3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Laboratory equipment

1.5/2.0 ml safe lock tubes (Eppendorf), 0.2 ml PCR tubes (Alpha)
Adhesive film (AB gene-Cat.No. AB-0588)
ADInstruments PowerLab/4s
Aerator
Amplifier (Neurolog system, Digitimer Ltd.)
Anaesthetic agent: 100 µl contained 10 µl Rompon 2 %, 90 µl Ketavet 100 mg/ml (Pfizer).
Applied Biosystems PRISM 7700 Sequence Detection System
Automated dispenser (Pipetboy)
BDK Laminar Flow Hood
BioRad Mini Transblot Apparatus
BioRad PowerPac 300
Centrifuge (Roth, Eppendorf or Heraeus)
CRO: Tektronix TDS 1002
Cryostat
Digitimer Ltd. NeuroLog Amplifier
Dissection Lamp (Leica)
Dissection Microscope (LeicaWILD M28, Leica MZ6)
Dissection microscope (Leica MZ 6)
Dissection scissors (FST-Cat No. 15000-10, 15024-10)
Electrical stimulator (Neurolog)
Electrophoretic apparatus (In house made, AB gene or OWI Separation Inc.)
Electroporation cuvette (Eurogentec)
Eppendorf Thermomixer Compact and 5436
EquiBio Easyject Electroporation Apparatus
Forceps (FST-Cat. No. 11295-20, 11295-51)
Forma Scientific -80°C Freezer
Forma Scientific Steri-Cult 200 Incubator
General glass wares (Schott, Fischer brand or Simax)
Gilson Minipuls 3 Peristaltic Pump
Glass rod for prodding (In house made)
Hamamatsu Digital Camera C4742-95
Harnischmacher Labortechnik DNA Electrophoresis Chambers
Heating block, 96 well (MJ Research PTC 200)
Heidolph Duomax 1030 and Promax 1020 Shakers
Heraeus Biofuge 13
Heraeus Megafuge 1.0

Heraeus Biofuge 15R
Herolab E.A.S.Y 429K Digital Camera
Herolab UVT 2035 Transilluminator 302nm
Hybridization plates (Multiscreen TM- MAHVS4510)
Ikamag Reo Magnetic Stirrer
Incubator (Unitherm Hybridizationsoven or Thermo Hybaid)
Insect needles
Julabo MP and Medingen Waterbaths
Kleindieck Nanomotor
Lamp (Leica KL750)
Leica DM 500B with Metamorph software.
Leica DM RBE Upright Fluorescence Light Microscope
Leica KL 750 Fiber Optic Light Source
Leica MS5 dissecting microscope
Magnetic Stand (Kanetec)
Mettler Toledo 320 pH Meter
Microscope for set up (Leica MS5)
MoTil System (TSE Systems)
Needles (Sterican)
PCR machine (MJ Research PTC 200 with 48 or 96 well heating blocks or PerkinElmer Gene Amp PCR system 2400)
PCR product Purification Plates (Qiagen -Cat. No. 1019567)
Pharmacia Biotech Ultrospec 1000 Spectrophotometer
Pipettes and multichannel pipettes (Eppendorf or Biohit)
Plastic 15 and 50 ml tubes (Falcon)
Plastic Boxes (Roth)
Power Pack (Bio-Rad)
Quartz cuvette (Hellma -Cat. No. 105.202-QS)
Reagent reservoirs (Matrix Tech Corporation-Cat. No. 8093 or Costar 50 ml)
Recording electrode
Refrigerator (Liebherr)
Scientific Industries Vortex-Genie 2
Shaker (Heidolph or from New Brunswick Scientific)
Shaving machine (Philips Philishave)
Slide holders (Roth)
Slides and coverslips (Roth or Menzel-Gläser)
Spectrophotomer (Pharmacia Biotech)
Stratagene UV Stratalinker 2400
Syringes (Braun)
Tektronix TDS 220 Two Channel Digital Real Time Oscilloscope
Tray with rubber (In house made)

Uni Equip Unitherm Hybridization oven 6/12

Vacuum manifold (Millipore Multiscreen Resist Vaccum Manifold-Cat. No. MAVM0960R)

Water bath (Lauda, Medingen or Julabo)

Weigh machine (Sartorius)

3.1.2 Chemicals and enzymes

Agarose	Gibco Life Technologies, Karlsruhe
Ampicillin	Sigma, Deisenhofen
Bacto Agar	Difco, Becton Dickinson, Sparks, MD; USA
Bacto trypton	Difco, Becton Dickinson, Sparks, MD; USA
Bacto yeast extract	Difco, Becton Dickinson, Sparks, MD; USA
Bromophenol blue	Sigma
Chloramphinicol	Sigma
dATP, dCTP, dGTP, dTTP sodium salt	Amersham Pharmacia
DNA molecular weight standards	MBI Fermentas
DNA Pfu-Polymerase	Promega
DNA Taq-Polymerase	PE Applied Biosystems
Dnase (RQ1 Dnase)	Promega
DTT	Serva
E. coli DNA Polymerase I	Invitrogen
E. coli RNase H	Invitrogen
EDTA (Titriplex® III)	Merck
Ethidium Bromide, 1% solution	Fluka
Formaldehyde	Sigma-Aldrich
Formamide	Sigma-Aldrich
Glucose	Merck
Glycerol	Merck
HEPES	Sigma
IPTG	Amersham Biosciences
Isopropanol	Merck
β-Mercapto-ethanol	Sigma
Paraformaldehyde	Sigma-Aldrich
Phenol	Carl Roth
Restriction Endonucleases	New England Biolabs; Amersham, Roche
Reverse Transcriptase (Superscript II)	Gibco Life Technologies
RNA polymerases (T7, SP6)	Invitrogen, Promega
SAP (Shrimp Alkaline Phosphatase)	Roche
Sigmacote	Sigma
T4 DNA Polymerase	Invitrogen
T4-DNA Ligase	Promega

Taq-Polymerase	Invitrogen
TEMED	Sigma-Aldrich
Tris	Merck
Triton X-100	Sigma-Aldrich
TRIzol	Invitrogen
Tween-20	Pierce Chemical, P/N 28320

Salts, acids, bases and solvents not mentioned in the table were *pro analysi* quality from Merck (Darmstadt) or Sigma (Deisenhofen).

3.1.3 Kits

DIG RNA Labeling Kit (SP6/ T7)	Roche
GeneClean III Kit	Dianova
PCR purification kit	Qiagen, Hilden; Roche, Germany
Plasmid Mini- und Maxipreparation	Qiagen, Hilden
QiaEx Gel Extraction Kit	Qiagen
Qiagen Plasmid Midi Kit	Qiagen
Qiagen Plasmid Mini Kit	Qiagen
RNeasy Kit	Qiagen, Hilden
TA-Cloning Kit (pGEM-T Easy and pGEM-T)	Promega, Mannheim
RNA Transcript Labeling Kit, Affymetrix, P/N 900182	Enzo
Phaselock	Eppendorf
Suppression subtractive hybridization kit	Clontech
Advantage 2 DNA polymerase kit	Clontech
BD PCR-Select™ cDNA subtraction Kit	Clontech (BD Biosciences)
PCR-Select Differential Screening Kit	Clontech (BD Biosciences)
T7Select®10-3 OrientExpress™ cDNA Cloning System, Random Primer	Novagen

3.1.4 Consumables

Eppendorf tubes	Eppendorf
Hybond-N	Amersham Biosciences
PCR plates, 96-well	Abgene, Surrey, UK (AB gene-Cat.No. AB-0800)
Polypropylene tubes 15 ml and 50 ml, sterile	Greiner Labortechnik GmbH, Frickenhausen
Size standard, DNA marker PhiX174 DNA/BsuRI	MBI Fermentas, Germany

Sterile filters	Cellulose Nitrate membrane, pore size 0.2 µm, Nalgene, Hamburg

3.1.5 Cells and antibodies

DH5α chemocompetent cells	Promega, Mannheim
BLT-5615	Novagen, Germany
Primary antibodies	Molecular Probes, Invitrogen
	R&D systems, USA
	Santa Cruz Biotechnology Inc., CA, USA
Secondary antibodies	Molecular Probes, Invitrogen
	Sigma, USA
	Roche, Germany

3.1.6 Solutions, buffers and media

10x TBE buffer(1 litre)	108 g Tris base
	55 g Boric acid
	40 ml 0.5 M EDTA, pH 8.0
10x TBS	100 mM Tris
	9% NaCl
	pH adjusted to 7.4 with HCl
50x TAE buffer (1 litre)	242 g Tris base
	57.1 ml Glacial acetic acid
	100 ml 0.5 M EDTA pH 8.0
Antibiotics (1000x)	50 mg/ml Ampicillin, 50 mg/ml Carbenicillin, Streptomycin 50mg/ml
Blocking buffer	1x TBS
	3% BSA
LB agar	LB medium
	15 g/l Bacto agar
LB medium (1 litre)	Bacto-tryptone 10 g
	Bacto-yeast extract 5g
	NaCl 10 g
	pH adjusted to 7.2; autoclaved
OR2	82.5 m M NaCl
	2.5 mM KCl
	1 mM $MgCl_2$

	1 mM $CaCl_2$ 1 mM Na_2HPO_4 5 mM HEPES, pH 7.4 pH adjusted with NAOH to 7.8
OR2:L15	50% OR2 50% L15 0.4% BSA Penicillin/streptomycin 1X
PBS	Ready to use PBS Dulbecco w/o Mg^{2+} , Ca^{2+}
Ringer soultion	82.5 m M NaCl 2 mM KCl 1 mM $MgCl_2$ 1 mM $CaCl_2$ 10 mM HEPES, pH 7.4
SDS-PAGE running buffer	25 mM Tris-HCl pH 8.3 190 mM Glycine 0.1% SDS
SIF (Synthetic Interstitial Fluid)	2 mM $CaCl_2$ 5.5 mM glucose 10 mM HEPES 3.5 mM KCl 0.7 mM $MgSO_4$ 123 mM NaCl 1.5 mM NaH_2PO_4 9.5 mM Na-gluconate 7.4 mM saccharose set to pH 8.4 with 10 N NaOH; carbogene used for oxygenation during the experiment will bring it to pH 7.4
Solution I (resuspension buffer for bacterial culture)	100 mM Tris HCl, pH 7.5 10 mM EDTA 400 μg/ml RNAse I
Solution II (alkaline lysis of bacterial culture)	1 M NaOH 5.3% (w/v) SDS
Solution III (neutralisation buffer)	3 M Potassium-acetate, pH 5.5
Tail lysis buffer	0.05 M Tris (pH 8) 0.1 M EDTA (pH 8)

	0.1 M NaCl
	1% SDS
TBST	TBS
	0.05% (v/v) Tween 20
TE buffer	10 mM Tris-HCl
	1 mM EDTA; pH 8.0
Water, molecular biology grade	AccuGENE, Cat. No. 51200

Glycerol stocks plasmids clones were prepared in 25% glycerol and stored at –80°C.

3.1.7 Software

• BLAST	NCBI, Bethesda, USA
• AnalySIS 3.2 Software	Soft Imaging System, Münster, Germany
• ActiMot Analysis Software	TSE Systems, Germany
• Chart v5.2 for Windows	ADI instruments
• Lasergene Software	DNAStar Inc.
• 7000 System Software	Applied Biosystems
• Affymetrix analysis softwares (GCOS, DMT, etc)	Affymetrix

3.2 METHODS

3.2.1 General Molecular Biology Methods:

Standard molecular biology methods were used according to Sambrook and colleagues (1989) and Asubel and colleagues (1997) and are not described here in detail. All centrifugations were performed at 4°C unless otherwise mentioned.

3.2.1.1 Isolation of plasmid DNA

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

3.2.1.2 Transformation

3.2.1.2.1 Electroporation

Electrocompetent bacteria were thawed on ice for 5–10 min. For a single transformation 1 aliquot (50 µl) of bacterial suspension was mixed in a cold transformation cuvette with 3–5 µl of the ligation product, equalling approximately 1–10 ng of circular plasmid DNA. Immediately after the electroporation (U = 2.5 kV; C = 25 µF; R < 200 Ω; $t_{impulse}$ = 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 1 h. Then 100 µl were plated on an agar plate containing the appropriate selection antibiotic for the plasmid and grown at 37°C over night.

3.2.1.2.2 Chemical transformation

Chemocompetent bacteria were thawn on ice for 5-10 min. For a single transformation 1 aliquot (50 µl) of bacterial suspension was mixed with 3-5 µl of ligation product in an Eppendorff tube, equalling approximately 1-10 ng of circular plasmid DNA. Immediately after heat-shock (42°C for 30 s) the tube was placed on ice for 2 min. 950 µl of LB-medium was added and this suspension was incubated with shaking at 37°C for 1 h. After incubation, 100 µl were plated on an agar plate containing the appropriate selection antibiotic for the plasmid and incubated at 37°C over night.

3.2.2 Part I: Development of High throughput in situ hybridization:

3.2.2.1 Animals:

BDNF+/- animals denoted as “BDNF mutants” and wild type littermates denoted as “Control” were used for the experiments. The generation of the BDNF mice was originally described in Korte et al. (1995), and in Carroll et al., (1998). All genotyping was carried out using standard PCR protocols from genomic DNA isolated from tail biopsies.

3.2.2.2 Genotyping:

Genotyping for all animals were carried out using genomic DNA from isolated from tail biopsies by standard protocol (Sambrook et al.,). PCR reaction mix was made according to the manual (Taq Polymerase, Invitrogen – Cat. No.10342-020). 2 µl of template was taken in a reaction volume of 20 µl. For BDNF genotyping 2 primer pairs were used for + allele and – allele in separate reactions. For the detection of + allele, primer pair BD-2A (GTG TCT ATC

CTT ATG AAT CGC), BKO-1 (ATA AGG ACG CGG ACT TGT ACA) and for the detection of – allele BD-2A and 3' Neo (GAT TCG CAG CGC ATC GCC TT) were used. PCR condition consisted the steps, one 94^{o}C for 7 min, one 60^{o}C for 35 sec, one 72^{o}C for 2 min, X cycles of steps consisting one 94^{o}C for 30 sec, one Y^{o}C for 35 sec and one 72^{o}C for 2 min. Final extension is done at 72^{o}C for 7 min. For BDNF + allele detection (product size 470 bp), X=30 and Y=60^{o}C and for – allele detection (product size 420 bp), X=40 and Y=58.5^{o}C were used.

3.2.2.3 Construction of enriched cDNA library and plating.

The library was made by Shin JB. In brief, DRGs from all spinal levels from 2 mice heterozygote for BDNF and 2 WT mice were taken (on average 40 DRGs per animal) and RNA extracted using the Trizol method. This library was made by using a kit by Clontech (BD PCR-Select™ cDNA Subtraction Kit - Cat No. 637401) based on the principle of suppression subtractive hybridization (Diatchenko, et al., 1996, Gurskaya, et al., 1996). The PCR fragments were cloned in pGEM-T vector (Promega) and transformed in TOP10 cells (Invitrogen). The transformants were sent to RZPD for the colonies to be picked and cultured in 384 well plates. 2688 colonies picked and cultured in 384 well plates were re-plated onto 96 well plates for screening.

3.2.2.4 Whole mount *in situ* hybridization:

Whole mount *in situ* protocol on adult DRGs is based on a protocol developed for whole mount embryonic spinal cord (Carroll, et al., 2001) with some modifications. Mice were sacrificed by CO_2 inhalation and the spinal column was separated. DRGs from all the levels were dissected out into fresh 4% PFA prepared in calcium, magnesium free PBT (PBS + 0.1%(v/v) Tween-20) and fixed either by incubating at room temperature for 2 h or at 4^{0}C overnight with gentle rocking. After washing (10 min, with gentle rocking at RT other wise specified) twice with PBT, they are progressively dehydrated for storage by washing in 25%, 50%, 75% PBT-Ethanol and twice with 100% ethanol successively. These could be stored in 100% ethanol for several weeks. Fixed DRGs kept in 100% ethanol were distributed into 96 well plates (3 or 4/ well) with a porous bottom (Multiscreen™ - MAHVS4510), which can hold solutions and these solutions could be removed by suction. All the following steps were done in these plates. For the hybridization protocol, DRGs stored in 100% ethanol, were re-hydrated progressively by washing with 75%, 50%, 25% PBT-ethanol and twice with PBT successively. Proteinase K (10 µg/ml final concentration) treatment is done in PBT for 45 min at room temperature (RT). After washing in PBT, post-fixation is carried out by 4% PFA,

0.1% gluteraldehyde (available as 25% solution in water, Serva-23115) in PBT for 20 min at RT. Before changing to hybridization buffer HB - 1.3% v/v 20X SSC (3 M Sodium chloride, 0.3 M Sodium citrate, pH 7.0), 50% formamide, 2% Tween 20, 0.5% CHAPS, 5 mM EDTA, and 50 µg/ml yeast RNA), washing is carried out twice to remove the fixatives, rinsing with PBT-Hybridization buffer (1:1) and again washing with HB. Pre-hybridization is done in hybridization buffer for more than one hour at 70°C. For hybridization, DNase treated, purified and diluted labelled probe is added to the HB and incubated overnight at 70°C. Unhybridized probes were removed by rinsing and washing twice with pre-warmed HB at 70°C. Prior to the RNAse treatment, washing was done with pre-warmed TST-HB at 70°C and with TST (0.5 M NaCl, 10 mM Tris, pH 7.5, and 0.1% Tween 20) at RT. RNase treatment is done (10 µg/ml in TST) at 37°C for 30 min. To inactivate RNase, washed once with TST, once with HB at RT and incubated twice in HB at 65°C for 30 min. Washing with pre-warmed MABT-HB (1:1) at RT, rinsing twice with MABT (0.1 M maleate, 0.15 M NaCl, and 0.1% Tween 20, pH 7.5), washing once with MABT and incubating in MABT at RT for 1 hr is done before blocking step. Blocking is done by 20% goat serum in MABT for 1.5 h. After blocking, DRGs are incubated overnight at 4°C with anti-DIG antibody coupled with Alkaline phosphatase (Boehringer Cat No. 1093274, 1:2000 dilution) in the presence of 2% goat serum in MABT. Excess antibodies are washed off by rinsing thrice with MABT and incubating thrice in MABT at RT for 1 h each with gentle agitation. For the color development, DRGs are washed twice with B3 (0.1 M Tris, pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$, and 0.1% Tween 20, 5 mM Levamisole) and incubated with the substrate NBT/BCIP (Roche, Cat. No. 1681451, 1.5% v/v) in B3 with out shaking till optimum color development occurs (kept at RT and during the night kept at 4°C) with intermittent changes of the solution after every 12 h. After the color development, staining is stopped by washing three times with PBT at RT and post fixed in 4% PFA in PBT for 30 min at RT. DRGs are soaked in 4% PFA, 75% glycerol mix for two to three hours and mounted with the same solution, and observed under light microscope (Leica) using Metamorph® software.

3.2.2.4.1 Synthesis of ribo probes:

One µl of the bacterial culture is taken as the template in 20 µl volume of reaction mix prepared according to the instruction manual (Taq Polymerase, Invitrogen – Cat. No. 10342-020). M13 Forward (GTT TTC CCA GTC ACG AC) and M13 Reverse (CAG GAA ACA GCT ATG AC) primers were taken to amplify the inserts along with the flanking T7 and SP6 polymerase binding sites. The PCR conditions used were one step of 94^{o}C for 3 min, 30 cycles of 3 steps - 94^{o}C for 45 sec, 56^{o}C for 30 sec, 72 ^{o}C for 90 sec and a final extension step

72°C for 10 min in a 96 well heating block (MJ Research PTC 200). 96 well PCR plates (AB gene-Cat.No. AB-0800) were used with an adhesive film (AB gene-Cat.No. AB-0588) to prevent the evaporation. 1µl of PCR product is used for making each sense and anti-sense ribo probes using T7 (Invitrogen – Cat.No. 18033-019, Promega – Cat. No. P-2075) and SP6 (Invitrogen – Cat.No. 18018-010, Promega – Cat. No. P-1085) RNA polymerases. Reaction mix was made with DIG RNA labeling Mix (Roche-Cat. No. 11 277 073 910) according to the manuals. After incubating at 37°C for 2 h, DNase I (RQ1 RNase free DNase – Promega, Cat. No. M610A) treatment (3U/well) was done at the same temperature for 15 min to eliminate the DNA template. After inactivating the DNase I, the volume of the reaction mix is made up to 50 µl and the probe is purified using PCR product Purification Plates (Qiagen-Cat. No. 1019567) according to the maufacturer's instructions. The probes were eluted with 50 µl of RNase, DNase free molecular biology grade water (AccuGENE, Cat. No. 51200). 1:600 dilution of T7 made probes and 1:450 dilution of SP6 made probes were used for hybridization.

3.2.2.5 qPCR experiments:

3.2.2.5.1 RNA isolation:

Qiagen RNeasy mini kit (Qiagen, Germany, Cat. No. 79254) was used as per the instructions in the manual with on column DNAse I (Qiagen, Germany Cat. No. 74104) digestion. In case of RNA isolation from cells, after lysis, the lysate was passed through 20G needle for 10 times and then passed through QIAshredder (Qiagen, Germany Cat. No. 79654) before subsequent steps according to the instruction manual.

3.2.2.5.2 First strand synthesis:

1000 ng or 2000 ng of total RNA was used as a template for the reverse transcription in 50µl volume. The same protocol was followed as described in Superscript II RNAse H^- (Invitrogen) manual with random hexamer primers. The optional steps of addition of RNAse inhibitor and RNAse H treatment were avoided.

3.2.2.5.3 Experimental set up

Template for the Real-time pcr was 1:100 or 1:200 dilution of the first strand reaction mix for (1000 ng and 2000 ng respectively). All the samples to be compared were processed in parallel and 3 or 4 independent experiments were performed. Two different protocols were

used for the qPCR experiments. In the first protocol, primer probe mix was obtained from Applied Biosystems (Darmstadt, Germany) or the primers and probes were ordered separately (Biotez, Berlin, Germany). The separately ordered probes had FAM as the fluorescent dye in the 5' position and Tamra in the 3'. Probes in the primer probe mix from Applied Biosystems had FAM and a nonfluorescent quencher in the 5' and 3' positions respectively. All the primers used were HPLC purified. Primer pair and probe for some genes were designed using the primer designing software (Primer Express 1.5) in the machine. For others inventoried assays (Applied Biosystems) were ordered. The second qPCR protocol we followed was based on mouse Universal Probe Library (Roche Diagnostics, Germany, Cat. No. 04683641001[4683641]). HPLC purified primers were ordered from either Invitrogen (Germany) or TIB Molbiol (Berlin, Germany). PCR reaction was done with 96 well plates (MicroAmp; Applied Biosystems; Cat No. N801-0560) covered with optical adhesive covers (Applied Biosystems; Cat No. 4313663). The instruments used were ABI PRISM 7700 Sequence Detection System or ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Reaction conditions were as follows: one step of 50°C for 2 min, one 95°C for 10 min, and 40 cycles of 95°C for 15 s denaturation and 59°C for 1 min annealing and extension. The reference gene used for the experiments was prenylcysteine oxidase. It was chosen because no change was observed in three independent Affymetrix gene chip experiments (Anirudhan, Shin and Lewin, unpublished observations) to compare the expression level of transcripts in BDNF mutant and control DRGs. Reaction master mix was made with Real-time PCR Master mix (TaqMan Universal PCR Master Mix; Applied Biosystems; Cat. No. 4318157), probes, primers and template according to the instruction manuals. 5 µl or 9 µl volume of the templates were used in 20 µl reaction volume. All reactions were performed as pentaplates. The threshold cycle number for product detection (ΔC_T value) was used to calculate the relative expression levels.

3.2.2.6 DRG neuron primary culture:

Cultures of DRG neurons were prepared as described previously with slight modifications (Mannsfeldt et al., 1999). DRGs of all spinal levels from 3 adult mice aged between 8–10 weeks were removed and collected in sterile PBS. DRGs were incubated in 1 mg/ml collagenase IV. (Sigma, Germany) for 30 min at 37°C and then in 0.1% trypsin (Sigma) for 30 min at 37°C. After removal of trypsin, serum containing medium (10% heat-inactivated horse serum (Gibco, Germany, Cat. No. 26050-070), 20 mM glutamine, 0.8% sucrose, 100 units penicillin/100 µg streptomycin) was added to the DRGs. Tissue was dissociated by passing them through a fire-polished bore of a siliconized Pasteur pipette and cells were

plated on polyornithine/laminin-coated 6 cm plastic plates (polyL-lysine; Sigma; 500 μg/ml; and laminin; Gibco, Germany; 20 μg/ml). Cells were divided equally into 3 parts and were incubated in serum-containing medium with no BDNF and two different concentrations of BDNF (100 ng/ml and 500 ng/ml recombinant human BDNF from Peprotech, cat. No. 450-02) at 37°C, 5% CO_2 for 16 to 18 h (overnight).

3.2.3 PART II: Electrophysiological, anatomical, molecular biological and behavioral characterization of NT3+/-//NT4-/- double mutants (NT34 mutants).

3.2.3.1 Animals:

NT3+/- and NT4-/- mice were obtained from Jackson laboratories (Bar Harbor, Maine). They were bred to get NT3+/-// NT4-/- mice denoted as "NT34 mutants". Appropriate control strain was obtained from the Jackson laboratories (129S3, Catalog # 002448). They will be denoted as "Control". Generation of NT3+/- and NT4-/- was originally described in Ernfors, et al., (1994) and Liu., et al, (1995).

3.2.3.2 Genotyping:

Genotyping for all animals were carried out using genomic DNA isolated from tail biopsies using standard protocol. Reaction mix was made according to the manual (Taq Polymerase, Invitrogen – Cat. No. 10342-020). 2 μl of template was taken in a reaction volume of 20 μl. For NT3, three primers viz., oIMR-130 (CCT GGC TTC TTT ACA TCT CG), oMIR-131 (TGG AGG ATT ATG TGG GCA AC) and oIMR-132 (GGG AAC TTC CTG ACT AGG GG) in PCR condition 1. For NT4 four primers viz., oIMR-13 (CTT GGG TGG AGA GGC TAT TC), oIMR-14 (AGG TGA GAT GAC AGG AGA TC), oIMR-602 (GTC AGT ACT TCT TCG AGA CGC G) and oIMR-603 (AGC TGT GTC GAT CCG AAT CC) were used in PCR condition 2. PCR condition 1 consisted the steps, one 94^{o}C for 7 min, one 60^{o}C for 35 sec, one 72^{o}C for 2 min, 40 cycles of steps consisting one 94^{o}C for 30 sec, one 56^{o}C for 35 sec and one 72^{o}C for 2 min. For NT3, expected product sizes were 250 bp (for + allele) and 350 bp (for – allele). PCR condition 2 consisted the steps, one 94^{o}C for 3 min, 37 cycles of steps consisting one 94^{o}C for 45 sec, one 58^{o}C for 45 sec and one 72^{o}C for 1 min. Final extension is done at 72^{o}C for 7 min. For NT4, expected product size for + allele is 197 bp and for – allele is 280 bp.

3.2.3.3 Electrophysiology:

In vitro skin nerve preparation (Fig.07) based on the method described in Koltzenburg, et al., (1997) was used with modifications. In brief the mice were sacrificed after CO_2 inhalation. The hair from the lower limb was shaved off and the skin from toe to halfway between ankle and the knee innervated by saphenous nerve was dissected out along with the nerve, sufficient enough for *in vitro* recording. It was fixed with insect needles in the bigger chamber of the bath in a corium side up position to facilitate the oxygenation by carbogenated warm ($32^oC \pm 0.5^oC$) SIF infused in the bath at the rate of 15 ml/min. The nerve is pulled into a smaller recording chamber on to a glass mirror platform where it is "cleaned" to remove other tissues attached to the nerve, desheathed to remove the epineurium and teasing is performed without damaging the desheathed nerve to obtain filaments for single unit recordings. Mineral oil is filled in the smaller recording chamber where the recording silver electrode is in contact with the nerve and the level is maintained in such a way that the buffer does not comes in contact with the recording electrode as well as the nerve. 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.

3.2.3.3.1 Searching for the receptive field and classification of units:

To classify an afferent unit, mechanical force threshold of activation, conduction velocity and the shape of the action potential were taken into consideration. Using a blunt glass rod the corium side of the skin is prodded to find out the receptive field of a neuron. After locating the most sensitive spot, mechanical threshold is determined using calibrated von Frey hairs (nylon monofilaments with varying thickness and length by which bending forces from 0.4 mN to 32 mN are produced), which imparts only a specific amount of force irrespective of the hand movement towards the skin. Conduction velocity of the unit is calculated (D/L) after measuring the distance between the stimulating electrode and the recording electrode along the length of the nerve (D) and the latency (L). Latency is measured after stimulating the nerve electrically placing a Teflon-coated silver electrode with an un-insulated tip ($\varnothing <$ 0.5mm) to the most sensitive spot of the receptor filed. 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 50-500 µsec depending on the afferent under investigation. Latency is measured as the time between the stimulus artifact

and the resulting action potential. Units with a conduction velocity between 1 and up to 10 m/sec are classified as A-δ fibers, which are thinly myelinated. They are further classified as D-hairs (with a von Frey threshold force of 0.4 mN) and AM mechanoreceptors. If the conduction velocity is higher than 10 m/s, they are large myelinated fibers, which consists of rapidly adapting (RA) and slowly adapting (SA) neurons. The third category is C-fibers, which have a conduction velocity less than 1 m/sec, and they are un-myelinated.

3.2.3.3.2 Testing the units for their functional properties:

After classifying the fiber, they are analyzed for their stimulus response function and velocity response function. A nanomotor®, which could be controlled by a computer is used to deliver known displacement and velocity by which the probe indent the skin.

3.2.3.3.3 Stimulus response function:

The probe is placed on the most sensitive spot of the receptive filed and the response of the unit is measured after various indentations with increasing displacement ranging from 6 to 384 µm.

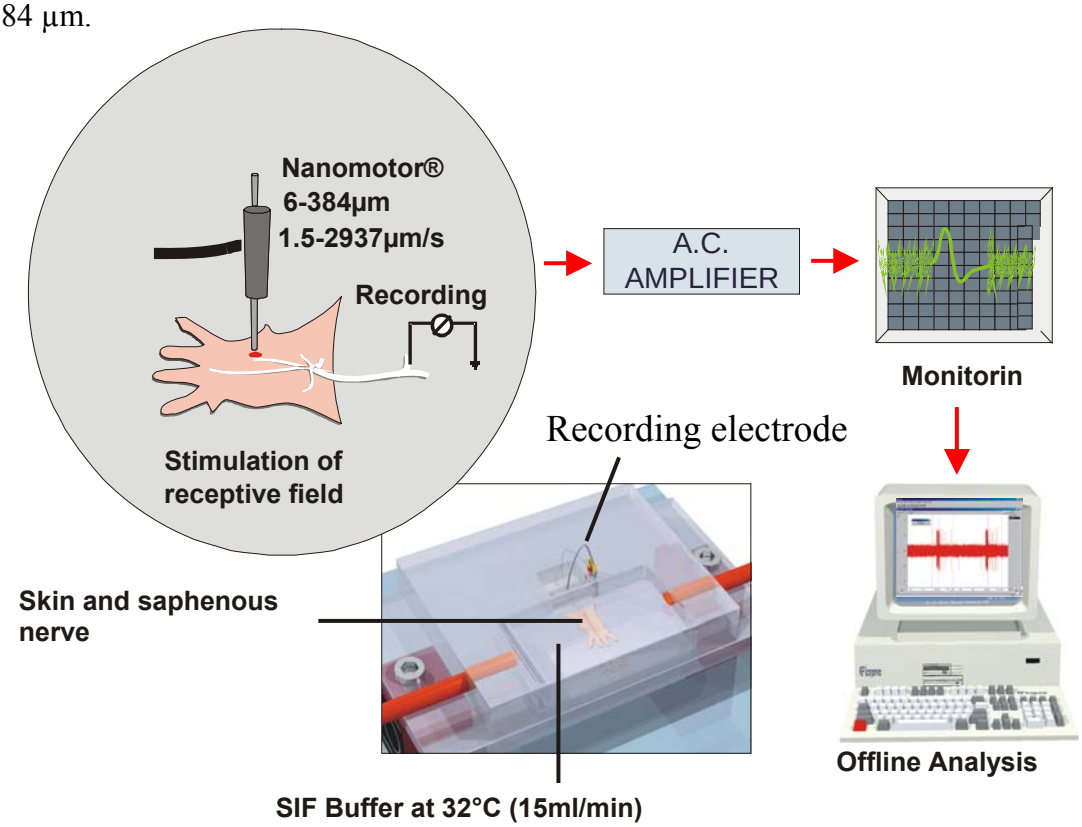

Fig:07. The skin nerve preparation – Overview

Adapted from Paul Heppenstall, Nevena Milenkovic and modified.

3.2.3.3.4 Velocity response function.

For low threshold mechanoreceptors, like RA, SA and D-hairs the response of the sensory unit with respect to different velocities of the probe movement ranging from 1.5 µm/sec to 2.9 mm/sec is recorded.

The stimulus response and velocity response of the mutant sensory afferents is compared to the WT sensory afferents.

3.2.3.3.5 Statistical testes used:

The two-way analysis of varience test was used by Prism software (version 4.03; GraphPad).

3.2.3.4 Electron microscopy procedure

Anesthetized mice were perfused with freshly prepared ice cold 4% formaldehyde in 0.1 M phosphate buffer. Part of Saphenous nerve in the thigh region was dissected from both left and right side, and postfixed in 4% formaldehyde/2.5% glutaraldehyde (EM-grade) in 0.1 M phosphate buffer for 3 days. They were washed in phosphate buffer and were processed in the common electron microscopy facility. In brief, there they were treated further with 1% OsO_4 in 0.1 M phosphate buffer for 2 h, were dehydrated in a graded ethanol series and propylene oxide and embedded in Poly/Bed® 812 (Polysciences, Inc., Eppelheim, Germany). Semithin sections (1 µm) 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 1k x 1k high-speed slow scan CCD camera (Proscan) at an original magnification of 1600x. Three ultrathin sections per nerve and genotype were analysed. On each ultrathin section, four images were taken each 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 calculated for the whole nerve by help of the area of the semithin section. Results reported for myelinated axons were calculated using the semithin sections.

3.2.3.5 Library construction:

I constructed an enriched library for the transcripts that could be altered by NT3 and (or) NT4 in NT34 mutant DRGs. The enrichment was done by suppression subtractive hybridization (SSH) described in Diatchenko, et al., (1996) (Fig.08). In the forward subtraction for the enrichment of down regulated transcripts in the NT34 mutant DRGs, control transcripts were adaptor ligated (tester) and transcripts from NT34 mutant DRGs were used for suppression (driver) of common transcripts during pcr amplification. For the reverse subtracted library, to

enrich the upregulated transcripts present in NT34 mutant DRGs, transcripts from control DRGs were used as drivers for the procedure.

Only cDNAs for the transcripts enriched by forward subtraction was ligated in to pGEM-T (Promega) and were transformed by electroporation. Transformants were sent to be picked, cultured in the 384 well plates and spotted on to high-density filters commercially (RZPD, Berlin). This will be termed as subtracted NT34 library for further discussion.

3.2.3.6 Selection of clones with enriched transcripts

Our previous experience using the subtracted BDNF library (enriched for transcripts down regulated in BDNF mutants), which is on a PCR based protocol, showed a high background of clones, which were not regulated. To select the clones in subtracted NT34 library that were enriched, high-density filters on to which it is spotted were screened using probes made from PCR products (subtraction procedure) obtained after both forward and reverse subtraction and corresponding unsubtracted controls. PCR-Select differential screening kit was used (BD Biosciences, Cat. No. K1808-1) for the selection of enriched clones. In brief, the secondary PCR products from forward and reverse subtracted subtraction as well as the corresponding unsubtracted PCR products were used as templates for making the probes. Random primer labeling using [α-^{32}P]dATP was used. Hybridizations were performed according to the manual and according to standard protocols described. Specific activities of the unsubtracted probes used were slightly higher than the subtracted probes. Clones that gave visibly higher intensity of signal with subtracted probes, comparing to the unsubtracted controls were selected to be replated. After manual comparison of the signal intensity, clones were selected and clone numbers identified from the spot positions. They were classified into down regulated, up regulated and common clones. It was difficult to exactly assign the clone position and in order to avoid missing clones of interest; selection was done in a lenient way.

Down regulated clones were the ones that gave higher signal intensity when using forward subtracted probes compared to its control. Up regulated clones were those that gave higher signal intensity with reverse subtracted probes compared to its control. The filters used were identical ones spotted with subtracted NT34 library, and because of the selection, which was done manually, there were some clones, which were selected from both the hybridizations. These were classified as common clones. By this procedure we could reduce the amount of clones to about 22% for further screening.

All the selected clones were replated in to a 96 well plate format commercially (RZPD, Berlin). They were replated in to the first 6 rows starting from A to F. The last two rows were

replated with clones that did not show any enrichment or there was no detectable signal. The idea was to have them as internal controls for the *in situ* hybridization experiment.

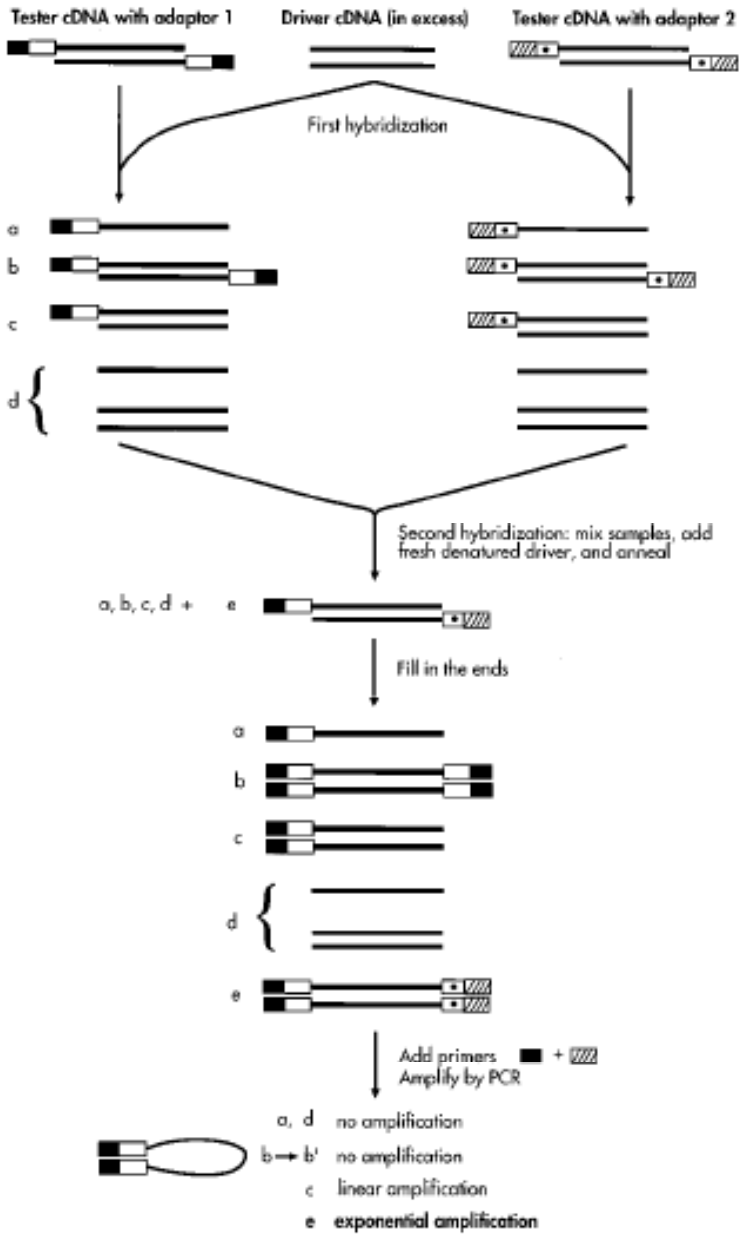

Fig.08. Scheme of the subtraction method. Solid lines represents the *Rsa*I digested tester or driver cDNA. Solid boxes represent the outer part of the adaptor 1 longer strand and corresponding PCR primer P1 sequence. Shaded boxes represent the outer part of the adaptor 2 longer strands and corresponding PCR primer P2 sequence. Clear boxes represent the inner part of the adaptors and corresponding nested PCR primers PN1 and PN2. Note that after filling in the recessed 39 ends with DNA polymerase, types a, b, and c molecules having adapter 2 are also present but are not shown. Driver is the transcript pool used for suppressing the amplification and the transcripts present in the tester pool higher in number get amplified. Adapted and modified from Diatchenko, et al., (1996).

3.2.3.7 Whole mount high through put *in situ* hybridizations and analysis of clones

Whole mount high though put *in situ* hybridizations were performed as described above and the clones with a restricted expression pattern were sequenced either using vector based T7 and (or) SP6 promoter primers. Sequences were aligned using SeqMan™II in DNA STAR package from Lasergene. Clones are annotated using BLAST either from NCBI or Ensembl.

3.2.3.8 Affymetrix® Gene chip® Experiments

Gene chip® micro arrays are manufactured using a combination of photolithography and combinatorial chemistry. Oligonucleotides of 25 bases called probes are synthesized on to the chips and the specific area where one single type of probe present is called a probe cell (Fig. 09). Probes are designed to detect complementary RNA or DNA in the sample.

Millions of copies of probes are present in one probe cell. Though probes are specific, multiple probes are used for sensitivity and reproducibility and a combination of probes called "probe set" are used to confer consistent discrimination between signal and background noise. As an internal control, each probe, which matches the target has a "mismatch" probe synthesized that differs with a mismatch at the center. Normally, target sequences binds to the probe but not to the corresponding mismatch probe. For each, transcript or DNA sequence to be targeted, multiple probes that uniquely bind to that sequence or transcript are selected. Targets are biotin labeled RNA and hybridized probe array is stained with streptavidin phycoerythrin conjugate. Presence of targets is detected by the light emitted at 570 nm and the amount of light is proportional to the target hybridized to the probe. Location of the probe is detected for the identification of the corresponding target.

In my experiment, 2 different pools of cRNA targets were used. cRNA targets from total RNA isolated from DRGs of control and the NT34 mutants were used to find out the comparative expression level of transcripts in control and NT34 mutant DRGs. Second pool of cRNA targets was made from SSH based enriched transcripts which were down regulated in NT34 mutant DRGs (Fig:10).

Protocol was followed according to the instruction manual and all the internal quality control criteria were met. In brief, DRGs from all levels of age matched control and NT34 mutant animals were dissected out and RNA prepared by Trizol® method according to the manufacturer's instructions. First strand synthesis and further steps were done till fragmentation of the cRNA probes. After this step, the probes were given to the common facility for hybridization and scanning. The raw data and comparative data were further analyzed to find out genes, which were regulated in two out of three independent experiments

while comparing the NT34 mutnat and control expression of transcripts in DRGs. In addition one round of experiment was performed with SSH based enriched pool of transcripts with the non-enriched pool of transcripts.

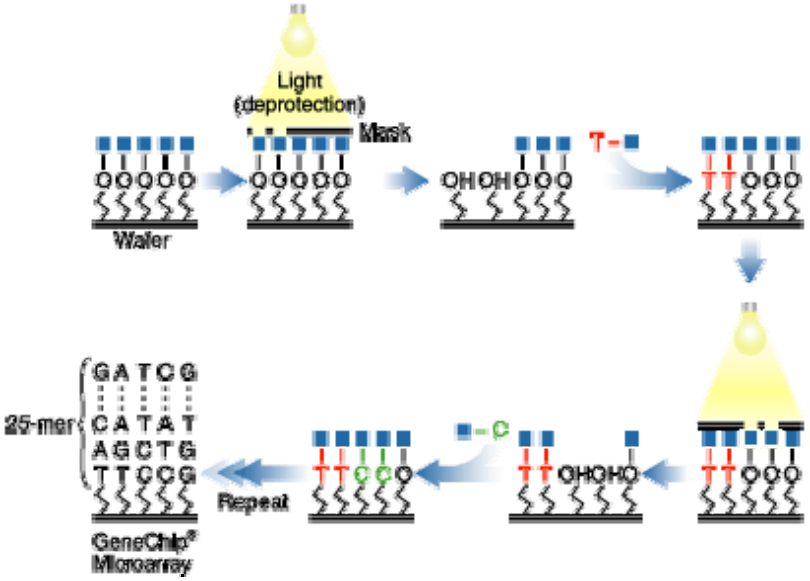

Fig. 09. Initially the surface of the quartz wafer is coated with a photolabile chemical compound, which prevents coupling of oligonucleotides to the surface. After deprotecting the compound through UV illumination on a desired area, desired nucleotide of the probe getting created is flooded to get it coupled to the surface. Coupled nucleotides also have a photolabile protecting group preventing them getting coupled to other nucleotides except after deprotection through UV illumination. Cycles of deprotection and coupling are repeated with all the nucleotides in a sequential manner till the whole probe gets synthesized in the microarray. Adapted from Affymetrix® site.

3.2.3.9 Software and method of analysis

The software used to analyze the data was Data Mining Tool Version 3.0 from Affymetrix.

3.2.3.10 qPCR experiments

qPCR experiments were done as described in the previous section. Only inventoried TaqMan pirmer probe mixtures from Applied Biosystems were used for the analysis.

3.2.3.11 Behavioral Test.

The MoTil system (TSE Systems, Germany) is a flexible system for studying the open field behavior 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

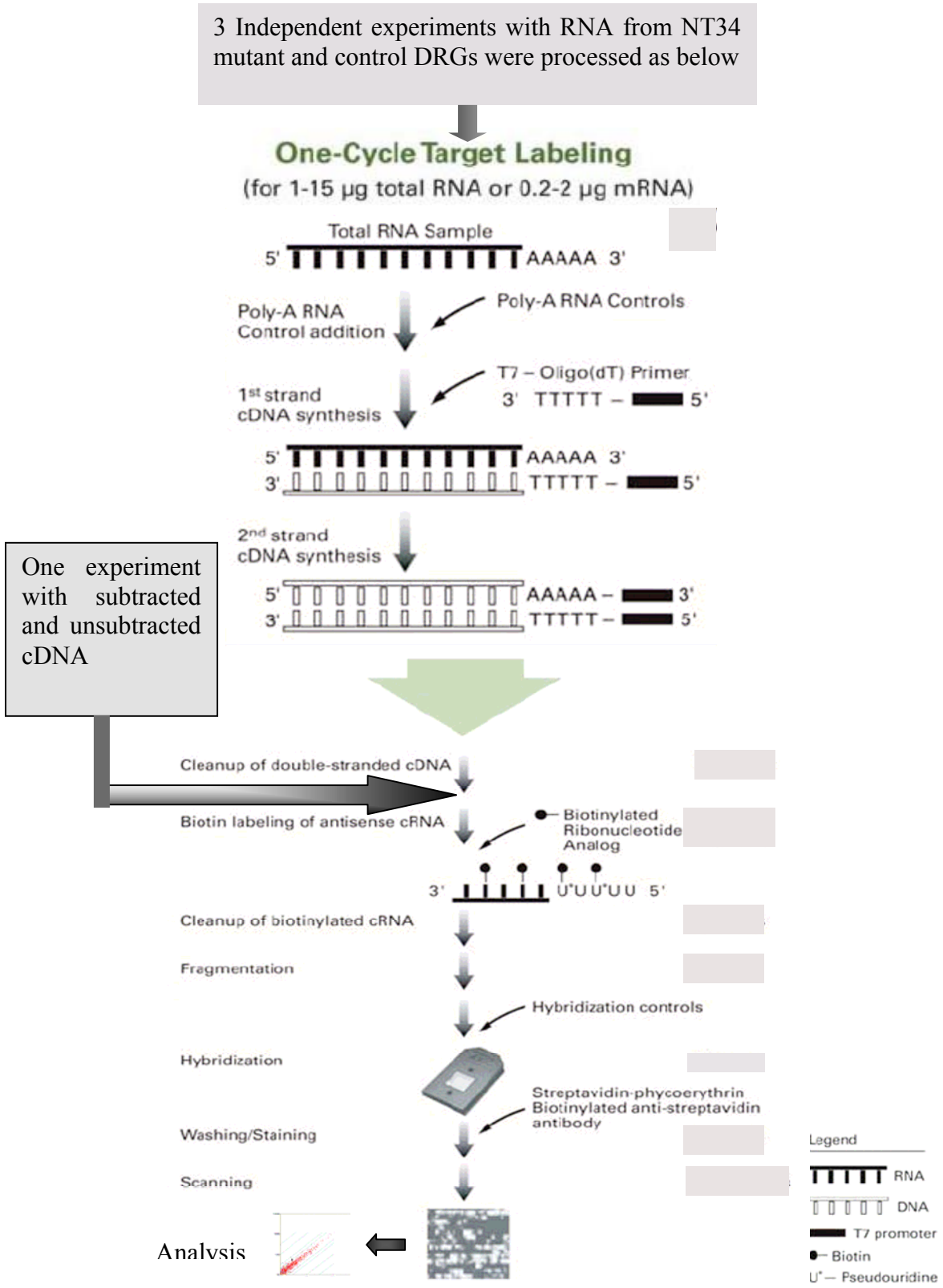

Fig: 10 Overview of Microarray experiments. Brief outline of the procedures involved in the affymetrix procedure used with both enriched and non enriched pool of transcripts. Adapted from GeneChip® Expression Analysis Technical Manual and modified.

10 msec 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, 28 mm 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).

3.2.3.11.1 Sand paper 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 50 mm 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 30 min the animals were exposed to these tactile cues for 90 min.

3.2.3.11.2 Tactile acuity test (Grid based)

Instead of using the sand paper as cues, we used grids that vary in spatial frequency similar to tactile acuity cubes usually used in human touch tests (Van Boven and Johnson, 1994). Two equivalent positions within the floor plate were chosen. They were symmetrically positioned along the middle axis of the box accommodating a putative tactile surface cue (plastic grid with 250 µm, 500 µm, 750 µm spacing) at one position and a blank control insert exhibiting the same tactile property as the floor plate at the other position. Mice were placed individually into the test cages in complete darkness. They were acclimatized to the test box for a period of 10min before one of the surface cues is introduced and left for 90 after wards in complete darkness and the activity is monitored. Each mouse was tested on individual grid cues separately performing one experimental trial per day. Light sensors are sequentially scanned every 10msec to determine x-y- coordinates and hence the exact location of the mouse known during activity measurement. The following frame configurations were used: size 300 x 400 mm; 6 x 12 infrared sensors, 28 mm sensor distance (Fig. 11).

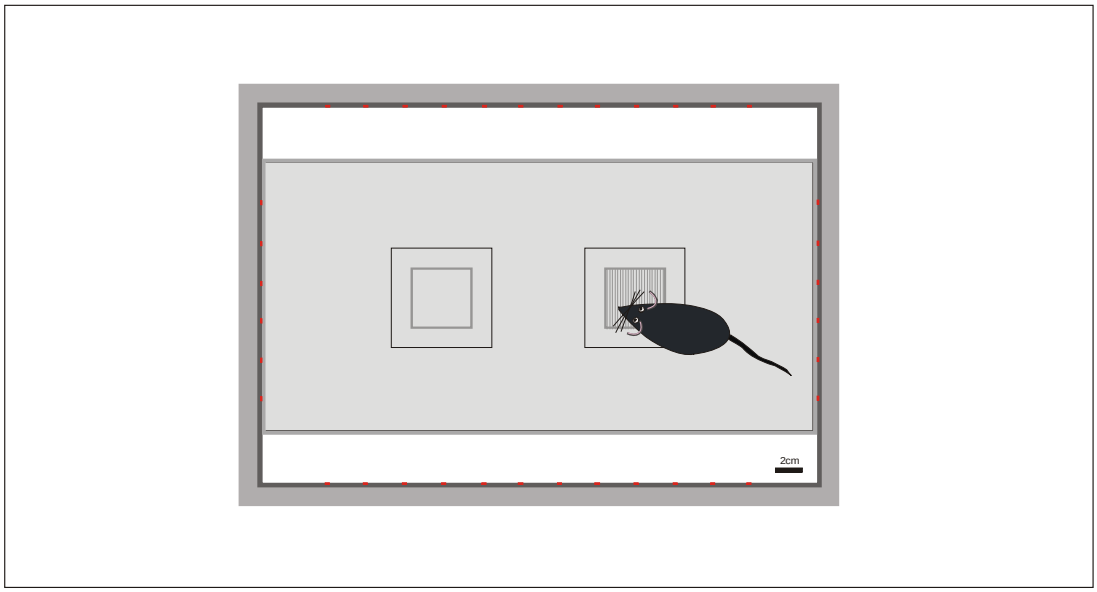

Fig. 11. One of the symmetrically arranged square positions (42 x 42mm in size) contains the tactile cue and the other the control surface. Area quadrants including the surrounding area of the surface were defined, which were 63 mm^2 in size. Adapted and modified from Christiane Wetzel.

3.2.4 PART III. Functional analysis of a candidate gene "Secreted phosphoprotein 1 (SPP1)" for its role in mechano-transduction

3.2.4.1 Animal strains

The homozygous mutants were obtained from The Jackson Laboratory (Bar Harbor, Maine). The strain name is B6.Cg-*Spp1*^{tm1Blh}/J (Stock no. 004936). The strain was developed as described in Liaw, et al. (1998). In brief, a targeting vector containing neomycin resistance and herpes simplex virus thymidine kinase genes was used to disrupt exons 4 through 7 of the targeted gene. The construct was electroporated into 129S6/SvEvTac derived TL-1 embryonic stem (ES) cells. Correctly targeted ES cells were injected into C57BL/6 blastocysts. The resulting chimeric animals were crossed to outbred Black Swiss, maintained on the mixed Black Swiss, 129S6 background and then backcrossed to C57BL/6 for 10 generations. The control mice used were C57BL/6. The homozygous mutant will be referred as "SPP1 mutant" and the control mice as "control".

3.2.4.2 Genotyping

Genotyping for all animals were carried out using genomic DNA from isolated from tail biopsies by standard protocol. To the PCR reaction mix (10x Buffer 1/10 final volume, $MgCl_2$ 2 mM, dNTP 0.2 mM, Primers oIMR 3340 (CCA TAC AGG AAA GAG AGA CC) 1.5 µM, oIMR 3341 (AAC TGT TTT GCT TGC ATG CG) 0.1 µM, oIMR 3342 (CGT CCT GTA AGT CTG CAG AA) 1.5 µM, Taq Pol .014U/µl) 5-20 ng of genomic DNA was added and PCR performed with the reaction conditions of 94^{o}C for 3 min, 35 cycles with steps 94^{o}C for 30 sec, 62^{o}C for 1 min and 72^{o}C for 1 min, and one final extension step 72^{o}C for 2 min. Wild type allele is recognized by the presence of 600 bp fragment and the presence of 500 bp allele denotes the mutant allele.

3.2.4.3 Behavioral Experiments

3.2.4.3.1 Measurement of mechanical withdrawal threshold

Mechanical withdrawal threshold was measured on both hind paws using an automated apparatus for applying reproducible touch (Dynamic plantar Aesthesiometer 37,400, Ugo Basile, Italy). It consists of a movable touch-stimulator, an elevated framed mesh on which the animal stands, a two-compartment enclosure and a microporcessor controlled electronic unit. The force applied by the stimulator is controlled and the amount of force applied when

the animal withdraws its paw is calculated. Mice were placed in their compartments on a metal mesh surface. After a period of time when the mice stopped exploring their surroundings, and remained calm in a resting position, the tests were initiated. With the help of an adjustable angled mirror in the equipment, the touch stimulator unit was placed beneath the selected hind paw, with the filament below the plantar surface of the mouse. When the unit was started, the electrodynamic actuator lifted the stainless-steel filament, which touched the plantar surface, and exerted a force less than the threshold of feeling. The force was then increased with a ramp of 0.5 g/second to the maximum of 10 g (20 secs). The force applied when the mice withdrew the paw from the filament was measured. Each paw was measured 5 times with an interval of >5 min between each measurement on 5 different days.

3.2.4.3.2 Measurement of thermal latency

Thermal latency was measured based on method described in Hargreaves K., et al., (1988) using Plantar Test 7371 (Ugo Basile, Italy). The plantar surface of the hind paw was exposed to the radiant heat source and the time taken to withdraw the paw is measured in seconds. Mice were put inside elevated Perspex boxes and were acclimatized. Testing was commenced when they are resting by placing the radiant heat source under the plantar surface of their hind paw. Paw withdrawal latency was measured in seconds automatically. Each paw was tested 5 times with an interval of >5 min on 5 different days.

3.2.5 PART IV: Finding molecules which may bind to extra-cellular domain of ASIC3 using a skin phage display library:

3.2.5.1 Preparation of oocyte and injection:

3.2.5.1.1 Collection of oocytes:

Adult female *Xenopus laevis* frog was anaesthetized by immersing it in to 0.35% Tricain solution for about 3.5 min and was laid on ice for dissection. An incision was (0.5 cm app) made on the ventral side of the abdomen cutting the skin and muscle layers. Oocytes were removed and was put in to OR2 medium. The layers of muscle and skin were suture and the animal was left to recover in water keeping its head above the surface.

3.2.5.1.2 Preparation of Oocytes:

Follicular tissue was removed by collagenase treatment for 3 h in calcium free ringer solution with 2mg/ml collagenase. To remove the collagenase, oocytes were rinsed twice and washed twice (5 min each) with OR2. Before incubating, these were rinsed twice and washed twice (10 min each) with OR2-L15 medium. Incubation was done at 18^0C overnight in OR2-L15 medium. Healthy oocytes without any damage were selected for the experiment observing under the microscope.

3.2.5.1.3 Preparation of capillaries for oocyte injection:

Capillaries were pulled with the apparatus using the preloaded program (Parameters used were heat: 570, pull: 170, velocity: 50 and time: 170). They were calibrated to deliver about 20 nl per injection.

3.2.5.1.4 Oocyte injection:

About 1 ng of RNA was injected per oocyte with an injection volume of 20 nl. After injection they were incubated overnight at 18^0C and the media was changed once. Unhealthy looking/lysed oocytes were removed.

3.2.5.2 Construction of skin phage display library:

3.2.5.2.1 Isolation of mRNA:

Total RNA was isolated form from the skin of 4 day old mice. Total RNA was isolated using Rneasy kit (Qiagen). mRNA was isolated from the total RNA using Oligotex direct mRNA mini kit (Qiagen).

3.2.5.2.2 Llibrary construction:

Phage display library was constructed using T7Select®10-3 OrientExpress™ cDNA Cloning System. In brief, 2 µg mRNA was used as the starting material and first strand synthesis was done using random primers as per the instructions in the manual. The cDNA thus obtained was ligated to *Eco*RI and *Hind*III directional linkers, digested and size fractionated to avoid fragments less than 300 bp. These fragments were directionally ligated to the vector arms (Fig. 12.), packaged and phages were amplified by liquid culture methods. After amplification, amplified phages were stored in at -70^0C with 0.1% (v/v) 80% gyecerol. BLT5616 cells were used for the amplification after inducing the culture (OD_{600} 0.5) with 1 mM IPTG for 30 min.

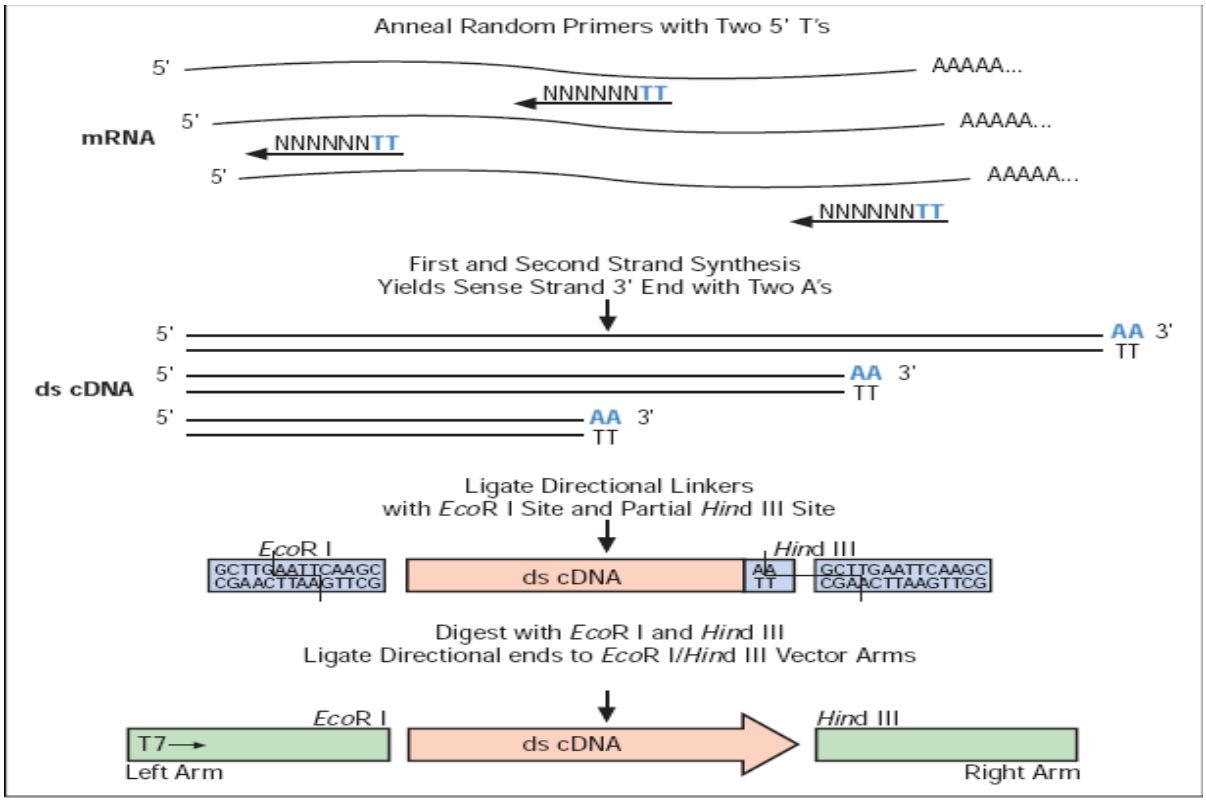

Fig. 12. Directional cloning of cDNA synthesized using random primers in "Orient Express". Adapted and modified from T7Select®10-3 OrientExpress™ cDNA Cloning System manual.

3.2.5.3 Adsorption protocol:

In total 4 rounds of adsorption and amplifications were performed. For the first round, 3 ml of phage solution (1.4 x 10^8 pfu/ml) was incubated with one oocyte at room temperature for 10 min in the presence of 2 mM Ca^{2+}. Further it was washed with 15 ml PBT for 10 times (3 min each) in the presence of 2 mM Ca^{2+} with gentle shaking. Oocyte was then squashed with the pipette and the lysate inoculated in to induced BLT5615 cells. It was incubated at 37^0C at 200rpm till lysis was observed. Lysate was centrifuged at 3663 g for 20 min and the supernatant was filtered through 0.45 µ membrane filter. As a control a noninjected oocyte was processed in parallel identically.

For the second, third and fourth rounds, equal amounts of phages were incubated with two oocytes and the procedure was followed.

3.2.5.4 Identification of adsorbed phages and sequence analysis:

Filtered lysates were plated as per the instruction in the manual and individual plaques were PCR amplified using vector based primers flanking the inserts. Products were electrophoresed to obtain the sizes and sent for sequencing after purification. Sequences were analysed for their similarity (DNA Star from Lasergene) and identified either by using tblast in NCBI or in Ensembl.