed in 2xSSC at 67°C, washed 4 times in 2xSSC at 67°C for 10min, once in 2xSSC at 67°C for 45min, once in 0.1xSSC at 67°C for 1 h and finally once in 0.2xSSC at RT for 10min.

For detecting the labelled probe on DRG sections slides were incubated first in B1 solution for 10min at RT, then for 1h in freshly made B2 solution to block unspecific bindings of the DIG antibody before adding anti-DIG antibody diluted 1:1500 in B2 solution and incubated overnight at 4°C.

Sections were washed 4 times at RT in B1 for 30min each and then equilibrated in B3 for 3x10min. B4-substrate reaction was used for colour development in the dark for one to several hours. Adding PBS at pH 7.5 stopped the reaction. Sections were finally mounted in Aqua-Polymount.

2.2.1.3 Quantitative Real Time PCR

Isolation and cleaning of total RNA from different mouse tissues was performed in accordance to the manufacturer's guide using TRIZOL Reagent (Invitrogen). For the first strand cDNA synthesis the following protocol was used:

1µl random hexamer primers
2µg total RNA
1µg dNTPs (10mM each)
ad 12µl destilled water

The mixture was heated to 65°C for 5 min and quickly chilled on ice. The content was collected by brief centrifugation and the following components were added:

4µl 5x first-strand buffer
2µl 0.1M DTT
1µl RNaseOUT^{TM} (40U/µl)

The contents were gently mixed and incubated at 42°C for 2min. One microlitre Superscript II^{TM} reverse transcriptase was added and the reverse transcription reaction was performed at 42°C for 50min. The reaction was terminated by heat inactivation of the enzyme at 70°C for 15min.

TaqMan Gene Expression Assays obtained from Applied Biosystems were used for quantitative Real Time PCR amplification reactions in accordance to the manufacturer's instructions. One single reaction mixture contained 1µl 20x TaqMan Expression Assay (containing amplification primer and probe sets), 9µl cDNA (converted from140ng total RNA) and 10µl 2xTaqMan Universal Master Mix. All reactions were carried out in triplicates.

Quantitative data on the absolute copy number per 100ng of total RNA were calculated from the threshold cycle determined for known quantities of plasmids containing *stomatin* or *slp3* cDNA. In some cases amplification threshold cycles of the gene of interests were compared to threshold cycles of the housekeeping gene *gapdh*. Real Time PCR reactions were run using the Applied Biosystems PRISM 7700 Sequence Detection System.

2.2.1.4 Genomic Southern blot

Isolation of genomic DNA from mouse-tail biopsy

About half a centimetre of the tail tips were obtained from adult mice. To digest the tail it was incubated in 700µl Tail lysis buffer containing ProteinaseK (15mg/ml) overnight at 55°C in a shaker (850rpm). The reaction tube was cooled on ice for 10min. To precipitate proteins 300µl NaCl (6M) solution was added, the mixture was briefly vortexed and incubated for 30 min on ice before spinning down the samples in an Eppendorf centrifuge (10'/15,000 rpm) at 4˚C. One millilitre of the supernatant was transferred into a new reaction tube, after adding RNaseA (10mg/ml) the suspension was incubated for 15min at 37°C in a shaker. DNA was precipitated with 1ml Isopropanol and centrifuged at 14,000 rpm at 4°C in an Eppendorf centrifuge. The pellet was washed with 1ml 70% ethanol (cold) and centrifuged at 10'/13,000 rpm at 4˚C for 20min. The dry pellet was resuspended in 50µl 1xTE-buffer. Yields up to 1.5µg genomic DNA are usually obtained with this procedure.

Restriction Digest

5-10µg of genomic mouse-tail DNA were used for a ScaI (5U/µg DNA) digestion overnight at 37°C. DNA restriction fragments were separated by gel electrophoresis using a 0.7% agarose gel.

Southern blot

The EtBr-stained gel was placed in a glass dish, and shaken gently in the following series of solutions:

Depurination solution: 15 minutes

Denaturation solution: 30 minutes

Rinse briefly in sterile distilled water.

Neutralisation solution: 2 X 15 minutes

20X SSC: 2 X 15 minutes

The upward capillary DNA transfer blot was prepared as described by Sambrook and Russell (Cold Spring Harbour, New York, 2001). Putting the blotting stack together in the order specified and eliminating any air bubbles between the gel and the nylon filter the transfer was started by soaking blotter papers in 20X SSC. The blot was left for at least 4 hours or overnight.

Hybridisation

The wet blot was incubated in 5ml of pre-warmed ExpressHyb solution for 30min at 65°C.

For probe labelling, linearised and purified DNA was denatured at 95°C for 5min and then cooled on ice for 2 min. 10ng/ml ExpressHyb solution and 5µl P_{32} dCTPs were added to 50ng denatured DNA and filled to a total volume of 50µl with and H_2O. The mixture was incubated for 30min at 37°C. The probe was cleaned up using Micro spin columns accordingly to the manufacturer's instructions. The clean probe was denatured (5-10 min, 95°C) and put on ice for 5min. One microlitre of the probe was put on filter paper for testing the P_{32} incorporation in a beta counter.

The probe was added to 1ml of the hybridisation solution denatured again for 5-10 min at 95°C. Blot hybridisation was performed for 1h at 65°C.

The hybridisation solution then was removed from the blot and the membrane was washed 3x 10 min at RT in Wash solution I and 2x 20 min at 50°C in

Wash solution II. The membrane was dried on tissue paper and exposed overnight at -80°C on commercial X-ray films.

2.2.2 Cell cultures

2.2.2.1 Maintenance

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.

2.2.2.2 Transfection

HEK293 cells were transfected using the lipofectamine system as described in the manufacturer's instructions. Cells were cultured in ∅10cm culture dishes overnight or until they reached 70-90% confluence. Serum-free transfection medium (OPTI-MEM) containing 0.5µg plasmid DNA/ml medium was mixed with a 10% lipofectamine in OPTI-MEM medium. The transfection mixture was incubated at RT for 30min.
Cells were rinsed twice in OPTI-MEM medium. The transfection mixture was diluted 1:10 in OPTI-MEM medium and applied onto the cells for 5 hours under normal cell culturing conditions before the transfection medium was exchanged with HEK293 medium. Cells were grown for 36 to 72h at 37°C as mentioned above.

2.2.2.3 Cultivation of sensory neurones

Mouse DRGs were dissected and collected in a 1.5ml tube in PBS on ice. DRGs were washed once with PBS before incubation with 10µl Collagenase TypeIV in 1ml PBS at 37°C for 30 min. DRGs were centrifuged briefly (170 x g), the supernatant was removed and DRGs were incubated with 100µl 0.5% Typsin in 1ml PBS at 37°C for 30 min. The supernatant was removed and 1ml D-MEM/F12 medium was added. The suspension was passed through 1-2 different siliconised Pasteur pipettes to

dissociate them into single cells and centrifuged at 170 x g for 4min. The supernatant was removed and DRGs were resuspended in 1ml DRG medium. Cells were plated on poly-L-Ornithin and laminin coated coverslips (about 60-120µl of cell suspension per coverslip) to let the cells attach to the coverslip. After 4 hours additional 150µl of the DRG medium was added to the coverslips. Cells were grown for 24h at 37°C in a Steri-Cult 200 incubator.

2.2.3 Protein chemistry

2.2.3.1 Protein isolation

Medium was removed from the cells and passed through a centrifugal filter device (YM-30/YM-100 from Centriplus) by centrifugation at 3,000 x g for 30min at 4°C. Proteins then were concentrated by TCA protein precipitation prior to diluting in 5x Lämmli buffer. Probes were heated to 100°C for 5min and could be stored at –20°C until needed.

HEK293 cells were collected and quickly homogenised in homogenisation buffer on ice. Samples were centrifuged at 30,000 x g for 30min at 4°C. The supernatant containing soluble proteins was collected and the pellet homogenised in TX-100 protein extraction buffer. Samples were centrifuged at 30,000 x g for 30min at 4°C and supernatants containing the plasma membrane proteins again collected. Appropriate amounts of protein containing supernatants then were diluted with 5x Lämmli buffer and heated to 100°C for 5min. Probes were stored at –20°C until needed.

2.2.3.2 SDS-PAGE

Utilizing a SDS polyacrylamid gelelectrophoresis (SDS-PAGE) in a mini-apparatus (Mini Protean 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 protein bands was determined by comparing them to a molecular mass standard (BioRad).

2.2.3.3 Western blotting

Proteins were transferred from the SDS gel to a nitrocellulose membrane for 1h at 150V at 4°C in the Mini Transblot Apparature from BioRad.
Membranes were quickly rinsed with distilled H_2O and protein bands detected with a 3% Ponceau red solution. After washing twice with H_20, the membrane was transferred into WB blocking buffer for 1h at RT to quench unspecific bindings. Primary antibodies were usually diluted 1:500 in blocking buffer and incubated overnight at 4°C. Membranes were washed several times. The appropriate secondary antibody, which always was conjugated with horse radish peroxidase (HRP), was diluted 1:2000 in WB blocking buffer and applied to the membrane for 1h at RT. Protein bands were detected using SuperSignal® ULTRA chemiluminescent substrate from Pierce and visualized on Kodak Scientific Imaging Film X-Omat™ Blue XB-1.

2.2.3.4 Cleavage of GPI anchored proteins

Cultivated HEK293 cells were washed 2-3 times with PBS containing protease inhibitors (PI) from Sigma (Protease Inhibitor Cocktail 1:100). Cells were harvested in 500µl PBS+PI and centrifuged at 1,500 x g for 2min at 4°C. The supernatant was removed and the pellet was homogenized in 1ml PBS+PI using a Dounce homogeniser. The suspension was centrifuged at 100,000 x g for 15min at 4°C for collecting the plasma membranes.
Plasma membranes were suspended in 100µl 20mM Tris/HCl pH 7.5 to a protein concentration of 10mg/ml and incubated in 10-40 U/ml PI-PLC from *T. thuringiensis*

for 60min at 37°C by placing the reaction tube into a Test Tube Rotor (Snijders) for the cleavage of GPI anchored proteins. For the mock digestion PI-PLC was pre-incubated in 100µl 20mM Tris/HCl pH 7.5 supplemented with 3mM $ZnCl_2$ for 30 min at 4°C to inactivate the enzyme. Plasma membranes were then suspended in this buffer and treated as described above. Membranes were pelleted by centrifugation at 100,000 x g for 30 min and supernatants were analysed for PON2 release from the membrane using SDS gelelectrophoresis.

2.2.3.5 Immunocytochemistry

Immunostaining of cultivated neurones

Cultivated DRG neurones were fixed in 4% paraformaldehyde in PBS for 10 min and then washed with PBS. Next, neurones were permeabilised with 0.05% Triton X-100 in PBS for 5min and then washed with PBS. Non-specific binding was blocked by incubating the neurones in 3% normal goat serum in PBS for 30min. Cells were incubated with the primary antibodies (diluted 1:200 in 3% goat serum) overnight at 4°C. The staining was detected using Cy3-coupled secondary antibodies (dilution 1: 5,000, incubation for 1 h at RT, wash 3x with PBS. Cy3 light emission was captured with the XF22 filter (excitation 535nm, emission 605DF50, Omega Optical).

Immunostaining of tissue sections

Animals were anaesthetised with sodium urethane 0.1 mg/ml (0.1-0.5 ml or more) and perfused with 4% paraformaldehyde in 0.1M PBS, pH 7.4 and 4°C. Immediately after perfusion the skin was dissected and post-fixed in the perfusion fixative at 4°C for 4 hours.

Tissue was immersed in 25% sucrose in PBS for 1-3 days until the skin sank to the bottom of the sucrose solution. Fresh sucrose solution was replaced daily.

Skin sections were cut on a freezing microtome into 40 µm sections perpendicular to the skin surface.

Skin sections were pre-incubated in 1% serum albumin (BSA) and 0.3% Triton X-100 in TBS for 1h and incubated overnight at RT with PGP9.5 antibody 1: 2,000

diluted in TBS with 0.3% Triton X-100 and 5% normal goat serum. Skin sections were washed by rinsing slides in excess TBS for 30 min and incubated for 1 hour at room temperature with Cy-3-conjugated secondary antibodies diluted 1:800 in TBS containing 0.3% Triton X-100 and 5% normal goat serum.
Skin sections were washed twice in excess TBS for 30 min and then once with water before mounting them in Aqua-Polymount. Cy3 light emission was captured with the XF22 filter (excitation 535nm, emission 605DF50, Omega Optical).

2.2.4 Electrophysiology

The in vitro skin nerve preparation

Adult mice were sacrificed using CO_2 inhalation and the hair from the hindlimb was removed. The saphenous nerve up to the lumbosacral plexus and a piece of the innervated skin reaching from the toes up to halfway between the ankle and the knee were excised. To facilitate oxygenation of the tissue, the skin was placed corium-side up in an organ bath, where it was fixed with insect needles, and superfused with 32°C warm oxygen-saturated synthetic interstitial fluid (SIF) at a flow rate of 10ml/min. The saphenous nerve was pulled through a gap to the recording chamber and laid on top of a small mirror that served as the dissection plate. The aqueous solution in the recording chamber was overlaid by mineral oil in such a way that the interface of the two phases was located just below the surface of the mirror. Dumont's 55 forceps were used to desheath the nerve, carefully removing its surrounding epineurium, and to tease small filaments from the nerve so that the activity from single units could be recorded by placing the individual strands of the nerve onto the silver recording electrode installed in the chamber's wall.

Electrophysiological recordings were performed using the NeuroLog^{TM} system from Digitimer Ltd., which included a low-noise differential amplifier for recording from identified units. The recordings were visualized on a connected Tektronix TDS 220 two-channel digital real time oscilloscope, while simultaneously acquired on a PC by a PowerLab/4s converter from ADInstruments. The receptive fields (RF) of individual units were identified by manually probing the corium-side of the skin with

a mechanical search stimulus, a blunt-end glass rod (Koltzenburg et al., 1997). Individual units were characterised by the constant shape of the action potentials they fired in response to stimulation, as the shape of an action potential depends on the composition of voltage-gated ion channels expressed by each neuron.

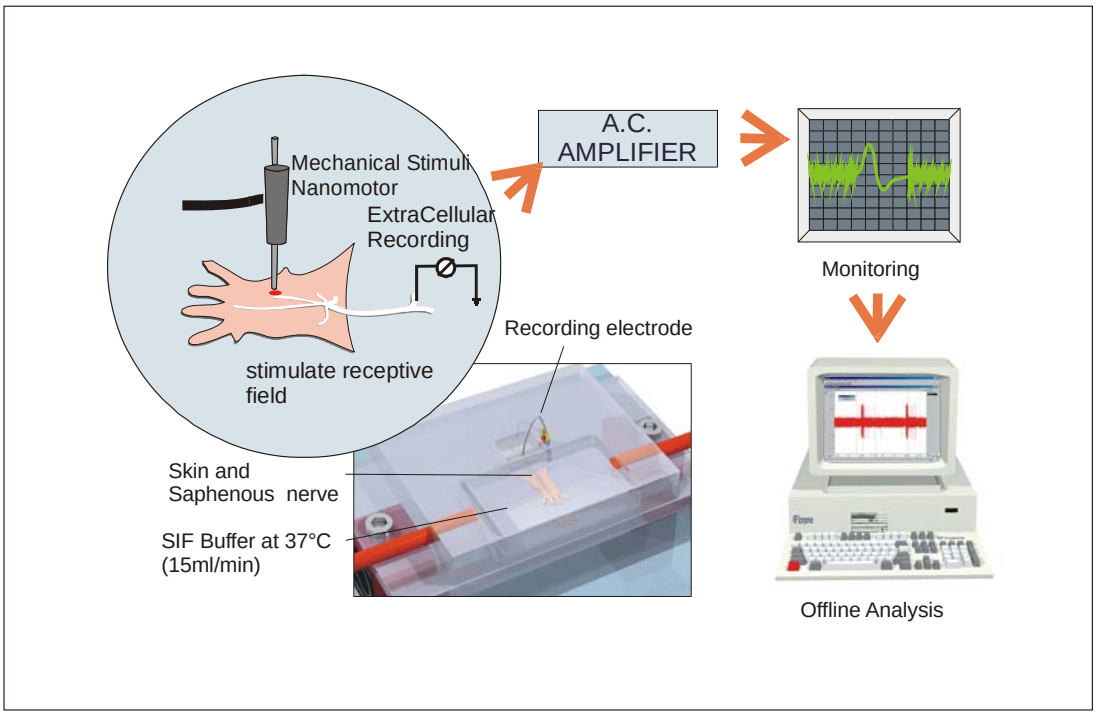

Figure 4 Overview of the skin-nerve preparation (courtesy of Paul Heppenstall)

Using the Chart v5.2 software from ADInstruments, the recorded action potentials of individual units were discriminated by their width (in µsec), height (in points) and conduction velocity. In this way, about 10-20 units could be characterised per experiment, so that their mechanical activation threshold, their conduction velocity, their stimulus-response function and spike frequency adaptation could be recorded.

Determining the mechanical activation threshold

The receptive field of a unit was identified using the glass rod. To determine the mechanical activation threshold, i.e. the minimal amount of force needed to elicit an action potential from that unit, calibrated von Frey hairs were used on the most sensitive spot of the RF. The von Frey hair nylon monofilaments were of various thickness, which accordingly produced bending forces from 0.4mN up to 32mN.

Determining the conduction velocity

A teflon-coated silver electrode with an un-insulated tip (∅ <0.5mm) was set on the most sensitive spot of the receptive field, and electrical pulses of constant current in square-wave pulses were used to excite the unit. The stimulus intensity was set at approximately two-times the threshold with a pulse duration of 50-500µsec depending on the afferent under investigation. The latency between the stimulus artefact and the resulting action potential was measured. To calculate the conduction velocity the distance between the stimulating and the recording electrode was divided by this latency. Units could thus be grouped into three classes: A-β fibres, which are thickly myelinated units, have a conduction velocity faster than 10m/sec, A-δ fibres are thinly myelinated units with a conduction velocity of 1-10m/sec, and non-myelinated C-fibres conduct slower than 1.2m/sec.

Electrical stimulation technique

The saphenous nerve was electrically stimulated with 1sec intervals of constant voltage pulses of 50 to 150µsec duration. In order to search for myelinated and non-myelinated fibres supramaximal electrical stimuli were applied to the nerve trunk using a tungsten microelectrode. When a single unit was isolated, its axon potential shape was monitored and its conduction velocity was determined prior to following the axon of the fibre progressively distal by electrical stimulation until the point was reached where the axon probably branches to form a RF in the skin. Subsequently, the putative innervation territory of the recorded fibre was explored using a mechanical search stimulus.

Measurement of mechanosensitivity

To determine the mechanical sensitivity of the sensory afferents of the saphenous nerve, the desired receptor was first identified by electrical stimulation. In order to determine the starting point, i.e. the mechanical threshold at which the unit could be reproducible excited, the nanomotor™ (Nanotechnik, Kleindieck) was placed over the most sensitive spot of the RF and moved slowly towards the skin.

In this way, the recording was started at threshold for each unit. For producing a stimulus response function an ascending series of increasing displacement stimuli was then applied to this spot (for detailed stimulus protocol see chapter 3.3.4). The elicited action potentials were acquired by PC and analysed using the Chart v5.2 software.

2.2.5 Electron microscopy

Mice were perfused with freshly prepared 4 % paraformaldehyde in 0.1 M phosphate buffer. Saphenous nerves were dissected and postfixed in 4% paraformaldehyde/ 2.5% glutaraldehyde in 0.1 M phosphate buffer for 3 days.

Following treatment with 1% OsO_4 for 2h nerves 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 1,600 x. 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 using the analySIS 3.2 software (Soft Imaging System, Münster, Germany).

2.2.6 Behavioural tests

2.2.6.1 Tactile acuity test

The MoTil system (TSE Systems, Germany) is a flexible system for studying the open field behaviour of small animals. A square base frame arranged at right angles to each other consists of two pairs of light sensor strips. Light sensors are sequentially scanned every 10msec to determine x-y- coordinates and therefore the exact location of the mouse during activity measurement. The following frame configurations were used: size 300 x 400mm; 6 x 12 infrared sensors, 28mm sensor distance. Mice were placed individually into the test cages in complete darkness. Movements of the animals were tracked by an automatic monitoring system (Actimot, TSE Systems, Germany).

Sandpaper-based test

Mice were tested twice. In the negative experiment, no tactile cues were inserted in any of the positions. In the positive experiment rough (roughness grade: 40) and smooth (opposite side of the sandpaper) sandpaper cues, which were 50 x 50mm in size, were placed randomly into two of four positions flush to the surface of the slightly roughened floor plate in the positive experiment. After a control period of 30min the animals were exposed to these tactile cues for 90min.

Grid-based test

Two equivalent positions (42 x 42mm in size) were symmetrically arranged and placed along the middle axis of the box. The tactile surface cue was inserted into one while the control insert was placed into the other position, thus both positions could directly be compared. Area quadrants including the surrounding area of the surface were defined, which were 63mm^2 in size. Mice were acclimatised to the test box for a period of 10min before one of the surface cues, which were Plexiglas inserts with varying grid spacing (blank, 250µm, 500µm, 750µm, 1mm, 2mm, 3mm, 6mm) was

introduced to the animals. Each mouse was tested on individual grid cues separately performing one experimental trial per day.

2.2.6.2 Rotorod test

The accelerating rotorod test (TSE Systems, Germany) is a behavioural test for motor coordination in mice. The apparatus consists of a 20cm-raised drum (∅ 10cm) split into 4 separate lanes. Adult mice were placed on the rotorod and the drum was turned on starting at an initial speed of 5rpm to allow mice to become acclimatised to the device. After this initial phase (30 sec) the rotorod was gradually accelerated from 5 to 60rpm over a course of 3min. The retention time, until a mouse fell from the device, was recorded. Each mouse was exposed to three experimental trials in succession. Experimental trials were repeated at the same time each day for 3 successive days. The mean time spent on the accelerating rod was calculated as a function of the test day.