# 7. Methods:
# 7.1. Methods related to molecular biology

### 7.1.1. Construct preparation.

For construct preparation, DNA fragments corresponding to the required coding regions were obtained either by restriction digestion of the available cDNA or amplified by PCR with a specific primer pair. PCR-amplified DNA and vector were subsequently digested by restriction enzyme/s to produce cohesive overhanging ends. Restriction-digested inserts and vectors were purified, ligated and the ligated product was transformed into *E.coli*. Transformed *E. coli* cells were selected on a plate as colonies by means of antibiotic resistance. Plasmid DNA was isolated from those colonies by using kits. Miniprep plasmid DNA was further analyzed for the positive construct by means of restriction pattern analysis, PCR amplification of insert and finally confirmed by sequencing.

### 7.1.2. Polymerase Chain Reaction (PCR).

In order to prepare different constructs in particular expression vectors, PCR reactions were carried out to precisely amplify the different coding regions with specific restriction sites at the ends. Specific inserts compatible for sub-cloning in different expression vectors were essentially prepared by digesting the PCR-amplified-DNA with restriction enzymes. Using miniprep-DNA isolated from *E.coli*, PCR reaction was also performed to screen and confirm the presence of insert in particular colonies grown on transformed plates after ligation. All PCR reactions were performed done following the conditions mentioned below. Taq DNA-polymerase (NEB) was used as an enzyme.

**Conditions of the PCR.**

| | |
|---|---|
| Forward Primer (100nM) | 1 µl |
| Reverse Primer (100nM) | 1 µl |
| Template | 0.5 µl |
| dNTPs (10mM) | 2 µl |
| 10x buffer | 2.5 µl |
| $H_2O$ | 17 µl |
| Enzyme | 1µl |
| Total Volume | 25 µl |

The mixture was subjected to a temperature change on a MWG thermocycler instrument as described below.

| Step | Process | Temperature and duration |
|---|---|---|
| **1.** | Denaturation | 95°C for 5 Min |
| **2.** | Denaturation | 94°C for 30sec |

| | | |
|---|---|---|
| **3.** | Annealing | 55°C for 30sec |
| **4.** | Extension | 72°C for 1 Min/Kb |
| **5.** | Repeat step 2 for 29 Cycles | |
| **6.** | Extension | 72°C for 10 minutes |
| **7.** | End and store | 4°C |

All PCR-amplified DNA were further confirmed by an agarose gel electrophoresis.

### 7.1.3. Restriction digestion of dsDNA.

For restriction digestion, approximately 1μg dsDNA was used in 20 μl restriction digestion mixtures. The restriction-digestion mixture contains specific restriction buffer at a concentration of 1X and doubly distilled autoclaved water. Depending on the enzyme activity, restriction enzymes were added at the ratio of 1 unit /μg of DNA. In some cases more enzyme was used. Depending on the activity of the enzymes, the reaction mixture was incubated at 37°C for 3 hours or over night. For doubled restriction digestion, a compatible buffer was selected according to the manufacturer's (NEB) instruction.

### 7.1.4. Ligation of dsDNA.

For ligation, restriction enzyme digested insert and vector dsDNA were first checked on an agarose gel and the bands were excised from the gel. The dsDNA was subsequently purified from the gel by using Qiagen kit according to the manufacturer's instruction. The purified insert and vector dsDNA were mixed with doubled distilled autoclaved water and ligation buffer so that the concentration of ligation buffer becomes 1x in the final solution. Finally T4-DNA ligase was added to the reaction mixture. The mixture was incubated over night at 16°C. For a better efficiency of ligation, insert and vector in the ligation mixture was maintained at a higher molar ratio (at least 10:1 for insert to vector). This enhances the probability of ligation reaction.

### 7.1.5. Agarose gel electrophoresis.

For the electrophoretic separation of dsDNA, a horizontal agarose-gel electrophoresis apparatus and TAE buffer was used if not mentioned otherwise. In brief, agarose powder is mixed with electrophoresis buffer to the desired concentration (0.8-1.2%, depending on the length of the dsDNA subjected to separation) and heated in a microwave oven until completely melted. Ethidium bromide (EtBr, at a final concentration of 0.1 to 0.5 μg/ml) was added to the gel at this point to facilitate visualization of DNA after electrophoresis. After cooling the solution to about 60°C, the molten gel was poured into a casting tray containing a sample comb and allowed to solidify at room temperature. Subsequently the comb was

removed from the solidified gel without destroying the bottom of the wells. The gel was inserted into the electrophoresis chamber, covered with electrophoretic buffer. Samples containing DNA mixed with loading buffer are then loaded into the sample wells and the apparatus was connected to a constant current source. Migration of the DNA in the gel can be judged by visually monitoring the migration of the tracking dyes, that is Bromophenol blue and Xylene cyanol present in the DNA loading buffer. After adequate migration, DNA fragment/s are visualized on an ultraviolet transilluminator (due to incorporation of ethidium bromide during the electrophoresis, DNA becomes fluorescent) and photographed by a camera attached with a gel-documentation system.

**Solutions and buffer required:**

**(1X) TAE buffer:** 40mM Tris/Acetic acid, pH7.8; 1mM EDTA pH 8.0

**(5X) DNA loading buffer:** 40% (w/v) Sucrose, 240mM Tris/Acetic acid, pH7.8, 5mM EDTA, pH 0.8, 0.25% (w/v) Bromophenol blue, 0.25% (w/v) Xylene cyanol FF

### 7.1.6. Competent *E.coli* cell preparation.

Competent *E. coli* cells were prepared as mentioned below. A single colony of bacterial cells was incubated in 3 ml of Luria-Bertani (LB) broth and grown over night. One ml of this culture was added to 100 ml of LB liquid medium and incubated at 37°C to make a broth culture. The cells were grown until an OD 0.4-0.5 was reached at 600 nm. The cells were incubated for 10 min on ice and centrifuged at 3000 rpm for 5 min at 4°C. The pellet was resuspended in 30 ml of ice-cold $CaCl_2$ (100 mM) solution and incubated for 30 minutes on ice. The suspension was further centrifuged for 5 min at 4°C. Finally the pellet was resuspended in ice-cold $CaCl_2$ (100 mM) solution supplemented with 10% glycerol, distributed in tubes and stored at -80°C.

**LB media:** 10g Bacto Tryptone, 5g Bacto Yeast extract, 10g NaCl dissolved in 1 litre of double distilled water and autoclave at 121°C at 15 lbs for 20mins.

### 7.1.7. Transformation of *E.coli*.

Bacterial transformation was done to amplify the cDNA, screen ligated colonies or for protein expression. Competent bacteria were thawed on ice. Approximately, 0.1µg plasmid DNA in 20 µl ligation mixtures was incubated with the bacteria for 20 min on ice. Bacterial cells along with the DNA were incubated at 42°C for 90 sec (heat-shock step) and then placed on ice for 1-2 min. Bacterial cells were subsequently incubated in 1000 µl of LB

broth (without any antibiotic) at 37°C for 1 hr on a shaker. After 1 hour of incubation, the cells were centrifuged at 5000 rpm for 5 min at room temperature and the pellets were resuspended in 100 µl of LB broth, distributed on LB-agar plate supplemented with antibiotic for selecting the transformed bacteria. Plates were incubated at 37°C overnight, and individual colonies appear on the plate. Each colony represents one transformed bacterial cell.

# 7.2. Methods related to protein chemistry.

### 7.2.1. Separation of denatured proteins by SDS-PAGE

For separating proteins electrophoretically, a SDS-PAGE with 10% acrylamide gels methods was used commonly on a Biorad mini-apparatus if not mentioned otherwise. The method is called SDS-PAGE due to the fact that sodium dodecyl sulphate (SDS), a strong anionic detergent is used to denature the proteins and a discontinuous polyacrylamide gel is used as a support medium to separate the denatured proteins according to their molecular size. The most commonly used system is also called the Laemmli method after U.K. Laemmli, who was the first to demonstrate this SDS-PAGE as a technique to separate proteins (Laemmli U.K., 1970).

In brief, to make the protein samples ready for SDS-PAGE separation, protein samples were completely denatured by first adding Laemmli protein loading buffer in 1:4 v/v (from a 5x stock of Laemmli protein loading buffer) and subsequently heating the mixture at 95°C for 5 minutes. SDS-PAGE cassettes were prepared by using a pair of clean glass plate (10 cm wide and 7 cm high) separated by a pair of spacer (0.75 mm thickness for thin gel or 1.5 mm thickness for thick gel). Approximately, 5 cm of the Cassettes were filled up with liquid separating gel mixture and allowed to polymerize within the cassettes. A thin layer of water was slowly added to the top of separating gel layer to make the top layer of separating gel smooth. After polymerization of the separating gels, stacking gel mixture was poured on the top of the separating gel and a 15-well comb was inserted within. After polymerization of the stacking gel, combs were removed slowly without disturbing the wells. Cassettes were inserted into the electrophoresis chamber vertically, filled with electrophoresis running buffer and denatured protein samples were loaded into the wells using a Hamilton syringe. The apparatus was connected to a constant current source (10 mAmp for thin gel and 20 mAmp for thick gel) for electrophoresis. Migration of the proteins in the gel can be judged by visually monitoring the migration of the tracking dyes that is Bromophenol blue added in the protein-loading buffer. When the dye front comes close to the end, the electrophoresis was stopped, the gel was fixed with fixer and the separated proteins in the gel were visualized by either Coomassie blue staining or by silver staining. In case of Western blot analysis, unfixed gel was used to transfer the proteins on a nitrocellulose membrane.

**Buffers required:**

**SDS-PAGE running buffer (1X):** 196mM glycine, 0.1% SDS, 50mM Tris-HCl (pH 8.3)

**Laemmli protein loading buffer (5X):** 62.5 mM Tris HCl (pH 6.8), 5% beta-mercaptoethanol (v/v), 50% Glycerol (v/v), 2% SDS (w/v), 0.1% (w/v) Bromo phenol Blue. Volume was made by adding water.

**Separating gel mixture:** 10% Bis-Acrylamide (v/v), 375 mM Tris HCl (pH 8.8), 0.1% SDS (w/v), 0.1% ammonium persulfate, 0.005% TEMED in water.

**Stacking gel mixture:** 4% Bis-Acrylamide (v/v), 125 mM Tris HCl (pH 6.8), 0.1% SDS (w/v), 0.1% ammonium persulfate, 0.005% TEMED in water.

30% Bis-Acrylamide stock solution, 10% APS stock solution, 100% TEMED solution, 20% SDS solution and 1.5 M Tris HCl (pH 8.8) solution (for separating gel)/or 0.5M M Tris HCl (pH 6.8, for stacking gel) were used to prepare separating and stacking solutions. APS and TEMED were added just prior to pouring the gels.

### 7.2.1. Coomassie staining of the protein bands in gel.

Commonly 0.1% Coomassie blue dye dissolved in 50% methanol, 10% glacial acetic acid was used to stain the proteins in a gel separated by SDS-PAGE. Acidified methanol precipitates the proteins. Staining was usually done over-night with agitation. The agitation circulates the dye, facilitating penetration, and helps ensure uniformity of staining. The dye actually penetrates the entire gel, however it only sticks permanently to the proteins. Destaining with acetic acid/methanol, also with agitation, washed out excess dye. It is most efficient to destain in two steps, starting with 50% methanol, 10% acetic acid for 1-2 hours, then using 7% methanol, 10% acetic methanol to finish. The first solution shrinks the gel, squeezing out much of the liquid component, and the gel swells and clears in the second solution. Properly stained - destained gels displayed a pattern of blue protein bands against a clear background. The gels were scanned in a scanner attached to a computer, and subsequently dried down on a Whatman paper on a vacuum drier for later analysis and documentation. The original dye front, consisting of bromophenol blue dye, disappeared during the process. Due to the fact that bromophenol blue acts as a pH indicator, it turned to light yellow colour under acidic conditions, prior to being washed out. Coomassie blue may not stain proteins that are present in very low amount. Coomassie blue dye has limitations to stain some other proteins too, especially those with high carbohydrate content.

### 7.2.3. Silver staining of the protein bands in gel.

Silver staining is much more sensitive compared to Coomassie blue staining. Therefore silver staining of proteins was essentially done to visualize the proteins that are present in very low amount as an alternative to Coomassie staining. For silver staining of the proteins in gel, first the gel was fixed in a fixer solution (50% MeOH and 10% Acetic acid) for 20 minutes, washed with doubly distilled water three times, each time for 10 minutes. Subsequently the gels were treated with 50% MeOH for 10 minutes, washed three times with

water, each time for 10 minutes. The gel was briefly (2 minutes) treated with $Na_2S_2O_3$ (0.02% w/v) and washed two times with water (each time for 1 minute). The gel was subsequently treated with $AgNO_3$ (0.1% w/v) for 20 minutes in the dark. After that the gel was washed again three times with water (each time for 1 minute). Subsequently, the gel was developed with developing solution (2%$Na_2CO_3$ and 0.04% Formalin) to visualize the protein bands. When the protein bands became visible in the gel, the reaction was stopped by adding 1% acetic acid. Care was taken to avoid over-developing of the proteins, which gives negative staining. Gels with visible protein bands were scanned soon and dried on paper. Double distilled water was used to prepare all the solutions. For mini-gels generally 50 ml volume of each solution was used for each step.

### 7.2.4. Western blot analysis

For Western blot analysis, proteins were first separated by SDS-PAGE and subsequently electro-transferred on a nitrocellulose membrane by semi-dry method. Briefly, unfixed gels were incubated shortly in a transfer buffer. Whatman paper and nitrocellulose membranes were also socked in the same transfer buffer. At first, the gel was placed on the membrane. Subsequently, two layers of Whatman papers were placed on both sides of the gel-membrane combination to make the transfer set. Air bubbles were removed from the whole transfer-set by rolling a glass rod over it. Finally this combination was placed on a transfer unit (Schleicher and Schuell) in such a way that the gel is connected to the cathode while the membrane is connected to the anode. The apparatus was connected to a power supply and the electro-transfer was done at a constant current of 50 mAmp (for a single gel) for 2 hours. Transfer of proteins from the gal onto membrane was confirmed by staining the membranes with Ponceau Red dye solution. Subsequently, the membranes were blocked with 5% non-fat milk dissolved in TBS-T buffer. Blocked membranes were incubated first with primary antibody in TBST buffer for 1 hour, washed 3 times with TBST buffer followed by incubation with secondary antibody in TBST buffer for 1 hour. Finally, the membranes were washed with TBST again and developed on ECL films (Amersham) with an ECL kit (Amersham Biosciences/GE Healthcare). In some experiments, blots were stripped-off by incubating the blots in a stripping buffer at 50°C for 30 minutes and reprobed again with a different primary antibody.

**Buffers and solutions required:**

| | |
|---|---|
| **Transfer buffer:** | 0.1% SDS, 20% (v/v) MeOH, 48mM TRIS7/HCl, 39 mM Glycine |
| **Ponceau Red solution:** | 2% (w/v) Ponceau S dye, 5% (v/v) Acetic acid |

| | |
|---|---|
| **TBS-T:** | 20 mM Tris, 150 mM NaCl. 0.1% (w/v) Tween-20 |
| **Stripping buffer:** | 1% SDS, 20mM TRIS/HCl (pH 6.8), 1% (v/v) β-Mercaptoethanol |

### 7.2.5. MALDI-MS*.

The proteins of interest were digested in-gel by trypsin according to standard procedures (Shevchenko et al. 1996). Measurements were performed using 2,5-dihydroxybenzoic acid (DHB) as matrix substance. A Bruker Reflex (Bruker Daltonics, Bremen, Germany) mass spectrometer was used to acquire peptide mass fingerprint spectra as well as fragment ion spectra obtained from post-source decay (PSD) of selected precursor ions. PSD spectra were assembled using the FAST method (Bruker Daltonics, Bremen, Germany). The search engines ProFound and PepFrag (available at http://prowl. rockefeller.edu) were used to match peptide mass fingerprints and fragment ion patterns to NCBInr database entries.

* All MALDI-MS analysis was done either by Dr. Mathias Dreger or by Dr. Peter Franke.

### 7.2.6. Cross-linking of proteins.

A protein mixture (1mg/ml) of equal amounts of αβ-tubulin dimer and MBP-TRPV1-Ct, in PEM buffer was adjusted to 0.2M triethanolamine (pH 8.1) buffer for cross-linking with dimethyl suberimidate (DMS, Sigma, 1 mg/ml). The reaction was carried out at RT for 1 minute to one hour and stopped by adding Tris-HCl (pH 6.8) to a final concentration of 50 mM. Samples were subjected to SDS-PAGE separation and Western blot analysis.

### 7.2.7. Biotinylation of tubulin

Purified tubulin (1 mg/ml) in 1 ml PEM buffer (50 mM pH 6.8, 1 mM EGTA, 0.2 mM $MgCl_2$) was labelled with 1mg/ml of EZ-Link Sulfo-NHS-LC Biotin at 4°C for 2 hours. The labelling reaction was terminated by adding Tris/HCl (pH 7.4) to a final concentration of 50 mM. Unreacted biotinylation reagent was separated from biotinylated tubulin by gel filtration with a G25 column.

# 7.3. Methods related to protein purification.

### 7.3.1. Purification of Tubulin

Alpha-beta tubulin dimers were purified from porcine brain according to Shelanski et al. (Shelanski et al. 1973). Tubulin purification was done from fresh brain tissue, as frozen tissue does not suite for tubulin preparation. Fresh porcine brains were collected from a slaughterhouse near Berlin. Fat bodies, hypothalamus region and blood clots with meninges were removed quickly from the brain tissue. Clean brain tissue was homogenized in PEM buffer (50 mM PIPES: pH 6.8, 1mM EGTA, 0.2 mM $MgCl_2$) with complete protease-inhibitor cocktail. Homogenization of the brain was done within 1 hour from the time of collecting porcine brain. The homogenate was subsequently centrifuged at 12K for 90 minutes at 4°C to obtain clear supernatant. 500ml pre-warmed glycerol was added to 1 L of the clear supernatant and incubated at 37°C in a water bath. ATP and GTP were added as solid to the final concentration of 1.5mM and 0.1 mM. Extra $MgCl_2$ was also added to make the final concentration at 4mM. A catalytic amount of taxol® was added to enhance the polymerization of microtubules. Within 1 to 1 1/2 hour a visible change in the extract was observed. The polymerization mixture was centrifuged at 20K for 60 minutes at 37°C to obtain the gelatinous pellet. This gelatinous pellet was further resuspended in the ice-cold PEM buffer, homogenized in glass-glass homogenizer, and incubated on ice for 30 minutes for cold-induced depolymerization of microtubules. This depolymerization mixture was subsequently centrifuged at 20K for 60 minutes at 4°C to remove the chunky pellets. This supernatant represents recyclable tubulin and associated proteins. Solid GTP at the final concentration of 0.5 mM and extra $MgCl_2$ at the final concentration of 4mM were added to this clear supernatant and incubated at 37°C for a second round of polymerization. Polymerization mixture was again centrifuged at 20K for 30 minutes at 37°C and polymerized microtubules were isolated. Polymerized microtubules were again depolymerized by resuspending in ice-cold PEM buffer and homogenizing in a glass-glass homogenizer. Depolymerization mixture was incubated on ice for 30 minutes followed by centrifugation at 20K for 30 minutes at 4°C to obtain the clear soluble extract with enriched tubulin dimer and minimum amount of microtubule-associated proteins. This clear mixture was run through a column prepared with phosphocellulose and eluted fractions with highly purified tubulin containing only small amount of MAPs were collected. Protein concentration of the purified tubulin was measured, distributed in aliquots, frozen with liquid $N_2$ and stored at –80°C for long-term use.

### 7.3.2. Preparation of high-speed detergent-extracted spinal-cord extract.

Rat spinal cord was homogenised in 20mM Hepes (pH 7.4), 1mM EGTA and 320 mM sucrose and protease inhibitor cocktail (Roche). After homogenisation, the extract was cleared by centrifugation at 48,000 × g for 30 minutes at 4°C, followed by solubilization with 1% (w/v) Tween-20 for 2 hours at room temperature (RT). The solubilized extract was centrifuged at 100,000 × g for 2 hours at 25°C in fixed angle rotor TFT65.13. The top layers that contained myelin and the bottom layers that contained insoluble material were discarded. Myelin-free cleared supernatant from the middle of the gradient was used for the pull-down assay.

### 7.3.3. Prokaryotic expression and purification of MBP-fusion proteins.

*E.coli* strain BL21DE3 was transformed by heat shock with plasmids containing the coding regions corresponding to cytosolic domains and fragments for TRPV1, TRPV2 and/or TRPV4. *E.coli* cells were induced to express the proteins by isopropyl thiogalactoside (IPTG) for 2 hours. Thereafter, the cells were harvested by centrifugation and subsequently lysed by freezing and thawing cycles in lysis buffer (20mM Tris/HCl, pH 7.4, 150mM NaCl, 0.2gm/ml sucrose, lysozyme, benzonase and protease inhibitor cocktail). The lysed extracts were cleared by spinning at 100,000 × g in a TFT.45 rotor for 2 hours. The cleared lysate was applied to the amylose resin and washed thoroughly. Bound protein was eluted with 10mM maltose in elution buffer (50mM PIPES, pH 6.8, 100 mM NaCl, 1 mM EGTA and 0.2 mM $MgCl_2$).

### 7.3.4. Protein estimation

The amount of protein present in samples ware determined either by Bradford method (Bradford, 1976) or by the BCA method (Pierce) following the manufacturer's instruction. Purified BSA solution (Pierce) was used to prepare the standard curve. All measurement was performed either in a spectrophotometer (Shimadzu) or a plate reader (Dynex) at 550nm.

# 7.4. Methods related to protein interaction.

### 7.4.1. MBP-Pull-down assay for identifying TRPV1 interacting proteins.

MBP-fusion proteins or only MBP alone were expressed in *E.coli* and the cleared cell lysates were applied to the amylose resin (NEB), and incubated for 1 hour at RT followed by washing. Approximately 1ml of amylose resin with the bound fusion protein was incubated with 10 ml of cleared spinal cord extract (1mg/ml protein) over night either in the presence or absence of $Ca^{2+}$ (5 mM). This was followed by washing three times with 10 ml each and constant buffer conditions. The proteins were eluted by 10mM maltose. Eluted samples were concentrated by TCA precipitation and analysed by 10% SDS-PAGE according to Laemmli (Laemmli 1970).

### 7.4.2. Other pull-down assays to characterize TRPV1-Tubulin interaction.

In experiments aimed at determining if there is a direct interaction between tubulin and TRPV1 fragments, 40 µl of amylose beads bound to different MBP-TRPV1-Ct, MBP-TRPV1-Nt and only MBP were incubated with 50µg of αβ-tubulin in PEM-S buffer (50 mm PIPES; pH 6.8, 1mM EGTA, 0.2 mM MgCl2 and 100mM NaCl) with (3mM) or without presence of $Ca^{2+}$. In a similar manner, MBP-TRPV2-Ct and MBP-TRPV4-Ct were used to characterize tubulin interaction with TRPV channels. Experiments aimed at determining if there is a direct interaction between actin or neurofilaments and TRPV1 fragments, 40 µl of amylose beads bound with MBP-TRPV1-Nt, MBP-TRPV1-Ct or MBP-LacZ were incubated with 30µg of purified actin, or 40 µg purified neurofilament preparations. Proteins were dissolved in PEM-S buffer and binding experiments ware carried out either in the presence or absence of free $Ca^{2+}$ (3mM).

In experiments aimed at determining the binding region of tubulin in the C-terminal of TRPV1 by pull-down experiment, 40 µl of amylose beads bound to MBP-fusion proteins representing different C-terminal deletions and fragments were incubated with 50µg of αβ-tubulin in PEM-S buffer with or without presence of $Ca^{2+}$ (3mM). Experiments aimed at determining the salt dependency of tubulin binding to the C-terminal of TRPV1, 40 µl of amylose beads bound to MBP-TRPV1-Ct were incubated with 50µg of αβ-tubulin in PEM buffer with NaCl at the concentrations of 150mM, 300mM and 500mM.

### 7.4.3. Immunoprecipitation assay

F11 cells were transfected with the construct coding for full-length TRPV1 by

lipofectamine (Invitrogen). Two days after transfection, cells were extracted with 1% (w/v) sodium dodecyl maltoside at RT for 2 hours. The extract was cleared by centrifugation at 28,000 × g for 30 minutes. The extraction buffer contained 20 mM PIPES pH 6.8, 1 mM EGTA, 1 mM $CaCl_2$, 0.2 mM $MgCl_2$, 1% (w/v) sodium dodecyl maltoside, and 150 mM NaCl along with the protease inhibitor cocktail and benzonase (Merck). 600 µg of cleared cell extract at a protein concentration of 3 mg/ml were incubated with 2 µg of antibodies. Immuno-complexes were isolated with protein-G-sepharose (20 µl) and separated by SDS-PAGE.

For immunoprecipitation of TRPV1 from spinal cord, 1g (wet weight) of spinal cord tissue from rat were homogenized in the same buffer as used in the homogenization of F11 cells, followed by extraction with 1% (w/v) dodecyl maltoside and clearance of the extract by centrifugation. 6 ml of cleared extract at a protein concentration of roughly 1 mg/ml were incubated with 20 µg of the N-terminus-specific anti-TRPV1 antibody, and immuno-complexes were collected with 25µl protein-G-sepharose. For competition experiments using the blocking peptide, a roughly 300 fold molar excess of peptide over antibody was used. The proteins were separated by SDS-PAGE, and transferred to a nitrocellulose membrane by electro-blotting.

### 7.4.4. Blot overlay

For blot overlay experiments, either native proteins were spotted directly or denatured and SDS-PAGE-separated proteins were transferred from gels to nitrocellulose membrane. Membranes were blocked for 1 hour with 5% fat-free milk in PIPES buffer (50 mM, pH 6.8) with 150 mM NaCl. Subsequently the membranes were washed with PEM-S buffer (50mM PIPES; pH 6.8, 1mM EGTA, 0.2 mM MgCl2 and 150mM NaCl) three times. Membranes were air-dried and incubated with different proteins (protein concentration 1 µg/ml) for 1 hour at RT. Proteins were diluted in PEM-S-T (PEM-S buffer with 0.1%Tween 20). After binding the membranes were washed with the PEM-ST buffer three times (each time for 10 minutes) and incubated with PEM-S buffer with 0.1% formaldehyde for 30 minutes for cross-linking the overlaying protein. Finally the membranes were quenched with 100mM glycine in TBS buffer and processed for western blot analysis for detecting the overlaying protein/s.

# 7.5. Methods related to microtubule interaction and dynamics.

### 7.5.1. Co-sedimentation assay with taxol®-stabilized microtubules, microtubule formation and cold-induced disassembly of microtubules.

Approximately 140 μg of purified αβ-tubulin dimer in a total volume of 90 to 100 μl were incubated in modified PEM Buffer (20mM PIPES, pH 6.8, 0.2mM $MgCl_2$ and 1 mM EGTA supplemented by 1 μM taxol®, 5 mM GTP and 1 mM ATP) for 30 minutes at 37°C, to form microtubules (MT). After MT formation, 5 μg of purified MBP-TRPV1-Ct, MBP-TRPV1-Nt, and MBP were incubated with taxol®-stabilized MTs for 40 minutes at RT either in the presence or absence of 1 mM $CaCl_2$, followed by centrifugal separation of pellet (MT) and supernatant (free dimer) at 70,000 × g/30min/35°C.

For microtubule formation under taxol®-free conditions, 40 μg of tubulin dimer were used in PEM buffer with 1 mM $CaCl_2$ (optional), 5 mM GTP, 1 mM ATP in the absence of taxol®, and incubated for 30 minutes at RT.

For cold-induced depolymerization of MT, at first the MTs were allowed to form in PEM-S buffer (PEM buffer with 0.1 M NaCl) without taxol® as described above. $Ca^{2+}$ and MBP-TRPV1-Ct, or MBP-TRPV1-Nt, were added along with the tubulin dimer during the first cycle of polymerization. Polymerized MT (pellets) were isolated by centrifugation, resuspended in ice-cold PEM-S buffer by repeated pipette aspiration, and kept on ice for another 30 min. This was followed by the centrifugal separation of dimers (supernatant) and remaining polymers as cold-stable microtubules (pellet) at 70,000 × g (30 minutes, 4°C).

# 7.6. Methods related to cell biology.

### 7.6.1. Cell culture and transfection.

F11 cells and TRPV1-F11 cells were cultured in Ham's F12 medium (Invitrogen) supplemented with 20% FCS (Invitrogen). HEK cells were maintained in DMEM medium with 10%FCS. HeLa Cells were maintained in a humidified atmosphere that contained 5% $CO_2$ at 37°C. For transient transfection, lipofectamine (Invitrogen) was used according to the manufacturer's instructions.

### 7.6.2. Generation of a stable TRPV1-expressing F11 cell line*.

The cDNA encoding TRPV1 was sub-cloned into pBICD4 (Liu et al., 2000) employing the EcoRI and NotI restriction sites of the vector. F11 cells were transduced with retroviral particles obtained from a triple transfection of HEK293T cells with plasmids BICD4-TRPV1, pVPack-GP, and pVPack-eco (Stratagene). CD4-positive cells were stained using a phycoerythrin-conjugated anti-CD4 antibody (clone EDU-2, Dianova) and isolated by flow cytometry on a Becton Dickinson FACS Vantage cell sorter. Expression of TRPV1 in this cell line was confirmed by western blot analysis and immunofluorescence analysis. This cell line will be subsequently referred to as TRPV1-F11 cells.

* (This cell line was generated in Heidelberg by B. Schwappach).

### 7.6.3. TRPV1 activation, isolation of cytoskeleton (*in situ* and biochemical) from mammalian cells.

In order to visualize the effect of TRPV1 activation on the cytoskeleton, cells expressing TRPV1 transiently or TRPV1-F11 stable cells were grown on glass cover slips for 2 days. Cells were washed gently with Hank's balanced salt solution (HBSS buffer, Invitrogen) at RT, incubated with HBSS buffer supplemented with 1mM $CaCl_2$ and RTX (100 nM) for 1 minute, and either fixed immediately or further extracted with membrane-permeabilization buffer for 1 minute before fixation. Membrane-permeabilization buffer contained 50mM PIPES, pH 6.8, 1mM EGTA, 0.2 mM $MgCl_2$, 10% glycerol, and digitonin (50 μg/ml), and complete$^{TM}$ protease-inhibitor cocktail (Roche). Quick extraction of cells in this buffer permeabilizes the membrane, but essentially retains the cell morphology intact, and is thus suitable for observation of the stable cytoskeleton (Lieuvin et al, 1994). For blocking the TRPV1, cells were incubated with 1μM I-RTX for 10 minutes and activation of TRPV1 by RTX was done in presence of I-RTX.

For biochemical analysis of the activated cells by western blot analysis, TRPV1-F11 cells were scraped from the culture vessels, collected by a brief centrifugation and resuspended in HBSS buffer. An equal volume of suspension was distributed into different tubes. Cells were activated by addition of an equal volume of HBSS buffer supplemented with RTX and $CaCl_2$. In control experiments, an equal volume of only HBSS buffer was added. For looking at the cytoskeleton status of the cells after TRPV1 activation, an equal volume of 2x membrane-permeabilization buffer was added and mixed gently (no homogenization) for 1 minute followed by centrifugal separation of supernatants and pellets at 1.5 K/ 5 minutes at RT.

### 7.6.4. Assessment for nocodazole-resistant microtubules from mammalian cells.

For isolation of nocodazole-resistant microtubules from mammalian cells, the transfected or non-transfected cells were incubated with 1μM nocodazole for 15 minutes at 37°C, washed with HBSS buffer at RT, extracted with membrane-permeabilization buffer and fixed with PFA. Fixed cells were immunostained for α- or β-tubulin specific antibody and immunoreactivity is attributed to nocodazole-resistant microtubules.

# 7.7. Methods related to immunocytochemistry and microscopy.

### 7.7.1. Immunocytochemistry.

Cells were grown and transfected on glass cover slips. Two days after seeding or transfection, the cells were fixed either with 2% paraformaldehyde at room temperature (RT) or with 80% methanol in PBS (phosphate-buffered saline) at -20°C for 10 minutes, permeabilized with 0.4% Triton X-100 in PBS for 5 minutes, followed by incubation with 100 mM glycine dissolved in PBS for 1 hour. The cells were blocked with 5% normal goat serum or bovine serum albumin (BSA). After incubating the cells with the primary antibody for 1 hour at RT, the cells were washed three times with PBS supplemented with 0.1% Tween-20 (PBS-T). The cells were further incubated with secondary antibody diluted in PBS-T buffer. After incubation with secondary antibody/ies, the cells were washed three times with PBST buffer. The cover slips were finally mounted onto glass slides with fluoromount G (Southern biotechnology).

Majority of the anti-tubulin staining reported here was done with the YL1/2 antibody if not stated otherwise. The mouse monoclonal antibody against β-tubulin was used to study the effect of TRPV1-Ct on microtubule stabilization. Alexa-594-labelled phalloidin was used to visualize the actin cytoskeleton. Images were taken on a confocal laser-scanning microscope (Zeiss Axiovert 100 M) with a 63x-objective and analysed by the Zeiss LSM image examiner software.

### 7.7.2. Live cell imaging.

Biological processes like the effect of TRPV1 activation on growth cone movement; microtubule dynamics in TRPV1-expressing cells and transport of TRPV1 to the growth cone were visualized by live cell microscopy. For that purpose, F11 cells were seeded on glass cover slips (24 mm, A. Hartenstein, Würzburg, Germany). TRPV1-GFP or TRPV1 and tubulin-CFP were expressed in F11 cells by transient transfection.

For visualizing the transport of TRPV1, F11 cells expressing TRPV1-GFP were maintained in complete medium. These cells were monitored for visualizing the TRPV1-GFP transport process. For visualizing growth-cone movement regulation by TRPV1 activation, TRPV1-GFP expressing F11 cells were used. RTX (100nM) and $Ca^{2+}$ (1mM) was added to the complete medium during imaging. For experiments aimed at monitoring the tubulin dynamics after TRPV1 activation, Tubulin-CFP and TRPV1 were co-expressed in F11 cells by co-transfection. Two days after transfection tubulin-CFP-positive cells were monitored for

this purpose. Most of these tubulin-CFP-positive cells reveal disassembly of peripheral microtubules upon TRPV1 activation by RTX. The antagonist 5'-iodoresiniferatoxin (5´-IRTX, 1μm) was used for blocking TRPV1 in some experiments. All live cell images were captured with the help of a time series programme. A confocal laser-scanning microscope (Zeiss Axiovert 100 M) with a 63x-objective was used to capture the images. All live cell imaging was done at room temperature. Images were analysed later with the Zeiss LSM image examiner software.

# 7.8. Methods related to embryonic culture.

### 7.8.1. Embryonic DRG explant culture.*

The effect of endogenous TRPV1 channel activation was assayed for growth cone collapse following methods developed by Raper et al (Raper and Kapfhammer, 1990). Briefly, explants dissected from mouse E12 or chick E7 DRG were grown at 37°C overnight on poly-L-lysine/laminin-1 (Tebu-bio) coated Petri perm dishes in DMEM medium (Invitrogen) supplemented with hNGF-β (20 ng/ml, Boehringer). The next day, cultures were incubated with 100 nM RTX for 20 min for TRPV1 activation. As control, cultures were pre-incubated with the antagonist 5'-IRTX (1 μM) for 10 min followed by addition of RTX. The cultures were subsequently fixed with 0.25% glutaraldehyde in PBS and stained for α-tubulin subsequently. Cultures were visualized by 20x magnification and neurites were scored as being either spread or collapsed in several spherical bodies (varicosities).

* All the embryonic explant studies were done at the Max-Delbrück Center (MDC, Berlin-Buch) along with Dr. Hannes Smith.