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

Biacore 2000

Biometra TRIO-Thermoblock PCR machine

BioRad BioLogic (FPLC)

BioRad Mini Protean II

BioRad Mini Transblot Apparatur

BioRad PowerPac 300

Cryostat

Digitimer Ltd. NeuroLog Amplifier

Eppendorf thermomixer compact and 5436

EquiBio Easyject electroporation apparatus

Forma Scientific -86°C freezer

Forma Scientific Steri-Cult 200 incubator

GeneChip fluidics station 400

GeneChip Hybridisation oven

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

HiLoad 16/60 Superdex200 Pharmacia Biotech (Gelfiltration column)

HP GeneArray Scanner

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

Leitz DM IL inverted microscope

Mettler Toledo 320 pH meter

Mitsubishi Video Copy Processor

Moulinex householdmixer

Paraffin HiTrap Heparin column 5 ml, Amersham Pharmacia Biotech

Pharmacia Biotech Ultrospec 1000 spectrophotometer

Sartorius Iso 9001 scale

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

2.1.2 Chemicals and reagents

REAGENT AND CHEMICAL	COMPANY
Acetylated Bovine Serum Albumin (BSA) solution (50 mg/mL),	Invitrogen Life Technologies, P/N 15561-020
Anti-streptavidin antibody (goat), biotinylated	Vector Laboratories, P/N BA-0500
APES	Sigma-Aldrich
Control Oligo B2, 3 nM	Affymetrix, P/N 900301
DIG RNA labeling mix	Roche
Flag-peptide	Sigma-Aldrich
Gelatine	Sigma-Aldrich
Glygogen	Promega Corporation
Goat IgG, Reagent Grade	Sigma-Aldrich, P/N I 5256
Herring Sperm DNA	Promega Corporation P/N D1811
Lipofectamine	Gibco

MES Free Acid Monohydrate Ultra pure	Sigma-Aldrich, P/N M5287
MES Sodium Salt	Sigma-Aldrich, P/N M5057
Paraformaldehyde	Sigma-Aldrich
Phenol/chloroform/isoamyl alcohol	Roth
Protein molecular mass standard	BioRad
R-Phycoerythrin Streptavidin	Molecular Probes, P/N S-866
Tissue Tek	Miles, Elkhart, Ind. USA
Triton X-100	Sigma-Aldrich
Triton X-114	Sigma-aldrich
Trizol	Roth
Tween-20	Pierce Chemical, P/N 28320

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

2.1.3 Buffers and solutions

Affymetrix

BUFFER AND SOLUTION	COMPOSITION
10 mg/mL Goat IgG Stock	Resuspend 50 mg in 5 mL PBS
100x stock of control cRNA	BioB (150 pM), BioC (500 pM), BioD (2,5 nM), Cre (10 nM), Herring sperm DNA (0,1 mg/ml), 1x MES, NaCl 0,925 M, Tween 20 0,01%, dissolved in DEPC water
12 x SSPE	3 M NaCl, 0,2 M NaH_2PO_4, 0,02 M EDTA
12X MES Stock	1.22 M MES free acid monohydrate, 0.89 MES sodium salt pH between 6.5 and 6.7
2X Hybridization Buffer	(Final 1X concentration is 100 mM MES, 1 M [Na+], 20 mM EDTA, 0.01% Tween 20) Stored at 2-8°C, and shielded from light
2X Stain Buffer	Final 1X concentration: 100 mM MES, 1 M [Na+], 0.05% Tween 20
5X RNA Fragmentation Buffer	(200 mM Tris-acetate, pH 8.1, 500 mM KOAc, 150 mM MgOAc)
Antibody solution mix	1X MES stain Buffer acetylated BSA 2 mg/mL

	Normal Goat IgG 0.1 mg/mL biotinylated antibody 3 µg/mL
Buffers for the heparin chromatography	BufferA: 50 mM Tris at pH 7,5 , 20 mM NaCl BufferB: 50 mM Tris at pH 7,5, 2 M NaCl
Gel filtration running buffer	50 mM Tris pH 7,5, 200 mM NaCl
Hybridisation mix	Fragmented cRNA 0.05 µg/µL Control oligonucleotide B2 50 pM 100 x control cRNA cocktail (bioB, bioC, bioD, cre) 1,5; 5; 25 and 100 pM respectively Herring sperm DNA 0.1 mg/mL Acetylated BSA 0.5 mg/mL Hybridization buffer 1x Minimal final volume: 300 µl
Non-stringent wash buffer	6X SSPE, 0.01% Tween 20
SAPE solution mix	1X MES stain Buffer acetylated BSA 2 mg/mL Streptavidin-Phycoerythrin (SAPE) 10 µg/mL
Stringent Wash Buffer	100 mM MES, 0.1 M [Na+], 0.01% Tween 20

In situ hybridization

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 (add dropwise last)
B1-buffer	0.1M Tris 0.15M NaCl pH 7.5
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)
Buffers for heparin chromatography	BufferA: 50 mM Tris at pH 7,5 , 20 mM NaCl BufferB: 50 mM Tris at pH 7,5, 2 M NaCl
Gel filtration running buffer	50 mM Tris pH 7,5, 200 mM NaCl
Hybridization 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
Neutralization buffer	200mM Na-acetate 1% acetic acid pH 6.0

Others:

BUFFER AND SOLUTION	COMPOSITION
10 x MOPS	200mM MOPS 50mM Na-acetate 10mM EDTA pH 7.0
10x TBS	0.5M Tris/HCl, pH 7.9 1.5M NaCl
4% paraformaldehyde	4g paraformaldehyde in 100ml PBS pH 7.4
5x Lämmlibuffer	60mM Tris/HCl, pH 6.9 10% SDS 10% β-Mercaptoethanol

	50% Glycerol 1.5% Bromphenolblue
5x Second strand buffer	Invitrogen Life Technologies
Acetate buffer	Na-acetate 10 mM pH 5
Biacore coupling reagents	N-ethyl-N`-(3-dimethyl-amino-propyl)-carbodiimide hydrochloride (EDC) (75 mg/ml water) N-hydroxy-succinimide (NHS) (11,5 mg/ml water) Ethanolamine-HCl (1M pH 8,5)
Buffers for heparin chromatography	BufferA: 50 mM Tris at pH 7,5 , 20 mM NaCl BufferB: 50 mM Tris at pH 7,5, 2 M NaCl
Gel filtration running buffer	50 mM Tris pH 7,5, 200 mM NaCl
Glycerino-solution	65% glycerol 0.1M $MgSO_4$ 2.5mM Tris pH 7.5
HBS-EP	0,01 M HEPES pH 7,4, 0,15M NaCl, 3 mM EDTA, 0,005% surfactant P20 (Tween 20), steril filtered and degassed
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 $CaCl_2$ 5.5mM 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 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
TE-buffer	10mM Tris pH 8.0 1mM EDTA

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)
pharmaca only where mentioned

2.1.5 Enzymes and molecular weight markers

ENZYME	COMPANY
Restriktionendonucleases	Amersham, Roche
RNA-polymerases (T7, Sp6)	Promega
DNAse (RNAse-free)	Roche
Superscript II reverse transcriptase	Gibco
Taq-polymerase	Gibco
Expand High Fidelity PCR system	Roche
Reverse transcriptase AMV	Roche
SAP (shrimp alkaline phosphatase)	Amersham
T4-DNA-ligase	Promega
100bp and 1kb ladder	Gibco
E. coli DNA Polymerase I	Invitrogen Life Technologies

E. coli RNaseH,	Invitrogen Life Technologies
T4 DNA Polymerase,	Invitrogen Life Technologies

2.1.6 Kits

KIT	COMPANY
Qiagen Plasmid Midi Kit	Qiagen
TriZol	Gibco
GeneClean III Kit	Dianova
QiaEx Gel Extraction Kit	Qiagen
TA-cloning Kit (pCRII)	Invitrogen
TA-cloning Kit (pGEM-T Easy)	Promega
OligoTex mRNA Kit	Qiagen
RNeasy Mini Kit,	QIAGEN, P/N 74104
RNA Transcript Labeling Kit, Affymetrix, P/N 900182	Enzo
phaselock	eppendorf
In Situ Cell Death detection kit, Fluorescein (TUNEL-Kit)	Roche
Suppression subtractive hybridization kit	clontech
Advantage cDNA amplification kit	clontech
TaqMan PCR Core reagent kit	Roche

2.1.7 Plasmids

PLASMID	COMPANY
pBKS (bluescript)	Stratagene
pGEM-T Easy	Promega
pcDNA 3.0	Invitrogen
pTracer	Invitrogen
pcDNA 6B	Invitrogen

2.1.8 Primers

PRIMER NAME	SEQUENCE (5´-3`)
T7– (dT)24 Primer	5´ggccagtgaattgtaatacgactcactatagggaggcgg-$(dT)_{24}$ -3´, 100 pmol/µL
CaV3.1 forward	tcaccaccttcaacatccca
CaV3.1 reverse	cccgtactctgtgtctccagg
CaV3.1 probe	6-FAM-tgggcccttcagctccatgca-TAMRA
CaV3.2 forward	gtgtggcgcacaccatagc
CaV3.2 reverse	tagttcatggtgcccaagcc
CaV3.2 probe	6-FAM-ctgctgccagcctcaagctggc-TAMRA
CaV3.3 forward	tgtctgtcacatccttcgcaa
CaV3.3 reverse	ggttctgcagggcctggta
CaV3.3 probe	6-FAM-caagcgccgtgccctaggcc-TAMRA
Kv1.1 forward	caagggctcccgtagtgttc
Kv1.1 reverse	gccaccttctccggaatca
Kv1.1 probe	6-FAM-cgagagtgcagcttatcgccatttgct-TAMRA
TrkB forward	tttccgccaccttgacttg
TrkB reverse	atgtcgcaggagcacgtga
TrkB probe	6-FAM-ctgacctgatcctgacgggtaatccg-TAMRA
GAPDH forward	ggcaaattcaacggcacagt
GAPDH reverse	Agatggtgatgggcttccc
GAPDH probe	6-FAM-acgccgagaatgggaagcttgtcatc-TAMRA

2.1.9 Antibodies

ANTIBODY	SEQUENCE AND COMPANY
BNC1_EC	polyclonal affinity-purified antibody against amino acids 382-396 of BNC1a, KTS AKY LEK KFN KSE
αCaV3.2	polyclonal antibody against amino acids 320-336 of CaV3.2, EAYGQPQAEDGGAGRNA (generated by Neosystem, Strasbourg)
M2 (anti-Flag)	Eastman Kodak Company

secondary antibodies	Dianova, Hamburg
Agarose beads with flag antibody	Sigma-Aldrich

2.1.10 Consumables

PRODUCT	COMPANY
Hybond-N	Amersham
Eppendorff tubes	Eppendorff
Cell culture dishes	Falcon
15ml and 50ml tubes	Falcon, Greiner
X-ray films	Kodak
Whatman filters	Schleicher and Schuell
Sterile filters	Nalgene, Millipore
Centricon 30	Millipore
MicroAmp Optical 96 well reaction plate	Applied Biosystem

2.1.11 Animals

C57/Bl6 mice were obtained from Vomholtgard and kept in the animal house of the MDC until they were used for experiments.

BDNF heterogygote mice were obtained from Patrick Carroll, Martinsried, Germany, and were bred in the animal house of the MDC.

NT-4 knock out animals were obtained from Jackson Laboratories (USA) and bred in the animal house of the MDC (originally made by P. Ernfors (Ernfors et al., 1994)).

2.2 Methods

2.2.1 Molecular biology

Standard methods were performed according to Sambrock and colleagues (1989) and Asubel and colleagues (1997) and will not be described in detail (agarose gel electrophoresis, bacterial cultures, ethanol precipitations, restriction digests, etc.).

2.2.1.1 DNA cloning

Gel extraction

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

Ligation

The final reaction volume for ligations was 10µl. 100ng of vector were used with the molar ratio of vector to insert being set at 1:3.

100ng vector
3x insert
1µl 10x buffer
1µl T4-DNA-ligase
ad 10µl with H_2O

3 to 5µl of ligation were then used to transform competent bacteria.

Preparation of Top 10 electro-competent bacteria for transformation

Top 10 cells were grown over night in 20ml of LB-medium at 37°C. Further 200ml of LB-medium were then added and the bacteria was grown to an OD_{600} = 0.5–0.8. Cells were cooled on ice for 20 min then centrifuged at 4°C at 4000g for 15min. The pellet was washed in decreasing amounts of 10% glycerol/LB-medium (50ml, 25ml, 7ml). After the final centrifugation the pellet was resuspended in 0.5ml 10% glycerol. Cells were aliquoted on dry ice into 50µl aliquots and stored at –80°C until use.

Electroporation

Electrocompetent bacteria were thawed on ice for 5–10min. For a single transformation 1 aliquot (50 µl) of bacterial suspension was mixed in a cold transformation cuvette with 3–5µl of the ligation product, equaling approximately 1–10ng of circular plasmid DNA. Immediately after the electroporation (U = 2.5kV; C = 25µF; R < 200Ω; t_{impuls} = 5 ms) 1ml of SOC-medium was added and this suspension was transferred to an Eppendorff tube. The cells were incubated in a shaker at 37°C for 1h. Then 100µl were plated on an agar plate containing the appropriate selection antibiotic for the plasmid and grown at 37°C over night.

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

Bacteria were grown over night at 37°C in 3ml of LB-medium containing the appropriate selective antibiotic. 1.5ml of cells were transferred to an Eppendorff tube and centrifuged at 14000 rpm for 30sec. The pellet was resuspended in 200µl of solution I, 200µl of solution II were added and the tubes were inverted several times to mix the entire content. Finally 200µl of solution III were added and the tube was again inverted several times and incubated for 10min at room temperature (RT). After centrifugation the supernatant was discarded. The DNA in the pellet dried for 5–10min at RT and finally resuspended in 50µl H_2O.

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 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, a biotech company on the research campus in Berlin-Buch and Seqlab, a biotech company in Göttingen.

PCR

DNA was amplified by PCR using the Expand High Fidelity PCR system. The reaction volume was 50µl.

1µl template

5µl 10 x buffer

2.5µl $MgCl_2$

1µl dNTPs (10mM)

1µl primer 3´

1µl primer 5´

38µl H_20

0.5µl Taq polymerase.

The following PCR profile was used:

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

The PCR product was applied to an agarose gel and bands of the expected size were gel-extracted and cloned into a TA-cloning vector, pGEM-T, in accordance to the manufacturer's instructions. The PCR-fragments were then analyzed by restriction digest and/or sequencing.

DNA constructs

All PCR-products were cloned into pGEM-T Easy or pGEM-T vector.

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 hybridization

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

1µg linearized DNA template

10µl 5x reaction buffer

5µl DTT

5µl DIG RNA labeling mix

1µl RNAse inhibitor

3µl RNA-polymerase

add DEPC-H_2O to 50µl (final reaction volume)

Leaving the reaction for 3h at 37°C, it was stopped by adding 2µl DNAse for 15min at 37°C.

For better penetration of the tissue the probe was hydrolyzed by adding two volumes of carbonate buffer for 45min at 60°C. The reaction was stopped by adding an equal amount of hydrolysis-neutralization buffer. Then the probe was purified by ethanol precipitation and resuspended in 100µl DEPC-H_2O. The concentration of the probe was estimated by performing a dot blot as described in the manual from Boehringer for non-radioactive in situ hybridization. Probes were then stored at –80°C.

Tissue was dissected and freshly frozen in Tissue Tek with dry ice. 10 µm thick cryosections were cut and taken up on slides coated with 2% APES (Aminopropylethylsilane). Sections were allowed to dry for 1-3 hours and could be stored up to several weeks at –80°C.

Sections were fixed in 4% cold paraformaldehyde for 10min and then washed 3x2min in cold PBS. The tissue was then deacetylated by placing into a stirring solution of 2.3ml triethanolamine in 200ml H_2O and then adding 500µl acetic anhydride dropwise. The sections were left stirring in this for 10min at RT. They were then washed 3x2min in PBS at RT.

A humid box was prepared for the prehybridisation by pouring a mix of 50% formamide and 4xSSC on the bottom of the chamber. Slides were covered with 750µl of hybridization buffer without probe and left for 2-3 hours at RT. For the hybridization solution, 150ng of DIG-labeled cRNA per ml hybridization buffer were combined, denatured at 85°C for 5min and then immediately placed on ice. The prehybridization solution was gently poured off the slides and 200µl of hybridization solution were added to each. Slides were covered with pieces of parafilm, to ensure an even distribution of the probe. The hybridization was performed in the humid box at 52°C to 58°C over night.

Slides were placed in 2xSSC at 67°C so that the parafilm could lift off without force. They were then 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, once in 0.2xSSC at RT for 10min.

The detection was started by placing the slides in B1 solution for 10min at RT. They were then transferred to a humid box with H_2O on the bottom, where they were laid flat and then covered for 1h with freshly made up B2 solution to block unspecific bindings of the DIG antibody. 250µl

of anti-DIG antibody diluted 1:1500 in B2 solution were added to each slide and left to incubate over night at 4°C.

Sections were washed 4 times at RT in B1 for 30min each and then equilibrated in B3 for 3x10min. Each slide was covered with 50 µl B4 for the substrate reaction with the color development commencing in the dark for 1 to several hours. The reaction was stopped by immersing the slides in PBS (pH 7.5). Sections were finally mounted in Aqua polymount.

2.2.1.3 TUNEL staining

Principle

One major feature of apoptotic cells is the cleavage of genomic DNA in double stranded, low molecular weight DNA fragments as well as single strand breaks (nicks) in high molecular weight DNA. These single strand DNA breaks can be labelled by using the catalytic activity of the terminal deoxynucleotidyl transferase (TdT), which normally catalyzes polymerisation of nucleotides to the free 3' ending and therefore also incorporates fluorescein labelled nucleotides.

Paraffin sections

DRG from all spinal levels were dissected, placed into a tissue cassette and postfixed in 4% paraformaldehyde over night. After incubation in water for at least 3 hours, DRGs were subjected to increasing concentrations of ethanol (70%, 80%, 90%, 96%, 3x 100 %, each for 2 h). Next, DRG were incubated twice in xylol for 20 min, placed in paraffin (60°C) for 2 hours and again in paraffin (60°C) over night. Finally the tissue was embedded into cutting molds. After a hardening time of several hours, paraffin embedded tissue was cut in 5 µm sections and placed onto glass slides (positively charged superfrost plus). Each glass slide contained in average 80 DRG sections.

Staining procedure

Paraffin embedded tissue was dewaxed (reverse xylene/ethanol series like described above, each step 5 min) and washed in PBS. In order to permeate the tissue, sections were incubated in 0,1 % Triton X-100 (in 0,1 % sodium citrate) for 5 min. After washing 3 times with PBS, 100 µl of the TUNEL reaction mixture was pipetted onto the slides and covered with a cover slip in order to equally distribute the reaction mixture and avoid evaporation. The reaction was placed into a chamber and incubated at 37°C for 1 hour. In order to remove the cover slip without shearing off the sections, glass slides were placed in PBS so that the cover slips floated off. Finally, slides

were washed three times in PBS, mounted with aqua polymount and examined under microscope.

2.2.1.4 Affymetrix® oligonucleotide array technology

Introduction

Microarrays are arrays of cDNAs or oligonucleotides of known sequence that are printed or synthesized on a glass or silicon surface and is therefore a further development of the DNA hybridisation filters that have been used for some time. But the latest high density microarray technology has provided the scientific community with the ability to assay the transcript level of entire genomes simultanously in response to a variety of stimuli. Two principally different types of arrays are used, the cDNA arrays, where whole cDNAs are spotted onto the chip surface, and the oligonucleotide arrays, where selected representative oligonucleotides are directly sythesized on the chip surface. The Affymetrix technology uses the latter method.

Oligonucleotide array

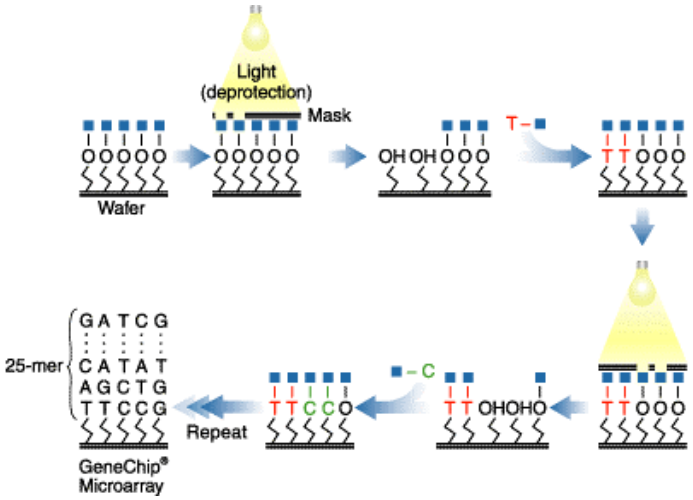

Figure 3: Scheme of the synthesis procedure of oligonucleotide arrays. The oligonucleotide arrays are generated by attaching synthetic linker molecules that have been modified with a photochemically removable protecting group to a solid support such as glass or silicon. By selective activation of protected dNTP using a photolithographic mask and UV light, the desired probes are synthesized (adapted from Affymetrix® company website).

Each gene on the array is represented by up to 20 different oligonucleotides spanning the entire length of the coding region of that gene. Moreover each of these oligonucleotides is paired with a

second mismatch oligonucleotide in which the central base in the sequence has been changed. The combination of probe redundancy and inclusion of a mismatched control sequence greatly reduces the rate of false positives obtained from this type of approach.

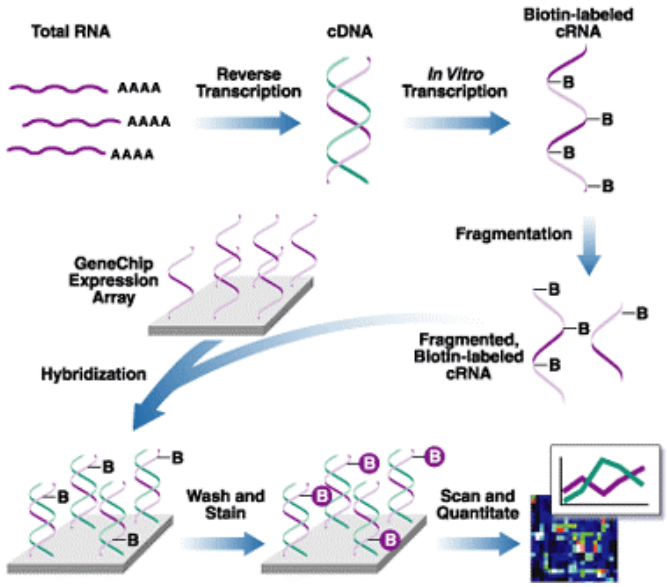

Figure 4: Overview of the Affymetrix experimental procedure (adapted from Affymetrix® company website)

For expression profiling-based comparisons, fluorescently labeled probes are generated from test and reference samples. For Affymetrix-based oligonucleotide arrays, fluorescent probes are generated by reverse transcribing total RNA using a primer containing a T7 polymerase site. Amplification and labeling of the cDNA probe is achieved by carrying out an in vitro transcription reaction in the presence of a biotinylated dNTP, resulting in the linear amplification of the cDNA population (approximately 30-100-fold). The biotin-labeled cRNA probe generated from test and reference samples is then hybridized to separate oligonucleotide arrays, followed by binding to a streptavidin-conjugated fluorescent marker. Detection of bound probe is achieved following laser excitation of the fluorescent marker and scanning of the resultant emission spectra using a scanning confocal laser microscope. The differential fluorescent signal is then represented as alterations in transcriptional profile between the two samples compared.

Dissection of DRG

After killing the mouse using CO_2 the whole spine was dissected. After laminectomy, DRGs from all spinal levels were dissected out and were frozen immediately on dry ice.

Isolation of RNA using Trizol and RNAeasy Kit

Isolation and cleaning of total RNA was performed in accordance to the manufacturers instructions. Briefly, frozen DRG were resuspended in 1 ml TRIZOL reagent and homogenized using a glass homogenizer. After adding 200 µl chloroform the homogenate was vortexed and centrifuged to separate the phases. The upper aeqous phase contained the RNA and was collected in a new tube. Next, total RNA was precipitated and washed with ethanol and resuspended in DEPC treated water. To ensure the purity of the total RNA, the RNAeasy kit (Qiagen) was used to clean the RNA once again. The recovery yield was always as good as 80-90 %.

Synthesis of double-stranded cDNA from total RNA

10-20 µg of total RNA from mouse DRG in maximal 10 µl DEPC-H_2O and

1 µl of T7-$(dT)_{24}$ primer (100 pmol/µl),

incubate at 70°C for 10 minutes for primer hybridisation and quick spin and put on ice.

add 4 µl First strand cDNA buffer

2 µl 0,1 M DTT

1 µl 10 mM dNTP mix

and incubate on 42°C for 2 minutes.

add 2-3 µl Superscript II Reverse transcriptase

and incubate on 42°C for 1 hour

For second strand synthesis the following reagents were added to the first strand reaction:

90 µl DEPC-treated water

30 µl 5x second strand reaction buffer

3 µl dNTP mix (10 mM)

2 µl DNA Ligase (5 U/µl)

4 µl DNA Polymerase I (10 U/µl)

1 µl RNAse H (2 U/µl)

to a final volume 150 µl.

The reaction mix was incubated at 16°C for 2 hours in a PCR thermal cycler. After the incubation time 2 µl T4 DNA polymerase (10U) was added followed by incubation at 16°C for 5 minutes. Finally, 10 µl 0,5 M EDTA was added to terminate the reaction.

The resulting double stranded cDNA was cleaned-up using a phenol-chloroform:isoamylalcohol extraction. After precipitation with 0,5 volumes of 7,5 M NH_4Ac and 2,5 volumes of absolute ethanol the pellet was washed once in 80% ethanol and dissolved in 12 µl DEPC-H_20.

In vitro transcription (IVT) (for expression profiling)

Biotin-labelled cRNA for hybridisation on Affymetrix gene chips was generated using the IVT kit from Enzo. The procedure was performed according to the users manual.

0,5 µg of dsDNA (or 5 µl of ds DNA)
2 µl 10x transcription buffer
2 µl Biotin-Mix
2µl RNAse Inhibitor Mix
2µl T7 Polymerase Mix

add to 20 µl with DEPC-H_20

The reaction mix was incubated for 4-5 hours and stopped by adding 1 µl of 500 mM EDTA. The resulting biotin-labelled cRNA was then purified by using the RNAeasy Kit from Qiagen. The volume of the elution buffer was 40 µl.

1 µl of the IVT product was kept for gel electrophoresis analysis.

Fragmentation of cRNA

The fragmenting of the cRNA ensures a better hybridisation to the oligonucleotides on the chip. About 20 µg cRNA was mixed with 5x fragmentation buffer and added with water to a final volume of 40 µl. After incubation at 94°C for 35 minutes the mix was put on ice. 1 µl of the fragmented cRNA was analyzed on a agarose gel along side with the unfragmented control for proper size distribution. The fragmentation procedure should produce a distribution of RNA fragment sizes from approximately 35 to 200 bases.

Preparation of the hybridisation cocktail

Following reagents were mixed for a standard array

20 µg fragmented cRNA
4 µl control oligonucleotide B2 (5 nM)
4 µl 100x control cRNA cocktail (BioB, BioC, BioD, cre)
4 µl herring sperm DNA (10 mg/ml)
10 µl acetylated BSA (20 mg/ml)
200 µl 2x MES Hybridisation buffer

add to final volume of 400 µl with DEPC-H_2O and keep at –20°C until the hybridisation procedure.

Hybridisation

The hybridisation cocktail was heated to 95°C on a heat block for 5 min. Meanwhile, the array was filled with 200 µl 1x MES hybridisation buffer and incubated for 10 min at 45°C with rotation. The denatured hybridisation cocktail was equilibrated by incubation at 45°C for 5 minutes and centrifuged at maximum speed to pellet insoluble matter. Finally the 1xMES hybridisation buffer in the array was replaced by the hybridisation cocktail and put into a rotisserie box in 45°C oven for 16 hours.

Analysis of expression data

All data analysis were performed using the Affymetrix software. Detailed protocols are extensively documentated in the manual and several other publications. The Affymetrix MGU74vs2 mouse genome chip set contains about 36 000 putative genes, 20 % known and 80% EST sequences. Briefly, each gene is represented by up to 20 perfect match (PM) and mismatch controls (MM). Decision about presence or absence of the selected gene and calculation of the abundance of the RNA was done by a algorithm developed by Affymetrix, which mainly used the PM:MM ratio, background subtraction and discarding of outliers. For my analysis, the average difference, the absolute call (present, absent, marginal) and the fold change (in comparison analysis) were taken into consideration.

2.2.1.5 Subtractive cDNA hybridisation

Subtractive cDNA hybridisation is a well established method to identify and isolate differentially regulated gene transcripts. In general, the cDNA from one group is subtracted from the other group, resulting in a subtracted library enriched with genes that are differentially expressed in the latter population. Several subtraction methods are described in the literature. We used a PCR-based subtraction technique called suppression subtractive hybridisation SSH (Diatchenko et al., 1996). Briefly, common genes of both groups hybridize with each other, whereas differentially expressed genes do not hybridize and are available for PCR amplification. A normalisation step also allows equal representation of high and low abundant genes so that the resulting subtracted library is not biased for high abundant genes. For this experiment a SSH kit (PCR Select, Clontech) was used and the procedure was performed according to the users manual. The subtraction protocol requires 1-5 µg of polyA+ - RNA as starting material. This

amount was not available, therefore a PCR-based cDNA amplification kit (PCR Smart, Clontech) was used. 1 µg total RNA was used to perform the ploy dT-based first-strand synthesis followed by a PCR amplification. The resulting dsDNA was directly used as starting material for the subtraction procedure. The resulting subtrated library consisting of gene fragments of an average length of 600 bp can be cloned into common plasmid vectors and sequenced. We used an alternative method to identify the genes in the subtracted library. The subtraction procedure results in the subtracted library and an unsubtracted control library, which contains all genes of both RNA pools. Gene fragments of both, the subtracted and the unsubtracted control library were in-vitro transcribed to biotin-labeled cRNA and hybridized onto Affymetrix oligonucleotide arrays.

2.2.1.6 Quantitative PCR

Total RNA was extracted from DRG of NT4 WT, NT4 ko (4 week) and NT4 ko (12 week) mice and cDNA was made. In preliminary experiments using serial dilutions of cDNA, the proper dilution of the cDNA was determined. 1 µl of this prediluted cDNA was used for the Real-time PCR.

CaV3.1: cDNA 1:4 diluted

CaV3.2: cDNA 1:4 diluted

CaV3.3: cDNA 1:4 diluted

Kv1.1: cDNA 1:40 diluted

TrkB: cDNA 1:20 diluted

GAPDH: cDNA 1:80 diluted

The primers and probes for all genes of interest were designed with PRIMER-EXPRESS software (Applied Biosystems). The PCR mix contained 1 µl cDNA template, 1× Taqman buffer, 5 mM $MgCl_2$, 200 µM each of dATP, dCTP, and dGTP, 400 µM dUTP, 1.25 units AmpliTaq Gold DNA polymerase, 0.25 units AmpErase UNG, primer and probe (300 nM each of primers and 200 nM probe for Kv1.1, TrkB and GAPDH, and 600 nM primers and 600 nM probe for the three T-type channels) in a total volume of 25 µl. Standard reactions were performed by using an Applied Biosystems PRISM 7700 Sequence Detection System. All experiments were carried out in triplicate.

2.2.2 Cell culture

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. To passage the cells, they were washed with PBS, which was pipetted up and down to dissociate the cells from the culture dish. They were then 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.

2.2.2.2 Transfection

HEK293 cells were transfected using the lipofectamine system as described in the manufacturer's instructions. Cells were cultured in ∅10cm culture dishes over night until they reached 70-90% confluency. The DNA was prepared prior to the transfection. Serum-free medium containing 0.5µg pDNA/µl medium was combined with a 10% Lipofectamin/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, i.e. 500µg/ml geneticin in normal cell medium. Selection medium was renewed every other day. After 1-2 weeks only transfected cells survived, having formed colonies in the culture dish. Using cloning rings, small glass rings, colonies were isolated from the surrounding medium and dissociated. They were then plated and grown separately for further analysis. Separated colonies were grown for 1 week before small samples of was taken for staining with either the antibody BNC1_EC or M2 to detect the expression of recombinant BNC1b or the mutated BNC1a. Staining of all cells was required to be even, thereby indicating their origin from a single cell. Clones that showed the highest immufluorescence were tested in Western blots for their expression and the transfected cells derived from the clone with the highest expression was kept as a permanent cell line.

2.2.3 Protein chemistry

2.2.3.1 Antibodies

Generation of the polyclonal antibody against CaV3.2 was carried out by a company (Neosytem, strasbourg). CaV3.2 shows high homology to the other two t-type calcium channels. The antibody was generated against a peptide that was specific for CaV3.2 and located in a putative extracellular region. Furthermore, the antibody should recognize both mouse and rat protein and should lack any recognition sites for protein modifications like phosphorylation or glycolysation sites (peptide: EAYGQPQAEDGGAGRNA). The peptide was synthesized and conjugated at the N-terminus to the carrier KLH (E at N-terminus for coupling reaction). The rabbit was immunized three times, once every 14 days. Blood samples were taken prior to the immunization and 11 days after the second and third injection and analyzed for their antibody titer using an ELISA test. Animals were sacrificed 4 weeks after a fourth injection with the antigen and a final bleeding was carried out. The serum containing IgG antibodies were purified using affinity adsorbtion column chromatography following a standard protocol as reported by Scopes (Scopes, 1982)

2.2.3.2 Protein isolation

HEK293 cells were collected in 1.5ml Eppendorf tubes and quickly homogenized in 1ml homogenization buffer on ice. Samples were centrifuged at 30 000g for 30min at 4°C. The supernatant was transferred and the pellet homogenized in 500µl of protein extraction buffer. Samples were centrifuged at 30 000g for 30min at 4°C. Supernatants were transferred into new tubes, diluted with 5x Lämmli buffer and heated to 100°C for 5min. Probes could then be stored at –20°C until needed.

2.2.3.3 SDS-polyacrylamide-gel electrophoresis

Utilizing a SDS-polyacrylamide-gel electrophoresis (SDS-PAGE) in a miniapparatus, the 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).

2.2.3.4 Western blotting

Proteins were transferred from the SDS gel to a nitrocellulose membrane for 1h at 150V at approximately 4°C 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 twice with H_20, the membrane was transferred into WB blocking buffer for 1h at RT to quench unspecific bindings. Primary antibodies, anti-flag was diluted 1:500 or anti BNC1_EC at 1:1 000, were diluted in 2ml blocking buffer and incubated over night at 4°C. Membranes were washed several times and the appropriate secondary antibodies (goat-anti-mouse for the monoclonal and goat-anti-rabbit for polyclonal antibody) conjugated with horse radish peroxidase (HRP) diluted 1:20 000 in WB blocking buffer were applied 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.

2.2.3.5 Immunocytochemistry

Cultured neurons were fixed with 4% paraformaldehyde in PBS for 10 min and then washed with PBS. Next, neurons were permeabilized with 0.05% Triton X-100 in PBS for 5min and then washed with PBS. Non specific binding was blocked by incubating the neurons in 3% normal goat serum in PBS for 30min. Then the blot was incubated with the primary antibodies (diluted 1:200 in 3% goat serum) over night at 4°C. The staining was detected using Cy2 and/or Cy3-coupled secondary antibodies (dilution:1:5000, incubation for 1 h at RT, wash 3x with PBS. Cy3 light emission was captured with the XF22 filter (excitation 535nm, emission 605DF50) and Cy2 and FITC were detected using the XF33 filter (excitation 485nm, emission 530DF30, Omega Optical).

2.2.3.6 Phase separation using TX-114 (Bordier, 1981)

Tissue (sciatic nerve, DRG, brain or skin from mouse hind limb) was homogenized using a regular household mixer (moulinex) at 4°C in Tris/Cl, pH 8,1, 1% Triton X-114, 10 mM EDTA. No other protease inhibitors were used. The suspension was clarified by centrifugation at 12 000 rpm at 4°C. The clarified lysate is subjected to temperature-induced phase separation by incubation at 37°C for 5 min, after which the detergent phase („the pellet“) was collected by centrifugation for 10 min at 20°C (1800 g = 3300 rpm). The pellet was resuspended again to the previous volume in 0,1 M Tris/HCl, pH8,1 at 4°C and mixed to restore a single phase. Another incubation at 37°C for 5 min and centrifugation at 20°C for 10 min separates the phases. The resulting lower detergent phase is enriched with GPI-anchored and transmembrane proteins. To further enrich for GPI-anchored proteins, the detergent phase was resuspended in Tris/Cl Buffer again, warmed up to 37°C. After phase separation, PIPLC (5 U/ml) was added to the suspension

and the suspension was incubated rigourously shaken at 37°C for 2 hours. PIPLC cleaves specifically GPI-anchored proteins which are bound to the detergent vesicles and releases them into the aqueous phase. After centrifugation at 20°C both pellet and supernatant was examined for protease activity using protease gels.

2.2.3.7 Protease Gels

A modified version of the protocol for non reducing SDS PAGE was used (Pittman, 1985). Samples were electrophoresed in SDS buffer (Lämmli, 1970) at 9 mA and 4°C in a polyacrylamide gel containinig 7,3% polyacrylamide, 0,31 % bisacrylamide and 0,11 % gelatine. Then the electrophoresis gel was incubated 3 x in 2% TX-100 in order to extract the SDS and restore the native conformation of most proteins. Gels were then incubated in 20 mM Tri/HCl, PH 8,0 at 37°C for 24 hours. During this time proteases in the gel digest the gelatine matrix. Finally gels were fixed and stained with 0,2 % coomassie. Proteases were visualized as clear bands against a background of coomassie staining.

2.2.3.8 FPLC

For heparin chromatography, the protein probe was centrifuged for 20 min at 3000*g*, and the supernatant (50 ml) was passed over a heparin column (HiTrap Heparin 5 ml, Amersham Pharmacia Biotech) using a fast protein liquid chromatography system (FPLC, BioLogic, Biorad). Heparin-binding components were eluted using a salt gradient: of 20 mM to 2M NaCl in 50 mM Tris and collected in fractions of 1 ml.

2.2.3.9 Gelfiltration

Protein fractions from heparin column were loaded onto a gelfiltration column (HiLoad 16/60 Superdex200, Pharmacia Biotech) and fractions collected. Running buffer was 50 mM Tris pH 7,5, 200 mM NaCl.

2.2.3.10 Binding studies using the Biacore™System

The Biacore system makes use of surface plasmon resonance (SPR), a physical phenomenon which arises when light is reflected under certain conditions from a conducting film at the interface between two media of different refractive index (for detailed description: Jönsson et al., 1991). In Biacore systems the media are the sample and the glass of the sensor chip, and the conducting film is a thin layer of gold on the chip surface. SPR causes a reduction in the intensity of reflected light at a specific angle of reflection. This angle varies with the refractive index of the sample, which again changes when molecules bind to the sensor surface. This

causes the SPR response, which is proportional to the amount of bound molecules. By plotting the response against the time, one can follow the binding or dissociation kinetics visualized as a sensorgram. Binding of soluble ligands to immobilized protein is measured in arbitrary units (RU). There is a linear relationship between the mass of the protein bound to the immobilized protein and the RU observed (1000 RU = 1 ng/mm^2 bound protein). The CM5 sensor chips that were used in our experiments contained a glass slide covered with a layer of gold. This gold layer again is covered with a layer of carboxymethylated dextran which provides a matrix for covalent attachment of proteins and other molecules as well as providing a hydrophilic environment where the interaction will take place.

First, recombinant BNC1a and BNC1b were purified using the permanent cell lines, that were generated by Sabrina McIlwrath (BNC1a-pCDNA3 in HEK293) and myself (BNC1b-pTracer in HEK293). Cells were grown to 70-90% confluency, washed three times with PBS and harvested in harvest medium containing 1% Triton X-100. In order to extract membranous proteins this mix was homogenized carefully in a glass/teflon homogenizer and stirred for 3 hours at 4°C. Recombinant BNC1a and BNC1b have a FLAG-tag at the N-terminus and therefore we used a affinity column with immobilized anti-FLAG antibody to purify the proteins. The column volume was adjusted to 2 ml, and 5ml of the extraction mix was applied onto the column twice. After the column was washed with 10 column volumes of TBS/0,1% TX-100, buffer containing flag-peptide was used to elute the bound protein from the column. The following step gradient was used for elution:

1.) 1 ml of 25µg/ml TBS/0,1% TX-100

2.) 1 ml of 50 µg/ml

3.) 1 ml of 100 µg/ml

This was done twice, resulting in 6 ml of eluate. Using Centricon centrifugation columns with a cut-off of 30 kDa (millipore) the eluate was concentrated to a volume of 500 µl. 50 µl of this solution was diluted with 2 volumes of buffer and used for the immobilization reaction.

For immobilisation of recombinant BNC1a onto Biacore biochips, the protein was coupled at concentrations of 200 µg/ml in 0.1 M sodium acetate, pH 5.0, by the aminocoupling procedure according to the manufacturer's protocol. Briefly, the carboxymethyl groups on the chip surface are first activated by injecting the EDC/NHS mixture. After a few washing steps, the probe (in this case recombinant BNC1a) can be injected into the chip, so that the protein can be coupled onto the chip surface. The immobilisation progress can be monitored by the sensorgram. After additional washing steps, the Biochip was ready for binding experiments. Samples were diluted 1:4 in HBS buffer and injected at a constant flow rate of 8-10 µl/sec for 15 min and binding

kinetics were monitored with the Biacore software. The the protein was washed off using the same buffer HBS and dissociation kinetics was recorded. For recovery of the chip, tightly bound ligands were washed of using 0,1 M HCl. Protein chips were kept dry at 4°C.

2.2.4 In vitro skin nerve preparation (Koltzenburg et al., 1997)

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 half-way 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 flowrate 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 with 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.

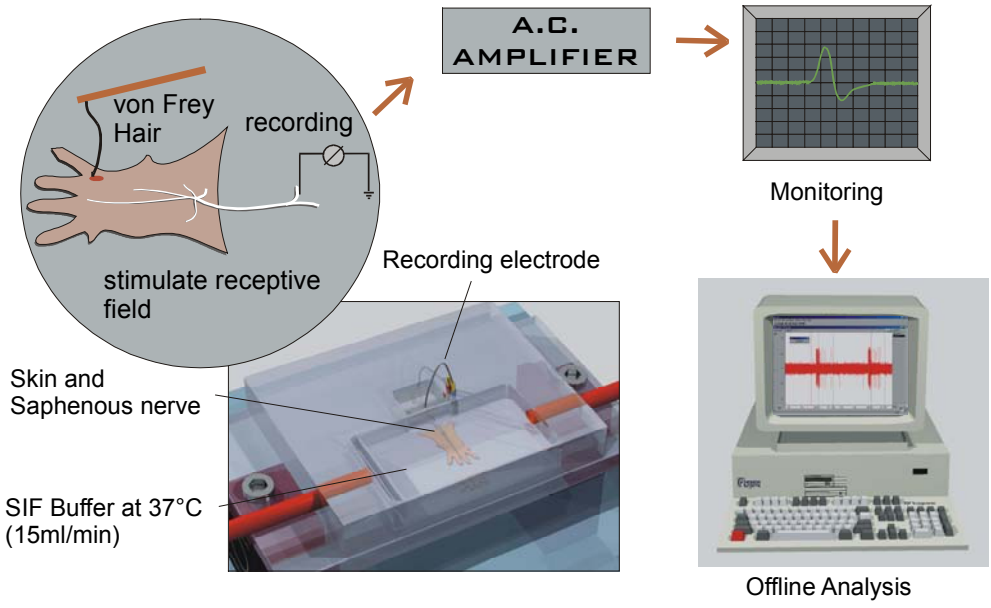

Figure 5: Overview of the skin-nerve preparation (courtesy of Paul A. Heppenstall)

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 with a Tektronix TDS 220 two channel digital real time oscilloscope

while simultaneously acquired on a PC with 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-ended glass rod (Koltzenburg et al., 1997). Individual units were characterized by the constant shape of the action potentials they fired in response to stimulation. Using the Chart v3.6.3/s 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 characterized 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

Using the glass rod, the receptive field of a unit was identified. 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 hairs, 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 uninsulated tip (∅ <0.5mm) was set on the most sensitive spot of the receptive field, electrically stimulating it with of constant current in square-wave pulses. 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 fibers, which are thickly myelinated units, have a conduction velocity faster than 10m/sec, A-δ fibers are thinly myelinated units with a conduction velocity of 1-10m/sec, and unmyelinated C-fibers conduct slower than 1m/sec.

Measurement of mechanosensitivity

To determine the mechanical sensitivity of the sensory neurons of the saphenous nerve, we first identified the desired receptor by using electrical stimulation and displaced the skin of the receptive field using a stepping motor (Nanotechnik, Kleindieck). The so called nanomotor™ has a constant forward driving velocity and can be operated by a computer software, making the

mechanical stimuli highly accurate and reproducible. The probe was placed over the most sensitive spot of the receptive field and moved slowly towards the skin. Once action potentials were fired by the unit, the probe was moved back by 9,6µm (corresponds 8 steps of 1,2 µm) to a fictive "zero", so that a 9,6µm indentation from there elicited action potentials from the unit. In this way, the stimulus-response function was started at threshold for each unit.
The RF was thus indented by 153µm for 2sec and then set to "zero" for 30sec. This was repeated every minute over 10 minutes. The elicited action potentials were aqcuired by PC and analyzed using the Chart v3.6.3/s software.

Spike frequency adaptation test

Using the so called spike frequency adaptation one can examine the ability of the receptor to follow electrical pulses of different frequencies and is a test of electrical excitability. We first identified a receptive field of a AM or D-hair mechanoreceptor and placed the stimulating electrode onto the most sensitive spot of the receptive field. Trains of suprathreshold current injections at increasing frequencies (2, 4, 10, 20, 40, 100, 200, 300, 400, 500, 600, 700, 800, 900 Hz) were applied for a short period (at least 10 stimuli) with 30 sec recovery time between the trains. For the analysis, the number of responding action potentials to the first 10 stimuli were considered.