# 3. Discussion:

While pain is a well-understood event as a physiological symptom, the mechanism of pain signalling is difficult to interpret in molecular, biochemical and cell biological level, mainly due to the complexity associated with this. In last few years, identification and characterization of few key molecules (for example, TRPV1) involved in pain pathway improved the knowledge about pain as a signalling event. However, pain, as a molecular mechanism remains unclear.

There is increasing evidence that the microtubule cytoskeleton, especially the integrity of the cytoskeleton plays a significant role in the transmission of nociceptive information (Dina et al. 2003; Topp et al. 2000; Alessandri-Haber et al. 2004; Tanner et al. 1998). A differential expression of cytoskeleton proteins in response to morphine (often used for pain relief) (Marie-Claire et al. 2004), an altered cytoskeleton morphology (Topp et al. 2000; Tanner et al. 1998), a differential expression/loss of a particular cell – all have been reported to be either a cause or a result of pain signalling. Additionally, for a long time it is known that microtubule cytoskeleton regulating drugs, namely Vincristine and paclitaxel (taxol®), two agents used as chemotherapeutics in the treatment of cancer, can produce a painful peripheral neuropathy (Polomano et al. 2001; Quasthoff et al., 2002).

Cancer pain is also a significant clinical problem because it is the first symptom of disease in cancer patients. Recently, involvement of TRPV1 in different kinds of cancer and cancer pain has been demonstrated (Lazzeri et al. 2005; Ghilardi et al. 2005; Sanchez et al. 2005; Vriens et al. 2004). All these reports in general suggested a functional role and an altered expression of TRPV1 in many cancerous conditions.

In this thesis work, identification of tubulin as a TRPV1 interacting protein and functional characterization of such an interaction re-enforces the importance of microtubule cytoskeleton in the pain transmission in many ways. Significance of tubulin as a TRPV1 interacting protein is discussed in details from structural point of view (chapter 3.1). Importance of tubulin-TRPV1 interaction as a cellular signalling event, as a link in different cancerous condition and the cross talk between TRPV1 and microtubule cytoskeleton is discussed in chapter 3.2. Finally, significance of tubulin - TRPV1 interaction in the formation of neuronal connection, especially in axonal growth cone movement and morphology regulation is discussed in chapter 3.3.

# 3.1. Tubulin as an interactor of TRPV1: Structural relevance.

### 3.1.1. Identification of C-terminal interacting proteins by a proteomics approach.

As TRPV1 acts as a non-selective $Ca^{2+}$ channel, activation of this channel results in the influx of $Ca^{2+}$. Both N- and C-terminal sequences form cytoplasmic domains are reported to be involved in the interaction with various proteins (table 3.1). The presence of three conserved ankyrin repeats within the N-terminal sequence also indicates that cytoplasmic domains are indeed involved in the protein-protein interactions. Desensitization of TRPV1 was reported to be a $Ca^{2+}$-dependent event. Involvement of PKC, PKA, CamKII and Calcineurin in the sensitization-desensitization of TRPV1 has been reported. Interestingly, $Ca^{2+}$ can modulate most of these reported interacting proteins directly (for example Calmodulin) or indirectly (like PKA, PKC, CamKII and Calcineurin). It is hypothesized that these interacting proteins are part of a larger signalling complex that recently has been termed as "signalplex" (Goel et al. 2005). It is believed that this signalplex is indeed modulated by $Ca^{2+}$.

In order to identify other members that constitute such a signalplex of TRPV1, a $Ca^{2+}$-dependent pull-down experiment was performed and a proteomics approach was taken to identify interacting proteins. Selection of MBP as a fusion-tag and expression of N- and C-terminal cytosolic domains of TRPV1 as MBP-fusion proteins results in good expression and more importantly in higher solubility of the fusion proteins. Subsequent pull-down experiments using MBP-TRPV1-Ct as bait and soluble spinal cord-extract as source of interacting proteins also turned out to be a successful approach to pull-down and identify

**Table 3.1. List of reported proteins interacting with the cytosolic domain of TRPV1.**

| Protein | Domain | Influence of $Ca^{2+}$ | Function |
|---|---|---|---|
| **PKC** | **C** | **Yes** | **Sensitization-desensitization** |
| **PKA** | **N** | **Yes** | **Sensitization-desensitization** |
| **Eferin** | **C** | **?** | **Signalling?** |
| **Calmodulin** | **N and C** | **Yes** | **Desensitization?** |
| **CamKII** | **C** | **Yes** | **Sensitization-desensitization** |
| **Syt IX** | **N** | **?** | **Exocytosis** |
| **Snapin** | **N** | **?** | **Exocytosis** |
| **TRPV2** | **C ?** | **?** | **Heteromer channel formation?** |

interacting proteins. It was observed that a number of interacting proteins interact predominantly in the presence of $Ca^{2+}$ (see figure 2.1). This indicates that many of these interactions are indeed $Ca^{2+}$-dependent and/or -sensitive. Identification of all these "potential" interactors is ongoing and Mathias Broser identified many of these proteins (table 3.2). Among all these identified proteins, some of them may be true interactors of TRPV1, and of functional significance. For example, identification of PKC isotypes in the pull-down sample not only reconfirms the interaction between TRPV1 and PKC as reported by many others (Premkumar and Ahern. 2000; Vellani et al. 2001; Numazaki et al. 2002; Crandall et al. 2002; Olah et al. 2002; Wang et al. 2004; Morenilla-Palao et al. 2004) but also indicates that purified MBP-TRPV1-Ct is able to form the signalplex when incubated with the soluble spinal-cord extract. Annexin VI might also be another true interactor for TRPV1. It has been reported that annexin II interacts with TRPV5 and TRPV6, other members of the TRPV subfamily, and regulates their functional expression (van de Graaf et al. 2003, van de Graaf et al. 2004). Very recently, phosphorylation of Annexin 1 by TRPM7 has been reported too (Dorovkov and Ryazanov, 2004).

A pull-down experiment with MBP-TRPV1-Nt as a bait to identify N-terminal interacting proteins was not very successful mainly due to the low expression and rapid degradation of the fusion protein during expression, which results in multiple smaller fragments. An attempt to express the N-terminal cytoplasmic domain of TRPV1 as 6X-Histidine tag was also not successful due to very low expression, low solubility and aggregate formation. To overcome this degradation problem, along with the MBP-tag at the C-terminus, an additional fusion tag like GST or 6-Histidine at the N-terminal end might be

**Table 3.2 . List of proteins identified in the MBP-TRPV1-Ct pull-down.**

| Protein | Methods used | Probable function |
|---|---|---|
| * **PKCγ** | MS, WB | signalling |
| * **PKCα** | WB | signalling |
| **α-tubulin** | MS, WB | transport, signalling |
| **β-tubulin** | MS, WB | transport, signalling |
| * **PUR α** | MS | RNA transport |
| * **Annexin VI** | MS, WB | channel insertion, signalling |
| * **Clathrin heavy chanin** | MS | endocytosis |

**Note:** The (*) sign indicate that the presence of these proteins in the MBP-TRPV1-Ct pull downed sample was identified either by MALDI-MS, or by western blot analysis, or by both methods. Further characterization is needed to confirm about these interactions. Mathias Broser in the research group of Prof. F. Hucho identified most of these proteins.

helpful. Using two different tags at two ends will result in the purification of only full-length product. Addition of MBP to the C-terminus is favourable as it provides better solubility to the fusion protein.

### 3.1.2.Tubulin as a $Ca^{2+}$-sensitive interactor of TRPV1.

A number of membrane receptors have been shown to interact specifically with either α- or β-tubulin (Table 3.3). Isoforms of the metabotropic glutamate receptors mGluR1 and mGluR7 (Ciruela and McIlhinney. 2001; Ciruela et al. 1999; Saugstad et al. 2002) $GABA_A$ receptor (Item and Sieghart, 1994) or subunits of the NMDA receptor (van Rossum et al. 1999) have been reported to interact with tubulin. Interaction of tubulin was also observed with GABAa receptor, VDAC channel and for $Na^+$-$K^+$-ATPase (Item and Sieghart. 1994; Carre et al. 2002; Vladimirova at al. 2002). A number of studies established a wide range of interactions between tubulin and different G-proteins and pointed to the functional implications of these interactions (Sarma et al. 2003; Popova et al. 1997; Roychowdhury and Rasenick 1997; Roychowdhury et al. 1999). Very recently, interaction of β-tubulin with TRPC1, another member of the TRP super family has been reported (Bollimuntha et al. 2005). Two other members of the same family, namely TRPC5 and TRPC6, have also been shown to contain tubulin as constituent of the "signalplex" (Goel et al. 2005).

**Table 3.3. Examples of transmembrane receptors interacting with tubulin.**

| Receptor | Function | References |
|---|---|---|
| **TRPV1** | MT dynamics, signalling | This work |
| **TRPV2** | ? | This work |
| **TRPV4** | ? | This work |
| **TRPC1** | Translocation | Bollimuntha et al. 2005 |
| **TRPC5** | Signalling | Goel et al. 2005 |
| **TRPC6** | Signalling | Goel et al. 2005 |
| **NMDA** | MT dynamics, Synaptic MT reorganization | van Rossum et al. 1999 |
| **VDAC** (Mitochondrial) | Apoptosis, Signalling? | Carre et al. 2002 |
| **$Na^+$, $K^+$-ATPase** | Development of brain? | Vladimirova et al. 2002 |
| **$H^+$-ATPase** | Glucose uptake in yeast | Campetelli et al. 2005 |
| **$GABA_A$** | Signalling | Item and Sieghart 1994 |
| **mGluR1** | Signalling | Ciruela et al. 1999 Ciruela and McIlhinney.2001 |
| **mGluR7** | Signalling | Saugstad et al. 2002 |
| **G-Protein coupled receptors** | Signalling | Popova et al. 1997 Roychowdhury and Rasenick 1997; Roychowdhury et al. 1999; Sarma et al. 2003 |

The study presented here not only proves the interaction of tubulin with the C-terminal cytoplasmic domain of TRPV1, but also with TRPV2 and TRPV4 (figure 2.20). Various transmembrane receptors interacting with tubulin are summarized in the table below (table 3.3). Very recently, glucose uptake by $H^+$-ATPase in yeast has been reported to be regulated by acetylated tubulin (Campetelli et al. 2005).

These interactions between tubulin and transmembrane receptors are not restricted to transport process. Tubulin interaction with transmembrane receptors often results in an altered microtubule stability-instability or dynamics. In many instances, tubulin-transmembrane receptor interactions are also involved in complex signalling events. With regard to the identification of β-tubulin as a TRPV1 interactive partner in a $Ca^{2+}$-sensitive screen, it is important to mention that tubulin has been shown to bind two $Ca^{2+}$ ions at its C-terminal sequence (Hayashi and Matsumura. 1975; Solomon. 1977; Serrano et al., 1986; Fong et al. 1988). A $Ca^{2+}$-dependent conformational change of tubulin has been detected (Soto et al. 1996). There are many examples also where tubulin interacts with other molecules in $Ca^{2+}$-dependent manner (Honda et al 2002). In addition, the amino-acid sequence (KLQRAITILDTEKSFLKCMRKA) from 797 to 819, located within the C-terminal cytoplasmic domain of TRPV1 is predicted to form a putative EF-hand motif. This sequence might also play a role in the $Ca^{2+}$-sensitivity observed in the interaction between TRPV1 and tubulin.

### 3.1.3. Tubulin binding site located within TRPV1.

By pull-down and microtubule binding experiments the C-terminal sequence of TRPV1 was observed to interact directly with soluble tubulin as well as with polymerized microtubules. In contrast, the N-terminus of TRPV1 was not observed to interact neither with soluble tubulin (see figure 2.7 and 2.8) nor with polymerized microtubules (see figure 2.10). Similarly, the N-terminus of TRPV1 fails to modify the properties of microtubules *in vitro* (see figure 2.12 and 2.14). In contrast with the N-terminus of TRPV1, over-expression of TRPV1-Ct results in formation of more stable and bundled microtubules *in vivo*. In agreement with these observations, TRPV1-ΔCt, a construct that lacks the entire C-terminal sequence (amino acid 681 to 838) of TRPV1 failed to stabilize tubulin at the neurite endings (see figure 2.47). From all these observations it seems that interaction with tubulin is exclusively mediated by the C-terminal sequence of TRPV1.

Further deletion within the C-terminus and the use of shorter fragments of the TRPV1-C-terminus indicate the presence of two small sequence stretches which can

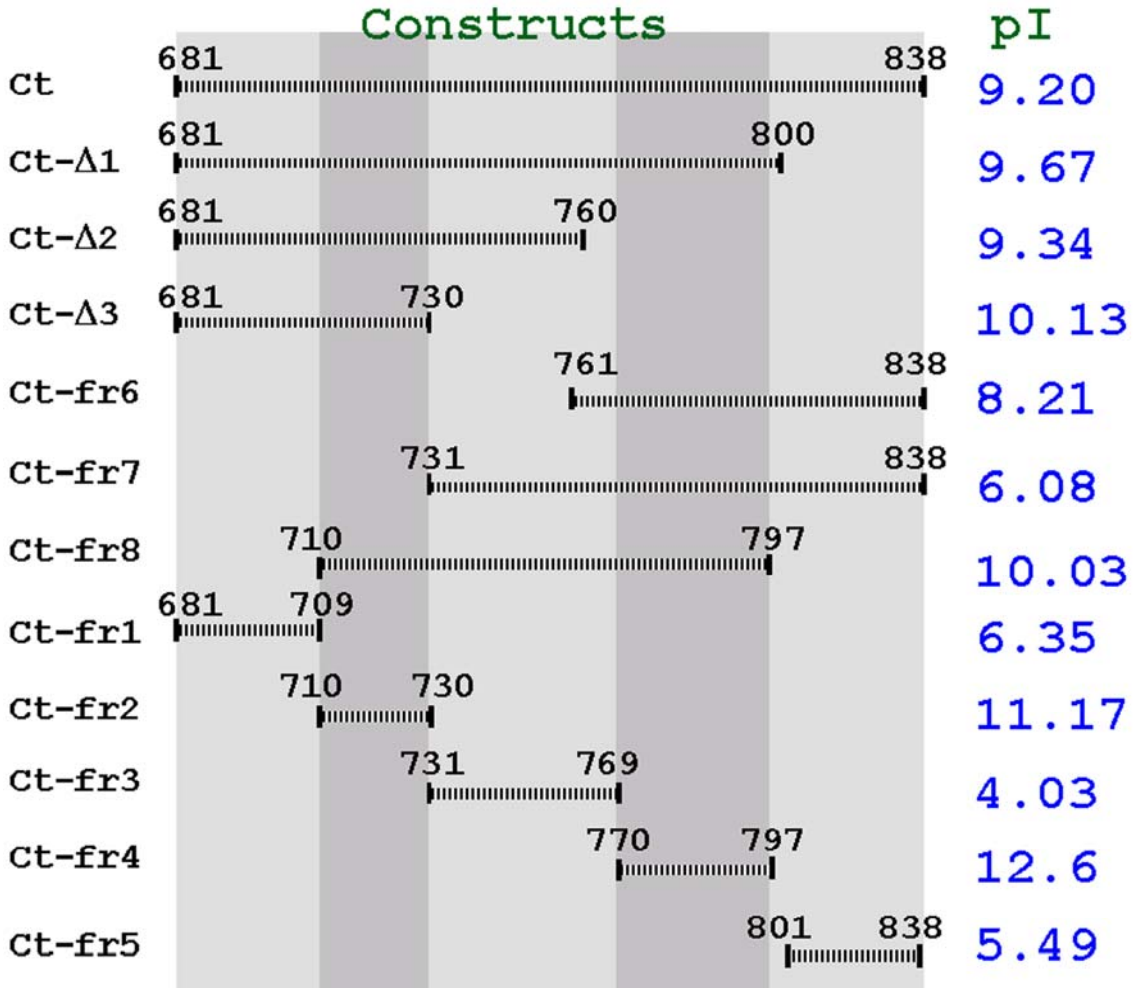


**Figure 3.1: TRPV1 may contain two tubulin-binding sequence motifs located within C-terminal cytoplasmic domains.** The C-terminal deletion constructs and fragments used to study tubulin binding are indicated. Corresponding isoelectric points are indicated on the right. Regions with basic pI are indicated with dark background and regions with lower pI are indicated by light background. All theoretical isoelectric points of the deletion constructs were calculated by using available software (www.expasy.org/tools/pi_tool.html).

| Species | Stretch 1 | Stretch 2 |
|---|---|---|
| | * * ** * * | ** * * * * * |
| Rat | -KSFLKCMRKAFRSGKLLQVGF- | -KRTLSFSLRSGRVSGRNWKNFALVPLLR- |
| Mice | -KSFLKCMRKAFRSGKLLQVGF- | -KRTLSFSLRSGRVSGRNWKNFALVPLLR- |
| Dog | -KSFLKCMRKAFRSGKLLQVGY- | -KRTLSFSLRSGRVSGRNWKNFSLVPLLR- |
| Human | -KSFLKCMRKAFRSGKLLQVGY- | -KRTLSFSLRSSRVSGRHWKNFALVPLLR- |
| Guinepig | -KSFLKCMRKAFRSGKLLQVGY- | -KRTLSFSLRSGRVSGRNWKNFALVPLLR- |
| Rabbit | -KGFLKCMRKAFRSGKLLQVGY- | -KRTLSFSLRSGRVSGRNWKNFALVPLLR- |
| Pig | -KSFLKCMRKVFRSGKLLQVGY- | -KRTLSFSLRSSRVAGRNWKNFALVPLLR- |
| Chicken | -SY-LNCLRRSFRSGKRVLVGI- | -KRNPSYCIKPGRVSGKNWK--TLVPLLR- |

**Figure 3.2. Two short sequence stretches located within the C-terminus of TRPV1 are conserved in mammals.** Shown is the sequence alignment based on TRPV1 sequences from different species. NCBI accession numbers are indicated. Rat (NP-114188), Mice (CAF05661), Dog (AAT71314), Human (NP_542437), Guinea pig (AAU43730), Rabbit (AAR34458), Chicken (NP_989903) and Pig (Ohta et al. 2005). Identical amino acids are shown in blue colour. Basic amino acids are indicated by asterisk (*).

positively influence tubulin interaction (amino acid number 710 to 730 for the 1st stretch and amino acid number 770 to 797 for the 2nd stretch respectively, see chapter 2.2). Interestingly, these sequence stretches are basic in nature (First sequence stretch 1 has pI of 11.17 and the second sequence stretch has pI of 12.6, respectively). The tubulin-binding capacity of these two short stretches is often suppressed by the presence of short neighbouring sequences that bear negative charges.

Binding capacity of the different smaller fragments and deletion constructs of TRPV1-Ct to tubulin and/or microtubules correlates well with two factors: The presence of these two short stretches and the overall pI of the constructs (figure 3.1, see also chapter 2.2). This is in full agreement with many observations that the majority of the tubulin interacting proteins contain short repeat sequences composed of basic amino acids. For example, microtubule-binding protein Tau isoforms contain three to four 18-amino acid long imperfect repeats separated by 13 to 14-amino acid long inter-repeat sequences (Goode et al. 2000, Himmler et al 1989). Doublecortin (Dcx), another microtubule-binding protein, contains two

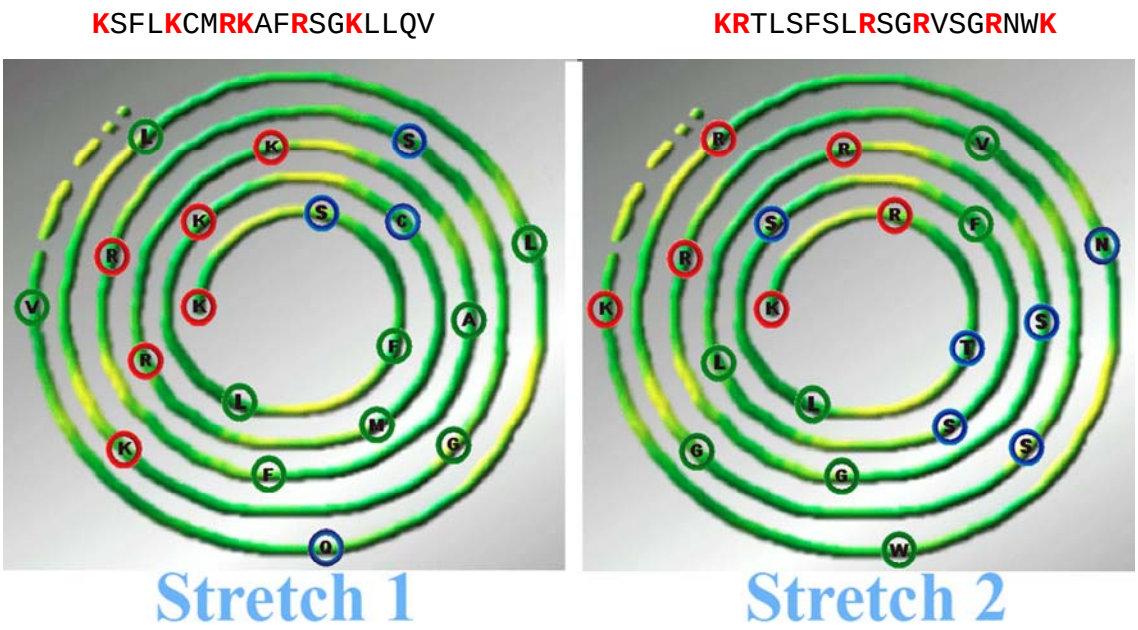


**Figure 3.3. Positively charged amino acids of stretch sequence 1 and 2 are clustered in one side of the helix.** Amino acids of stretch sequences 1 and stretch sequence 2 (in each case 19 amino acid-long, indicated in top) of the C-terminus of TRPV1 (rat) are plotted for helical distribution. Top-view of the helix is shown. HelixDrawV1.00 programme available at www.bioinf.man.ac.uk/~gibson/HelixDraw/helixdraw.html site has been used for this plotting. Positively charged amino acids (red) are marked with red circle, polar residues are marked with blue circle and hydrophobic residues are marked with green circles. Note the distribution of positively charged residues (red) in one side of the wheel.

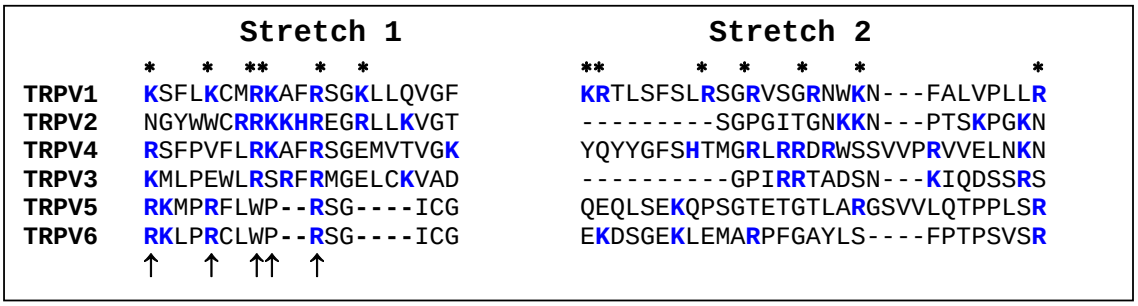


**Figure 3.4. The distribution of basic amino acids within the first stretch is conserved to certain extent in few other TRPV family members.** Shown is the sequence alignment of different TRPV members (based on sequences from rat species only). Alignment is done by using clustlaw software available in the expasy site. NCBI accession numbers are indicated. TRPV1 (NP-114188), TRPV2 (AAH89215), TRPV3 (NP-001020928), TRPV4 (NP-076460), TRPV5 (AAV31121) and TRPV6 (Q9R186). Basic amino acids are written in blue colour, and basic amino acids of TRPV1 are indicated by asterisk (*). Positions of basic amino acids that may represent the basis of a novel tubulin-binding motif are indicated by arrows.

repeat sequences with high pI (9.7 and 9.9) by which microtubule interaction is mediated (Taylor et al. 2000). Several microtubule-associated proteins (MAPs) also contain repeat sequences with basic pI important for microtubule binding (Noble et al. 1989; Al-Bassam et al. 2002; Lewis et al. 1988). Interestingly, the two short basic sequences present in TRPV1 are highly conserved in most of the known mammalian orthologous sequences (figure 3.2). The importance of these two stretch sequences in tubulin binding is further strengthened by the fact that all positively charged amino acids (located within these two stretches) are clustered in one side of the helix (figure 3.3). According to the model proposed by García-Sanz et al (see figure 3.7), the 1st stretch sequence is exposed and therefore easily accessible to interact (García-Sanz et al. 2004)**.** Taken together, it seems that TRPV1 has two tubulin binding sites at the C-terminal cytoplasmic domain. However it is not clear if these two sequences can bind tubulin independent of each other or the second sequence stretch favours the binding to the first one.

In addition, among members of the TRPV subfamily, similar sequences including the positively charged amino-acids within the first sequence stretch is conserved, especially in TRPV1, TRPV2, TRPV4, and TRPV3, but not so much in TRPV5 and TRPV6 (figure 3.4). The second sequence stretch is much less conserved in other members of the TRPV-subfamily (figure 3.4). In agreement with the presence of the first sequence stretch, both MBP-TRPV2-Ct and MBP-TRPV4-Ct bind to soluble tubulin and polymerized microtubules (figure 2.20). This may indicate that the first short basic amino-acid stretch of the TRPV1 represents a novel and unique tubulin-binding motif, which is also present in some other members of the TRPV subfamily.

### 3.1.4. Structure of the C-terminal cytoplasmic domain of TRPV1 and tubulin interaction.

So far no crystal structure or NMR structure of the C-terminal cytoplasmic domain of TRPV1 is available. This portion of TRPV1 does not contain any classical structural motif or domain that can be interpreted as a tubulin-binding domain. However, its structure has been predicted by two groups (Vlachova et al. 2003; García-Sanz et al. 2004) (figure 3.6 and 3.7). The structural prediction by Vlachova et al. is based on the high sequence homology (44%) of C-terminal cytoplasmic sequences of TRPV1 and the fragile histidine triad protein (FHIT), a tumour suppressor gene product (figure 3.5), for which a crystal structure is available. According to this structural model, the C-terminus of TRPV1 contains seven β- strands and two α-helices (Vlachova et al. 2003) (figure 3.6). Remarkably, FHIT was recently reported to be a tubulin-binding protein (Chaudhuri et al. 1999).

However, the structure predicted by García-Sanz et al. is different from what Vlachova et al. proposed. According to their model, the C-terminal cytoplasmic domain contains eight α-helices and three β-strands (figure 3.7). This structure is based on the similarity of TRPV1 with the HCN channel and also with the shaker-like $K^+$ channel (figure 3.8, García-Sanz et al. 2004). According to this model these two basic sequence stretches are partly unstructured and the first sequence stretch is exposed and accessible for interaction. Therefore interactions with other proteins in this sequence can stabilize this domain and thus make the interaction thermodynamically favourable.

```
1FIT    -SFRFGQHLIKPSVVFLKTELSFALVNRKPVVPGHVLVCPLRPVERFHD
C-tail  AQESKNIWKLQRAITILDTEKSFLKCMRKAFRSGKLLQVGFTPDGKDDY
          .    .    :: ::.:*.** **     **.. .*::*    : *  : .

1FIT    ---LRPDEVADLFQTTQRVGTVVEK--HFHG--TSLTFSMQDGPEAGQT
C-tail  RWCFRVDEVNWTTWNTN-VGIINEDPGNCEGVKRTLSFSLRSGRVSGRN
           :* ***      .*: ** : *.  : .*   :*:**::.*  :*:.

1FIT    VKHVNVHVLPRKAGDFHRNDSIYEELQKHDKEDFPASWRSEEEMAAEAA
C-tail  WKNFALVPLLRDASTRDRHATQQEEVQLK---HYTGSLKPEDAEVFKDS
          *:. :  * *.*.  .*: :  **:* :    .:..* :.*:  . : :

1FIT    ALRVYFQ
C-tail  MVPGEK-
          :
```

**Figure 3.5. Sequence alignment of the cytoplasmic C-terminal domain of TRPV1 from A690 to K838 and the template protein FHIT.** Identical and similar amino acids of the stronger groups are indicated with an *asterisk* and *colon*, respectively. These amino acids should conserve the structure with a probability of >95%. *Dots* indicate similar amino acids of the lower groups that should conserve the structure with a lower probability. (Adapted from Vlachova et al. 2003).

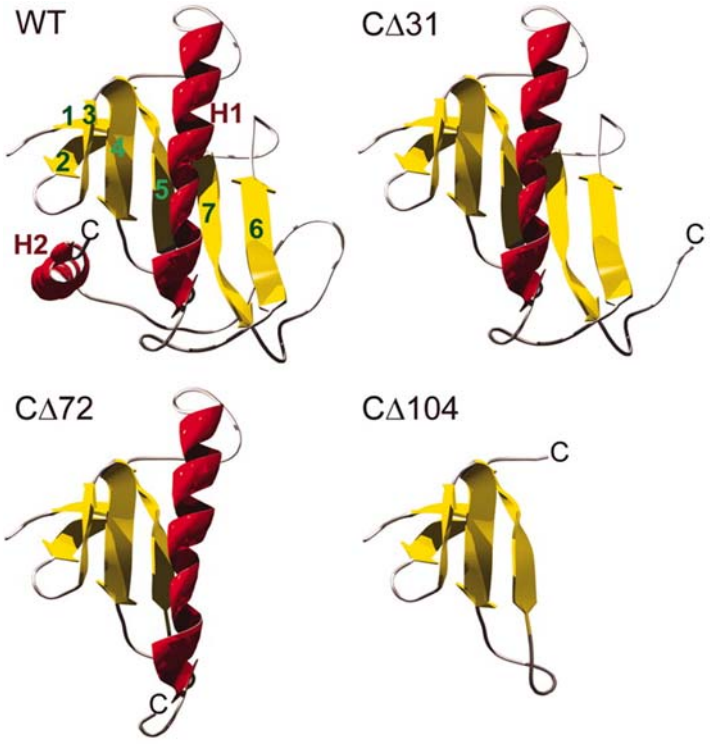


**Figure 3.6. Predicted structure of the complete C terminal of TRPV1 and the truncated mutants (According to Vlachova et al. 2003)**. WT, Ribbon diagram of the wild-type C terminus (residues A690-K838). Homology modeling predicts two α-helices (H1, H2) and seven β-strands (1-7). Antiparallel strands 1 and 2 form a β-hairpin, and strands 3-7 form a five-stranded antiparallel sheet. CΔ31, This mutant (residues A690-T807) lacks the α-helix H2. CΔ72, In this construct (A690-C766), secondary structural elements H2 and β-strands 6 and 7 are missing. CΔ104, the predicted structure of the truncated construct (A690-G734) consists only of β-strands 1-5.

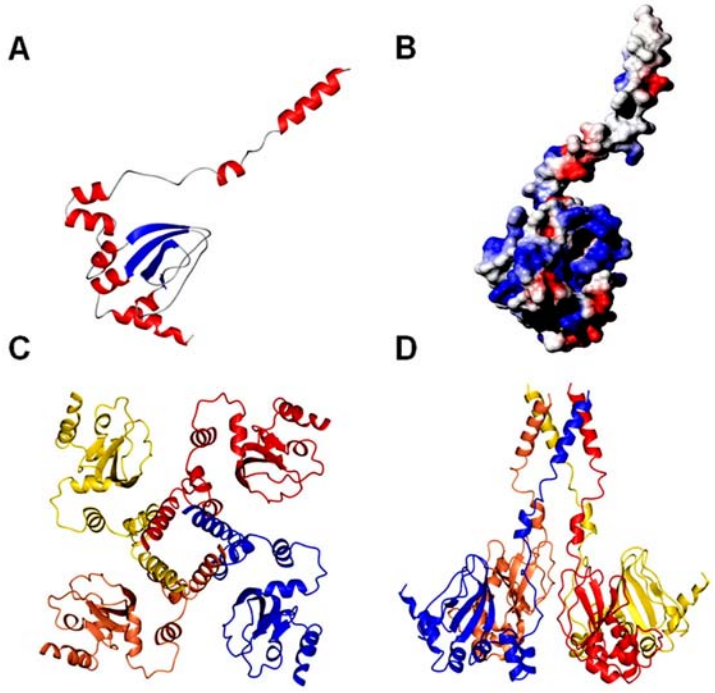


**Figure 3.7. Molecular model for the C terminus of TRPV1 (According to García-Sanz et al. 2004). a.** Molecular model of a TRPV1-C monomer. The structure of the mouse HCN2 C-linker and cyclic nucleotide binding domain construct bound to cGMP was used as a scaffold (PDB 1Q3E (PDB ). **b.** Electrostatic surface potential of monomeric TRPV1-C. **c-d.** TRPV1-C putative tetramer representation parallel to the fourfold axis of symmetry from the intracellular side and normal to the plane of the membrane, respectively.

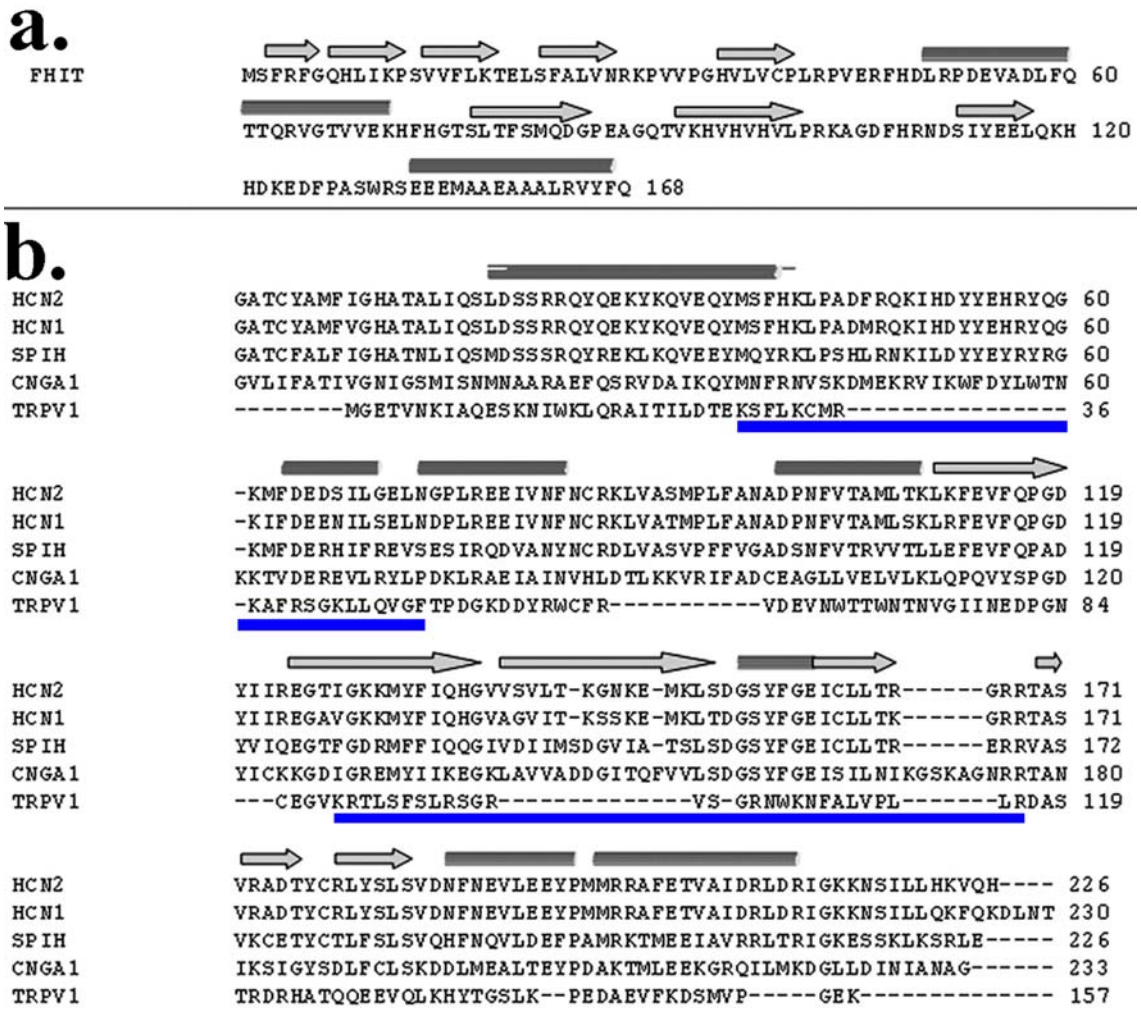


**Figure 3.8. TRPV1-C terminus contains structural similarities to C terminus of HCN channels. a.** Primary and secondary structures of the human FHIT protein (Swissprot P49789). **b.** Amino acid alignment of the C terminus of cyclic nucleotide-gated HCN2 (GenBank NP_001185), HCN1 (GenBank NP_066550), SPIH (GenBank CAD_43449), CNGA1 (Swissprot P29973) ion channels and TRPV1. Bars and arrows denote α-helical and β-strand secondary structure elements, respectively. Predicted elements for TRPV1 were compared with those present in the structure of HCN2. Adapted from García-Sanz et al. (2004). The two basic sequence stretches are underlined in blue.

### 3.1.5. Biological significance of the interaction between TRPV1 and microtubules.

Cross-linking experiments indicate that the C-terminus of TRPV1 interacts more specifically with the β-tubulin than with the α-tubulin (figure 2.15). Microtubule plus ends often end with protofilaments carrying β-tubulin at the end (see figure 3.15). The higher specificity of the TRPV1-Ct for β-tubulin and the ability to stabilize microtubules therefore indicate that the observed interaction may have a more profound effect at the level of a microtubule plus end rather than at the minus end. Interaction of microtubules with the C-

terminus of TRPV1 alters physico-chemical properties of microtubules and results in more stable microtubules *in vitro* and *in vivo*. The biological significance of such an effect fits well with the observation that at resting stage pioneer microtubules at the P-zone of growth cones are stabilized by TRPV1-enriched patches (see figure 2.45, discussed later in chapter 3.3).

### 3.1.6. Importance of the C-terminus of TRPV1 in channel function and regulation.

C-terminal sequence of TRPV1 is attributed to several other interactions and functions (figure 3.9). For example, the region comprising amino-acid residues 684 to 721 has been shown to be important for tetramerization (Garcia-Sanz et al. 2004). Amino acid 761 has been shown to be important for the capsaicin sensitivity (Jung et al. 2002). Vlachová et al demonstrated that the C-terminal tail of TRPV1 carries structural determinants rendering the receptor sensitive to heat and capsaicin. They could also demonstrate that deletion of distal 72 amino acids results in a decline of capsaicin-, pH-, and heat-sensitivity of the receptor (Vlachová et al 2003). Moreover, the C-terminal tail contains phosphorylation sites used by protein kinase C (Numazaki *et al*. 2002) and $Ca^{2+}$/calmodulin-dependent protein kinase II, and usage of this site affects the TRPV1 desensitization characteristics in the presence of $Ca^{2+}$ (Jung *et al*. 2004). Interestingly, two more serine residues (at position 774 and 820) located within the C-terminus of TRPV1 have been shown to be phosphorylated by

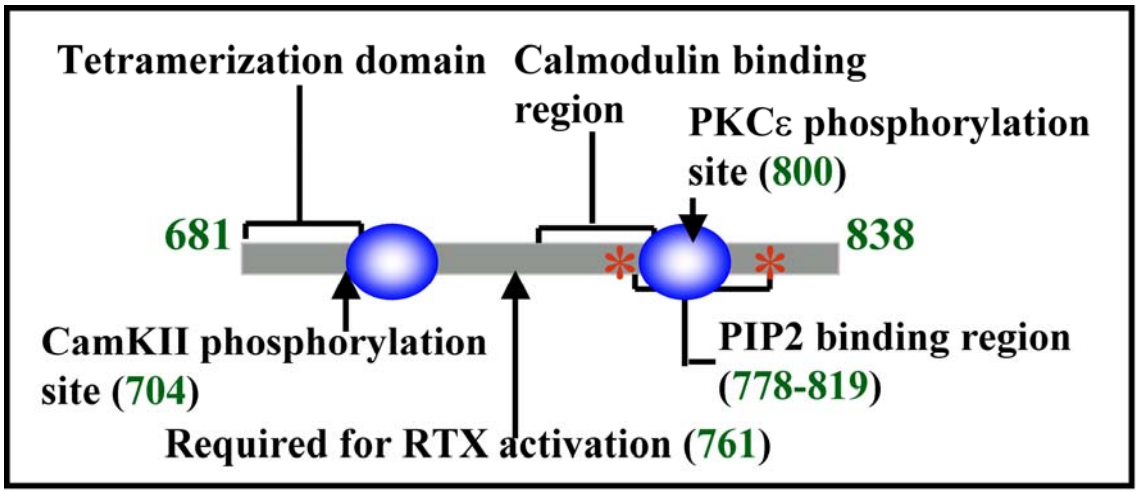


**Figure 3.9. C-terminus of TRPV1 contains structurally and functionally important residues.** Arrows and brackets indicate different important regions and residues located within the C-terminus. Numbers of amino acids are written in green. Blue areas indicate regions of tubulin binding. The exact binding site for Eferin is not known. Putative PKA and PKC phosphorylation sites (S774 and S820) are indicated by asterisk (∗) sign. The drawing is not exactly to scale.

PKA *in vitro* (Bhave at al. 2003; Mohapatra and Nau, 2003). Furthermore, Numazaki *et al* detected a short sequence within the C-terminal tail that is critical for receptor desensitization (Numazaki *et al*. 2003). They could also prove that this sequence carries a binding site for calmodulin. Furthermore, the C-terminal sequence of TRPV1 was demonstrated to contain a binding site for the phosphoinositide PIP2, which negatively regulates the ion channel activity (Prescott and Julius 2003). Recently, eferin, (EF-hands-containing Rab11/25-interacting protein) was reported to bind at the C-terminal cytoplasmic domain of TRPV1 (Lee SY 2005). However, the exact sequence where it binds has not been determined yet.

Notably, several functionally relevant structural determinants were all mapped to a certain sequence of the TRPV1 C-terminal tail, (amino-acid residues 760–800, based on the rat TRPV1 primary structure), which may represent a 'hot spot' for TRPV1 regulation. However, if tubulin binding to the C-terminus of TRPV1 alters any other interactions, is not explored yet. Taken together, it seems that the cytoplasmic domain interacts with a number of proteins and forms a “signalplex” anchored to the cytoskeleton.

# 3.2. TRPV1 activation and microtubule cytoskeleton.

### 3.2.1. Rapid disassembly of dynamic tubulin due to TRPV1 activation may be partly independent of direct $Ca^{2+}$ influx and may involve allosteric-proteins/enzymes.

Activation of TRPV1 results in a selective depolymerization of microtubules into soluble tubulin. This depolymerization affects primarily unmodified and tyrosinated tubulins, which are known to be part of the dynamic microtubules (Gundersen et al., 1984; Kreis. 1987; Wehland and Weber. 1987). The mechanism of tubulin cytoskeleton disruption upon TRPV1 activation, which was not the subject of this study, still remains unclear. It is known that $Ca^{2+}$ has a depolymerizing effect on microtubules *in vitro* (Karr et al., 1980; Job et al., 1981). Depolymerizing effects of $Ca^{2+}$ on microtubules were demonstrated *in vivo* and it has been shown that $Ca^{2+}$ leads to two distinct processes, the "dynamic destabilization" (a direct depolymerizing effect of $Ca^{2+}$ on microtubules) and "signal-cascade-induced fragmentation" of microtubules (Lieuvin et al., 1994).

In absence of any known specific mechanism, which can explain the reason behind TRPV1-activation mediated rapid disassembly of dynamic microtubules; several experiments indirectly indicate that this process is partly independent of $Ca^{2+}$ influx, and that enzymatic pathways/allosteric proteins might also be involved. For example, while microtubule disassembly due to TRPV1 activation could be perfectly blocked by using 5'I-RTX, a potent antagonist of TRPV1, a weak antagonist like capsazepine failed to do so. An excess of capsazepine was also not able to block the disassembly of microtubules. Addition of EGTA, a $Ca^{2+}$-chelator in the extra cellular medium along with $Ca^{2+}$ was also not successful in preventing disassembly of microtubules (data not shown). More importantly, a combination of both EGTA and excess capsazepine in the medium was also not effective. Disassembly of microtubules due to activation of TRPV1 is not dependent on the level of TRPV1 expression as stably transfected TRPV1-F11 cells that express a much lower level of TRPV1 exhibit the similar effect upon TRPV1 activation (see figures 2.30 and 2.31). All these findings indicate that activation of a very low number of receptors for a short time and a catalytic amount of $Ca^{2+}$ influx is able to recruit a profound effect resulting in rapid disassembly of microtubules. This indirectly suggests the possibility that this effect is partly independent on the amount of $Ca^{2+}$ influx through TRPV1 *per se*. In other words, it suggests a scenario of "amplification through a signalling cascade" rather than a direct effect of $Ca^{2+}$ on microtubules. In agreement with this notion, recently a $Ca^{2+}$-independent but TRPV1-activation-mediated cytotoxicity has been reported (Reilly et al. 2005). Remarkably, this cytotoxicity due to

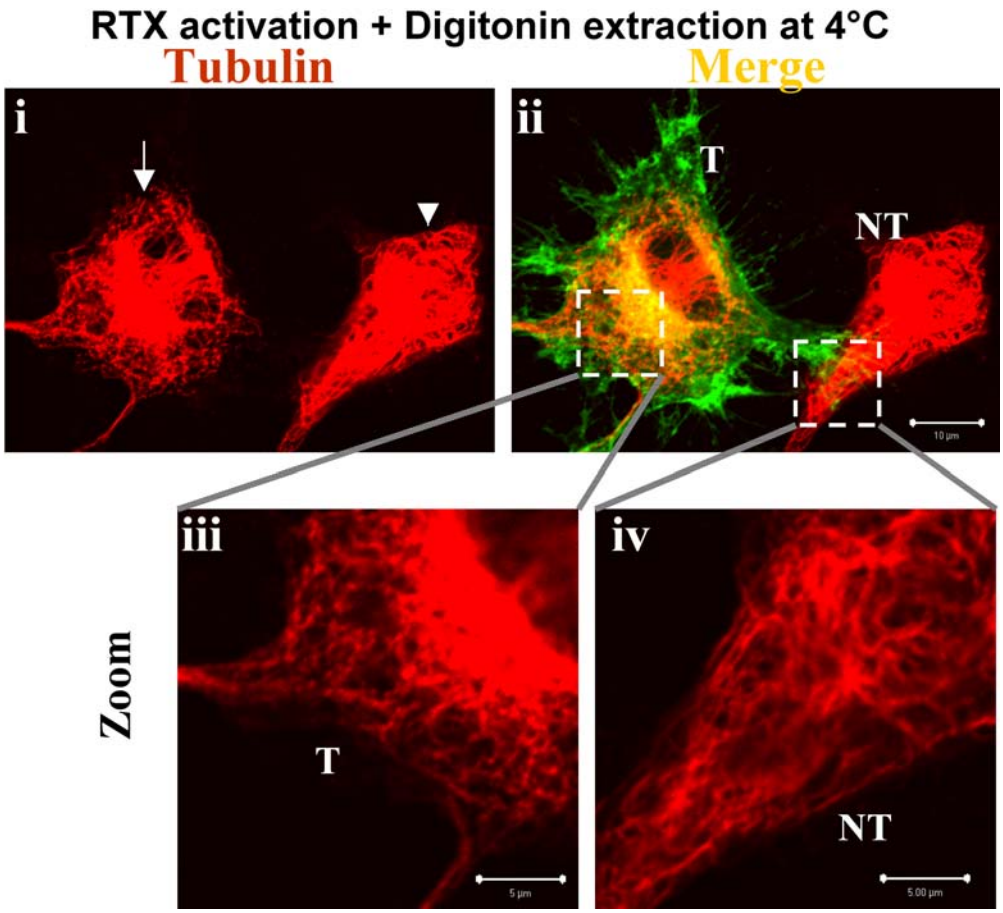


**Figure 3.10. Enzymatic/metabolic activity is required for MT disassembly by TRPV1 activation.** Activation of TRPV1 does not result in the disassembly of microtubules when activation is done at low temperature. Cells were first adjusted to room temperature to avoid cold shock. Subsequently cells were placed on ice for 5 minutes, and washed with ice-cold HBSS buffer. Cells were activated (1 minute) with ice-cold HBSS buffer supplemented with RTX and 1mM $Ca^{2+}$ and extracted with an ice-cold isotonic buffer containing digitonin. Cells were fixed immediately and processed for immunostaining to detect TRPV1 (green) and tubulin (red). Bright staining for TRPV1 distinguishes the TRPV1-expressing cells (indicated by arrow) from non-expressing cells (indicated by arrow head). No reduction in the tubulin staining of the TRPV1-expressing F11 cells was observed (ii). Enlarged areas (iii) of the TRPV1-expressing cell (indicated by dashed white box in ii) and the non-transfected cell (iv) reveal no change in the microtubule fibres even after activation with RTX. Fine filamentous microtubules all over the cell body and stable MTOC were clearly visible in TRPV1-expressing cells even after activation and extraction. Scale bar 10 µm (for i-iii) and 5 µm for (iv-v). See also figure 2.26-2.27 and 2.30-2.31).

TRPV1 activation could not be blocked by excess amount of Capsazepine and extra cellular EGTA, but could be blocked by 5'I-RTX. Secondly, involvement of allosteric proteins and/or enzymatic (metabolic) activities as signalling events in this disassembly of microtubules is supported by the fact that transient expression of TRPV1 does not result in the loss of

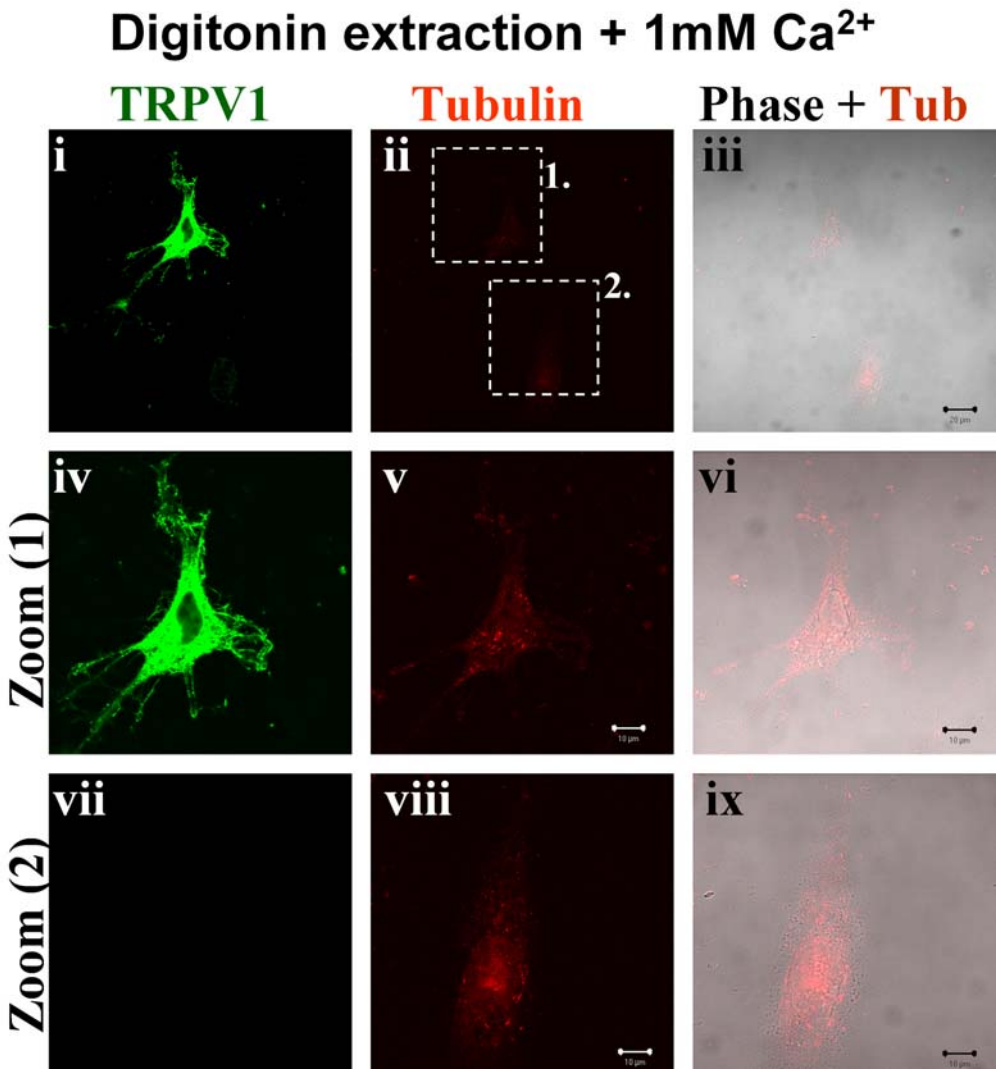


**Figure 3.11. $Ca^{2+}$-saturation results in complete disassembly of all microtubule-based structures including MTOC.** TRPV1 was expressed in F11 cells by transient transfection. Cells were first extracted with digitonin in an isotonic buffer for 1 minute and then incubated with the isotonic-buffer supplemented with 1mM $Ca^{2+}$ before fixing. Cells were subsequently immunostained for TRPV1 (green) and tubulin (red) with a rabbit anti-TRPV1 antibody and with rat monoclonal antibody YL1/2 respectively. Shown are the indirect immunofluorescence confocal images of F11 cells expressing TRPV1 and non-transfected cells (i-iii) and the enlarged areas depicting the TRPV1-expressing cell (iv-vi) and the non-transfected cell (vii-ix). Immunoreactivity for tubulin was almost absent in the cell body as well as in the MTOC structure in both transfected and non-transfected cells. Faint immunoreactivity was observed for tubulin in the MTOC when imaged at unexceptionally higher gain (v and viii). In right, superimposition of phase contrast and confocal immunofluorescence image for tubulin are shown (iii, vi and ix). Scale bar 20 µm (i-iii) and 10 µm (iv-ix). See also figure 2.26-2.27 and 2.29-2.31).

microtubules when activation is performed at low temperature (4°C). No significant difference in the microtubule pattern was observed between transfected and non-transfected cells under this condition (figure 3.10). Though the open-probability of TRPV1 is much

reduced at low temperature (Babesa et al. 2002), it is expected that in cells over-expressing TRPV1 a small number of receptors will open within a time frame of 1 minute upon RTX application. However, allosteric protein and other enzymatic activities as well as metabolic activities are expected to be low at low temperature. This indicates that TRPV1 activation does not alter the microtubule cytoskeleton when enzymatic and/or allosteric protein activities are low. This may suggest requirement of an additional step/s (other than a direct effect of $Ca^{2+}$) involving enzymatic or metabolic activity. However, $Ca^{2+}$ influx may have a synergistic effect. In this regard it is important to mention that some of the TRPV1 activation-mediated events could not be blocked by EGTA or with the weak antagonist Capsazepine (Reilly et al. 2005). This suggests that certain TRPV1 activation-dependent events are $Ca^{2+}$-independent.

Thirdly, TRPV1-expressing cells after activation and detergent extraction retain intact MTOC, a structure made of stable microtubules. Residual anti-tubulin immunoreactivity at the cell body is often visible. However, the status of the microtubules and MTOC is different when the cells are first extracted with digitonin and then incubated with an isotonic buffer supplemented with 1mM $Ca^{2+}$ for 1 minute. Under these conditions of $Ca^{2+}$-saturation, loss of microtubules is much more drastic and all cells reveal no tubulin immunoreactivity at all (figure 3.11). Even at the perinuclear region no tubulin immunoreactivity was observed, indicating a complete loss of MTOC structure due to $Ca^{2+}$-saturation. This again indicates that the actual concentration of $Ca^{2+}$ within TRPV1-transfected cells after activation does not exceed the threshold $Ca^{2+}$ level that is necessary for MTOC disassembly.

Finally, there are possibilities by which a catalytic amount of $Ca^{2+}$ influx can trigger a rapid disassembly of microtubules. Previously a number of studies demonstrated that calmodulin could cause for a rapid disassembly of microtubules in presence of a very catalytic amount of $Ca^{2+}$, but not in absence of $Ca^{2+}$ (Job et al. 1981; Lee and Wolff. 1982; Yamamoto et al. 1983; Keith et al. 1983). An activation of $Ca^{2+}$-dependent proteases (for example calpine) may occur upon TRPV1 activation, which triggers proteolysis of structural proteins as a downstream effect (Chard et al., 1995). Activation of different caspase pathways (mainly Caspase 8) due to TRPV1 activation was also reported (Sancho et al. 2003; Shin et al. 2003). Presence of fragmented microtubules all over the cell (see figure 2.27) suggest that katanin, a specific enzyme that cleaves microtubules may also be involved in this process (McNally and Vale, 1993). However, Western blot analysis with TRPV1 activated cell extracts, I observed no smaller fragments of tubulin, actin or neurofilaments (see figure 2.33). This suggests that these structural proteins are most likely not the prime targets for proteolytic cleavage.

### 3.2.2. The microtubule cytoskeleton as downstream effector for TRPV1 activation.

TRPV1 not only interacts with soluble tubulin in a $Ca^{2+}$-sensitive manner, but also with polymerized microtubules. Interactions of TRPV1 with polymerized microtubules also modulate the physico-chemical properties of microtubules. In addition to that, the integrity of the tubulin cytoskeleton is indeed affected by TRPV1 activation.

Activation of TRPV1 results in a selective depolymerization of microtubules into soluble tubulin. This depolymerization affects primarily unmodified and tyrosinated tubulins, which are known to be part of the dynamic microtubules (Gundersen et al. 1984; Kreis. 1987; Wehland and Weber. 1987). Interestingly, some microtubules remain attached to TRPV1 after activation of the ion channel. This is due to a stabilizing effect of the C-terminal portion of TRPV1 on the microtubules. This is supported by the finding that over-expression of the C-terminal fragment of TRPV1 alone in F11 cells leads to bundling and stabilization of microtubules, which makes them detergent- and nocodazole-resistant. Destabilization of microtubules by TRPV1 activation and stabilization of microtubules by the C-terminus of TRPV1 indicate that a fine balance exists between TRPV1 and the microtubule cytoskeleton. Therefore TRPV1 displays characteristics that render it a good candidate for a molecule causing remodelling of the microtubule cytoskeleton in pain transmission. Interestingly, activation of TRPV1 does not affect the stability of the neurofilament cytoskeleton, and affects the actin cytoskeleton only to a certain extent as some actin appears in the soluble fraction after TRPV1 activation (see figure 2.28 and 2.33). This increment of actin in the soluble fraction is not surprising as within the cell both actin and microtubule filaments are interconnected (Griffith and Pollard. 1978; Griffith and Pollard. 1982). In contrast to the microtubule cytoskeleton, both actin and neurofilament cytoskeletons remain as intact polymers in TRPV1-activated and detergent-extracted cells (see figure 2.28). This indicates that the tubulin, but neither the actin nor neurofilament cytoskeleton acts as an effector downstream of TRPV1.

Interestingly, regulation of the microtubule cytoskeleton by TRPV1 activation may have profound biological significance in systems where the microtubule cytoskeleton plays an important role. For example, neuronal growth-cone morphology and movements are highly regulated by TRPV1, and its activation (discussed later in details, see chapter 3.3). Furthermore, an altered urinary bladder function, especially altered bladder stretching in TRPV1-knockout mice (*trpv1*-/-) has been reported (Birder et al. 2002). It has been suggested that this altered urinary bladder function is due to the ability of TRPV1 to associate and regulate some mechanosensory complex which is not recapitulated in TRPV1-knockout mice. Regulation of the microtubule cytoskeleton, in particular disassembly of

microtubules by TRPV1 activation may well explain the reason behind the altered stretching properties of urinary bladder in TRPV1-knockout mice. Moreover, Maccarrone et al. recently reported (Maccarrone et al. 2005) that TRPV1 is present in the sperm acrosome and also throughout the tail region (figure 3.12). Behaviour and motility of the sperm is known to be regulated by its microtubule cytoskeleton. TRPV1 activity is reported to be important for sperm function (Maccarrone et al. 2005). However, if TRPV1 activation may play a role in regulating sperm-tail motility through microtubule cytoskeleton is remaining to be explored.

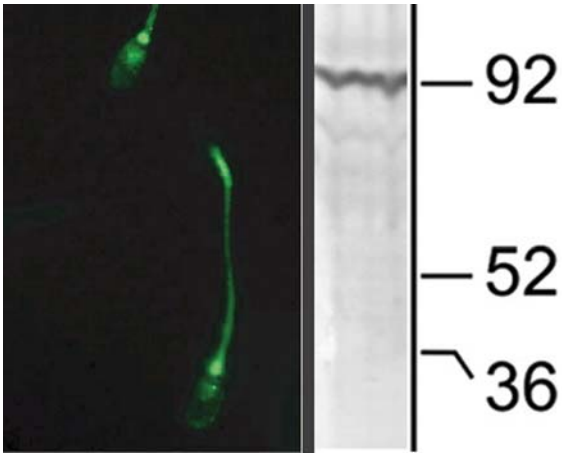


**Figure 3.12. Presence of TRPV1 in sperm tail.** Immunofluorescence and Western blot analysis of boar sperms with anti-TRPV1 antibody. Taken from Maccarrone et al. (Maccarrone et al. 2005)

### 3.2.3. Involvement of cytoskeleton in pain pathway.

There is increasing evidence that the cytoskeleton plays a significant role in the transmission of nociceptive information (Dina et al. 2003; Topp et al. 2000; Alessandri-Haber et al. 2004; Tanner et al. 1998). For example, prolonged stimulation of responsive neurons with capsaicin has been known for a long time to induce neuronal cell death (Jansco et al. 1984). In fact, retraction and degeneration of sensory neurons, which may well involve events that affect the cytoskeleton integrity, may be the basis for the analgesic effect of topical capsaicin-cream treatment (MacMahon et al. 1991). Cytotoxicity due to TRPV1 activation and subsequent deletion of TRPV-expressing neurons was also reported for other parts of the nervous system (Karai et al. 2004; Kim et al. 2005). Many other studies also demonstrated the direct link between a progressive cytotoxicity and subsequent cell death with the TRPV1 activation (Sancho et al. 2003; Agopyan et al. 2003; Agopyan et al. 2004; Puntambekar et al. 2004; Reilly et al. 2005; Qiao et al. 2005; Kim et al. 2005; Amantini et al. 2004; Contassot et al. 2004; Movsesyan et al. 2004; Hartel et al. 2005). However, if these cytotoxicity and cell death is due to disassembly of the microtubule cytoskeleton has not been examined.

This apparent specificity for the microtubule cytoskeleton is remarkable in the light of the well-established role of microtubule-active drugs in neuropathic pain. Vincristine and paclitaxel (commonly known as taxol®), two agents used as chemotherapeutics in the treatment of cancer, can produce a painful peripheral neuropathy (Polomano et al., 2001; Quasthoff et al. 2002; Hudes at al. 1992). Notably, both vincristine and taxol® regulate microtubule stability. Interestingly, it has been also reported that TRPV1 is involved in bone-cancer pain (Ghilardi et al., 2005). The precise mechanism that links the vincristine and paclitaxel action to peripheral neuropathy, however, is currently unclear. On the other hand, there are reports that the integrity of the cytoskeleton plays an important role in the development of persistent pain states in certain experimental paradigms (Dina et al., 2003). Furthermore, TRPV4, another member of the TRPV family of ion channels, has been reported to be essential for the development of pain in a rat model of paclitaxel-induced neuropathy (Alessandri-Haber et al., 2004). In summary, all these reports suggest that the effect of TRPV1 on the microtubule cytoskeleton may well be a previously unknown aspect in TRPV1-mediated pain transmission, particularly in the context of physio-pathological situations.

### 3.2.4. Regulation of TRPV11 channel function by microtubule drugs: Is TRPV1-tubulin interaction functional both ways?

Taxol® (paclitaxel), a well-known drug that provides stability to the microtubules was observed to be effective against microtubule disassembly due to TRPV1 activation. In presence of taxol®, activation of TRPV1 by RTX does not result in the disassembly of microtubules (data not shown). Presence of taxol® also results in significant delay in the formation of varicosities after RTX application to the mice DRG explants (data not shown). Though initially this suggested that taxol®-stabilized microtubules are more resistant against TRPV1-activation-mediated-microtubule-disassembly phenomena in general, subsequent electrophysiological experiments (in collaboration with Prof. Thomas Baukrowitz and Dr. Hariolf Fritzenschaft at Zena, Germany) indicate that taxol® significantly blocks the TRPV1 channel opening. A very fast decrease of the capsaicin-induced current was observed in presence of taxol® (data not shown). This inhibition of capsaicin-induced currents by taxol® is proved to be a dose-dependent phenomenon (figure 3.13). If taxol® can modulate the heat-evoked and low pH-mediated currents of TRPV1 too, have not been tested yet. Interestingly, other microtubule drugs like nocodazole and vincristin also reveal a change in the capsaicin-

induced currents to a certain extent (data not shown).

However, the exact mechanism by which taxol® exerts this effect is not clear. All these drugs are known to bind at different sites of the tubulin molecule (Nogales E. 2001). Binding of these drugs changes the conformation of tubulin dimers considerably and this conformational change ultimately results in altered microtubule dynamics. One possibility may be that taxol®-induced conformational change of TRPV1-associated tubulin inhibits the TRPV1 channel opening. Another possibility may be that taxol®, being a small molecule, can directly block the pore of the TRPV1 channel.

Nevertheless, the changes in capsaicin-induced currents produced by microtubule drugs clearly indicate the existence of a bi-directional regulation between TRPV1 and microtubule cytoskeleton. However, if inhibition of TRPV1 currents by taxol® is actually mediated by a change in the tubulin conformation or an effect due to direct channel blocking remains to be explored. It is also not known if this inhibitory effect of taxol® is specific for TRPV1 or if taxol® can exerts a similar effect on other TRP channels. It is not clear if other members of the TRPV subfamily, especially TRPV2 and TRPV4, which interact with soluble tubulin and polymerized microtubules, are affected by taxol®. In this regard it is important to mention that TRPV4 has been reported to be essential for the development of pain in a rat model of taxol®-induced neuropathy (Alessandri-Haber et al., 2004).

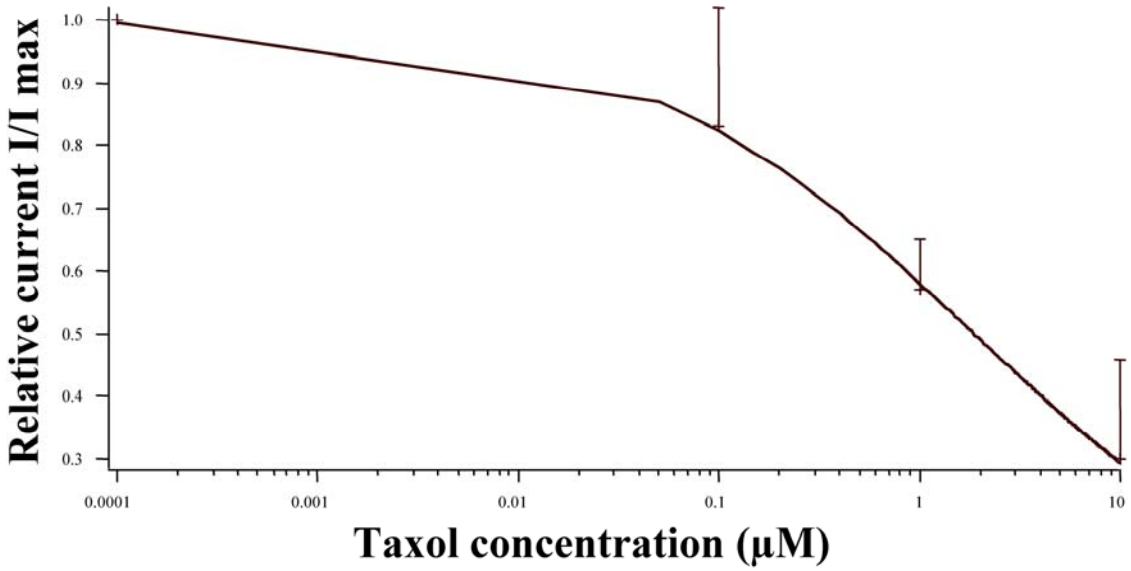


**Figure 3.13. Dose response curve for taxol®-mediated inhibition of capsaicin-induced currents.** A patch-clamp of Xenopus oocyte membrane expressing TRPV1 was tested for the effect of taxol® on capsaicin currents. Taxol® inhibits the capsaicin-induced currents in a dose-dependent manner. The calculated $k_i$ is 1.8 µM. The measured Hill coefficient is 0.55. Data kindly supplied by Hariolf Fritzenschaft (Jena, Germany).

An influence of the microtubule cytoskeleton on the intercellular $Ca^{2+}$ homeostasis and different cellular functions has been demonstrated in many studies. Several reports indicate that many of the transmembrane receptors interact with tubulin, and their functions are largely regulated by the tubulin-receptor complex (see table 3.3). More interestingly, many studies reported that drugs that work on microtubules could alter the properties of the different ion channels and receptors controlling the biological functions directly. With respect to taxol® particularly, it has been shown that cytochrome C release from isolated mitochondria can be induced by addition of taxol® (Andre et al. 2000). Muscarinic receptor-linked phosphlipase D activation was also reported to be inhibited by addition of taxol® (Chae et al. 2005). In a similar manner, colchicine (another drug known to works on microtubule cytoskeleton) was reported to inhibit the GABA-evoked currents (Weiner et al. 1998). This effect of colchicine is independent on the status of microtubule and suggested as a direct effect on GABA receptor.

### 3.2.5. Role of TRPV1 in cancer and cancer pain.

Cancer pain is a significant clinical problem because it is the first symptom of disease in cancer patients. Recently, the involvement of TRPV1 in different kinds of cancer and cancer pain has been demonstrated. Using mice as a model system, Asai et al showed the involvement of TRPV1 in heat and mechanical hyperalgesia in cancer pain (Asai et al. 2005). Lazzeri et al. reported a progressive loss in the expression pattern of TRPV1 during progression from normal urothelium to transitional cell carcinoma of human bladder (Lazzeri et al. 2005). Recently, Ghilardi et al. demonstrated that TRPV1 is involved in bone cancer pain (Ghilardi et al. 2005). Sanchez et al. could demonstrate the expression of functional TRPV1 in the prostate epithelial cancer cell lines PC-3 and LNCaP as well as in human prostate tissue (Sanchez et al. 2005). Vriens et al. reported the expression of TRPV1 and functional implication of such an expression in human hepatoblastoma (HepG2) cells (Vriens et al 2004). Very recently, involvement of TRPV1 in the pancreas cancer has been reported by Hartel et al. (Hartel et al. 2005). They also demonstrated an increased expression of TRPV1 in pancreas cancer. Interestingly, use of RTX to eliminate these pancreas-cancer cells was effective and proposed as potential chemotherapeutics in pain management. In summary, all these reports in general suggest an expression in many cancerous cell lines and a functional role of TRPV1 in different cancer-mediated pain.

# 3.3. TRPV1 and cytoskeleton: Importance for neuronal connection formation.

### 3.3.1. TRPV1 at the growth cone. Important for path finding.

It has been shown that basal $Ca^{2+}$ activity and frequencies of $Ca^{2+}$ waves generated by $Ca^{2+}$ channels present in neurites and growth cones have a strong regulatory influence on neurite initiation, extension and direction (Gomez and Spitzer. 2000; Gomez et al. 2001; Spitzer et al. 2000; Henley and Poo. 2004; Robels et al. 2003; Wen et al. 2004). With respect to the $Ca^{2+}$ activities and frequencies, presence of TRPV1 at certain neurites and growth cones is of functional importance as it modulates the cytoskeletal re-organization, by directly stabilizing the microtubules or destabilizing them by means of $Ca^{2+}$ influx/signalling upon activation.

Growth cones are well-organized dynamic structures involved in path finding. The fact that most of the pioneer microtubules present at the P-zone of growth cones as well as at filopodial structures are dynamic, "capturing" and "stabilizing" these dynamic microtubules

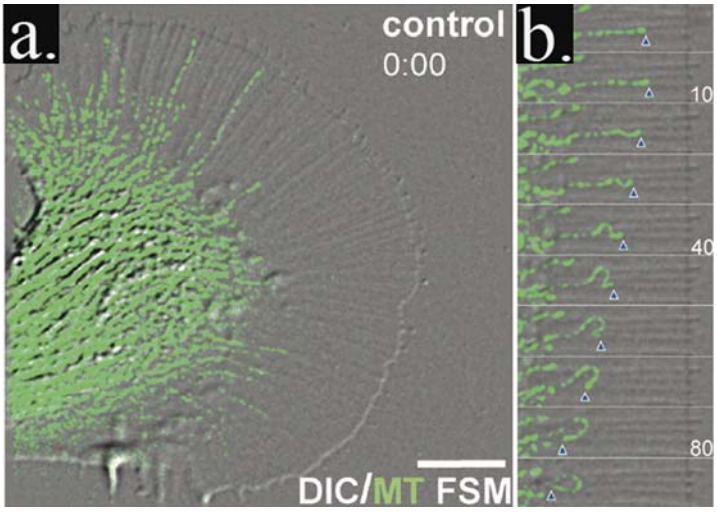


**Figure 3.14. Dynamic microtubules are present at the P-zone of growth cone. a.** Phase-contrast image and fluorescent image of dynamic microtubules (green) of a growth cone are superimposed. A fluorescent-speckle-microscope captured images depicting the microtubules. Though most of microtubules are restricted at the C-zone, few dynamic microtubules normally extend to the peripheral zone (P-zone) of the growth cone. **b.** Microtubules at the P-zone are very dynamic. Time series images of a microtubule at the P-zone of growth cone just after adding taxol® are shown. Addition of taxol®, a drug that stabilizes microtubules and reduces microtubule dynamicity, results in disappearance of microtubules from the P-zone due to retrograde actin flow. Plus end of a particular microtubule in different time periods are indicated by a blue triangle. Time is indicated in seconds. Image taken from Suter et al. 2004.

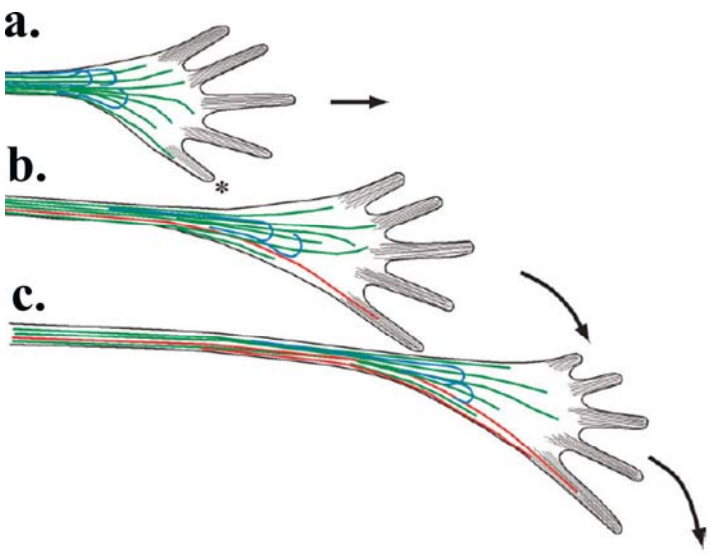


**Figure 3.15. Schematic representation of growth-cone turning by stabilizing the pioneer microtubules at the P-zone.** Drawings (a-c) represent sequential status of a growth-cone movement (indicated by arrows) with respect to the axon (left side) in response to a guidance cue (indicated by ∗). The distal plus-end of a microtubule is seen traversing the P-domain and lying alongside a bundle of actin filaments (grey colour) in the proximal part of a filopodium. Stabilization of this pioneer microtubule leads to the subsequent growth-cone turning. Dynamic microtubules are drawn in red while stable microtubules are drawn in blue and green. Image adapted from Gordon-Weeks P.R. (2004).

are the essential and initial steps for growth cone turning and subsequent path finding process (figure 3.14 and 3.15) (Gordon-Weeks P.R. 2004; Rodriguez et al. 2003; Dent and Gertler. 2003; Idriss H.T. 2000). Localization of TRPV1 at the P-zone as enriched patch, tubulin binding and the ability to stabilize microtubules through the C-terminus provide an ideal situation where TRPV1-enriched-patches could be involved in this "capturing" and thereby can stabilize pioneer microtubules and steer the growth cone direction.

TRPV1-enriched patches at the P-zone not only show enhanced immunoreactivity for unmodified tubulin, but also the presence of modified tubulins. Therefore TRPV1-enriched patches contain both dynamic microtubules and "aged" microtubules (Idriss H.T. 2000) (see figure 2.45 and 2.46, see also figure 3.16). This indicates that TRPV1-enriched membrane patches provide stability to the dynamic microtubules and may be involved in the capture of pioneer microtubules. Once some of these pioneer microtubules are captured, the rest of the extending microtubules and other cytoskeletal elements follow the track of the pioneer microtubules. Presence of neurofilaments in TRPV1-enriched patches further supports this hypothesis mainly for two reasons: firstly, the observed incorporation of neurofilaments is considered to be a relatively late event (Chan et al. 2003). Secondly, within growth cones, neurofilaments are dynamic and have a higher reversal rate (Uchida and Brown. 2004).

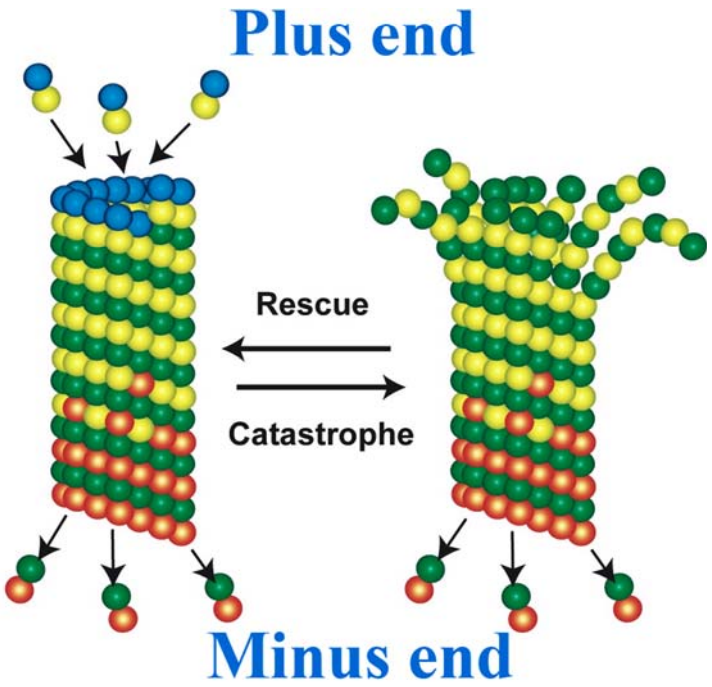


**Figure 3.16. Microtubules are polarized filamentous structures, which undergo a dynamic change and different post-translational modifications.** Microtubules are polymeric structures made of αβ–tubulin dimer. Addition of tubulin dimers (α/GTP-β) to the growing end and dissociation of tubulin dimers (α/GDP-β) from the other end give rise to polarity (plus end for the growing end and minus end for loosing end respectively) to the microtubules and a GTP-cap at the plus end. Microtubules are often subjected to rapid dynamic change termed as "catastrophe" by which microtubules lose tubulin dimers from their plus end. Different post-translational modifications generate microtubules with different physico-chemical properties by altering the "stability-instability" of microtubules. Shown here are, GTP-bound β-tubulin as blue circle, GDP-bound β-tubulin as green circle, non-modified α-tubulin as yellow circle and de-tyrosinated α-tubulin as orange circle. Figure taken from Dent and Gertler. (2003).

Activation of TRPV1 results in fast retraction of expanding growth cones. Growth-cone retraction is a process involving both the actin and microtubule cytoskeletons. More specifically, the force generated by the motor proteins is involved in this process (figure 3.17) (Ahmad et al. 2000; Baas and Ahmad. 2001). Disassembling microtubules produce a retrograde force that can also contribute in this retraction process (Grishchuk et al. 2005). In fact, a fast retraction of growth-cones is a proof for the presence of a functional actin cytoskeleton (in absence of a functional microtubule cytoskeleton) that can push back the growth-cone through its retrograde force (figure 3.17). As activation of TRPV1 significantly affects the organization of the microtubule cytoskeleton, but not of the actin cytoskeleton (this study, chapter 2, see figures 2.28 and 2.33), it can be hypothesised that the growth-cone retraction upon TRPV1 activation is not only dependent on $Ca^{2+}$ influx, but also due to the

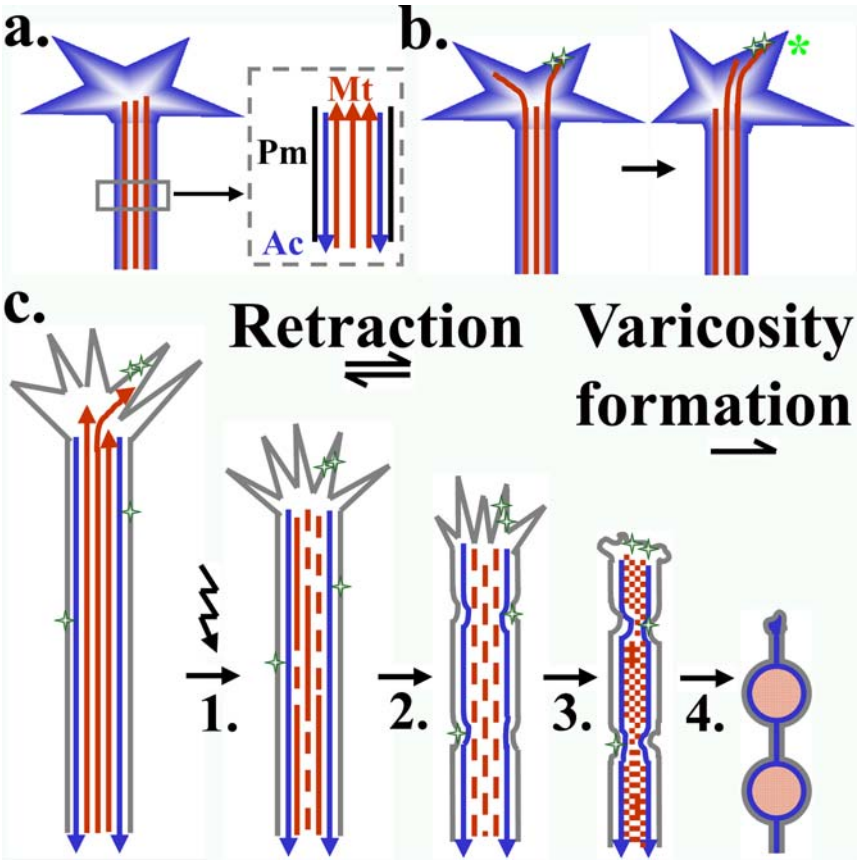


**Figure 3.17. Proposed model of growth cone and neurite movement regulation by TRPV1 through cytoskeletal reorganization. a.** Presence of microtubule cytoskeleton (red) and actin cytoskeleton (blue) at the neurite and at the growth cones are shown schematically. Both antrograde force from microtubule cytoskeleton (upside arrow) and a retrograde force provided by actin cytoskeleton (down side arrow) determine the net axonal-growth and movement. **b.** Majority of the axonal microtubules is restricted to the central-zone (C-zone) of the growth cone. Few dynamic microtubules at the P-zone are selectively stabilized by TRPV1-enriched patches (indicated by green stars). This may have an implication in the turning of the growth cone in response to a signal (indicated by green asterisk). **c.** TRPV1 activation-mediated growth cone retraction and varicosity formation is dependent on the status of microtubule cytoskeleton. *Stage 1:* Activation of TRPV1 (indicated by arrow) results in partial disassembly of microtubules, leading to the retraction of growth cone. *Stage 2:* Further disassembly of microtubules leads to the further retraction and initiates varicosity formation. *Stage 3:* Complete disassembly of microtubules results in a stage where further retraction is no more possible. *Stage 4:* Force from functional actin cytoskeleton and complete disassembled microtubules results in the varicosity formation. Strong agonist like RTX exerts retraction for a short duration and subsequently formation of extensive varicosities, indicating that activation of TRPV1 by RTX results in quick and irreversible shift to stage 3 and 4. In contrast, transient and mild activation by endogenous ligands like NADA results in retraction for a longer time but rarely forms varicosity (data not shown), indicating that activation of TRPV1 by NADA most likely results in slow and reversible shifting to stage 1 and 2 rather than in stage 3 and 4.

rapid disassembly of microtubules. Several lines of evidences support this hypothesis. Firstly, after TRPV1 activation diffuse immunoreactivity for tyrosinated tubulin is found at the P- and T-zones of growth cones (see figure 2.49). Secondly, long neurite-like structures frequently showed substantial beading (see figure 2.50) or varicosity formation. Varicosity formation is widely accepted as a classic morphological symptom of microtubule depolymerization (figure 3.17) (Ahmad et al. 2000). Finally, RTX application to embryonic DRG explants (stage E12, mice) also results in varicosity formation (see figure 2.51).

Remarkably, by live cell imaging, it was observed that RTX-responsive growth cones from mice DRG explants reveal an initial retraction before forming the varicosities (data not shown). Formation of varicosities was rapid and synchronous (within a single neurite all the varicosities formed at a time and not one after one). Once the RTX-responding growth cones start retracting, further addition of 5'IRTX, an antagonist of TRPV1, was not able to prevent the growth cone collapse or varicosity formation. The collapsed growth cones do not recover even in hours after adding 5'IRTX. These indicate that RTX-activation-mediated growth cone collapse is probably irreversible. However, considering the strong affinity of RTX to TRPV1, and difficulties to wash-off RTX efficiently (RTX is very hydrophobic) it is not possible to conclude if TRPV1 activation-mediated growth-cone retraction is fully irreversible. However, if activation of TRPV1 for a very brief period (less than 1 minute) by mild-endogenous compounds results in a more reversible retraction of growth cones, has not been tested yet.

Growth-cones are capable of recognizing different stimuli and integrating different cues in terms of $Ca^{2+}$ transients (Gomez and Spitzer. 1999). In recent years, a number of studies indicate the presence and functional importance of other TRP members, mainly from the TRPC subfamily in growth cone movement and functions (Montell C. 2004; Gomez T. 2005; Li et al. 2005; Wang et al. 2005; Shim et al. 2005; Bezzerides et al. 2004; Greka et al. 2003) (figure 3.18). Data presented here also indicate that stimuli specific for TRPV1 can regulate the morphology and movement of TRPV1-positive growth cones. The importance of TRPV1 in growth-cone movement is strengthened by the fact that functional TRPV1 is expressed endogenously in DRG at early embryonic stages (E12). This observation matches well with the observation made by Funakoshi et al. who described expression of TRPV1 in mouse DRG at embryonic stage 13 and 14 (Funakoshi et al. 2006). In this respect, it is important to mention that TRPV1 is activated by certain endovanilloids and endogenous components belonging to the lipoxygenase pathway (Van Der Stelt and DiMarzo. 2004; Chu et al. 2003; Huang et al. 2002). The list of endogenous components that can modulate TRPV1 is increasing rapidly (see table 1.4). More interestingly, NADA (N-Arachidonoyldopamine),

one of the endovanilloids, which has a relatively high potency for TRPV1, is present in adult brain and DRG (Huang et al. 2002). OLDA (N-Oleoyldopamine), another endogenous product of arachidonic acid metabolism is also reported to be present in the central nervous system and to activate TRPV1 (Chu et al. 2003). Preliminary experiments suggest that NADA application to the DRG explants results in growth cone collapse, growth cone retraction, and rarely varicosity formation (data not shown). However, if NADA, OLDA and other such endogenous compounds are present in the DRG at an embryonic stage is presently not known.

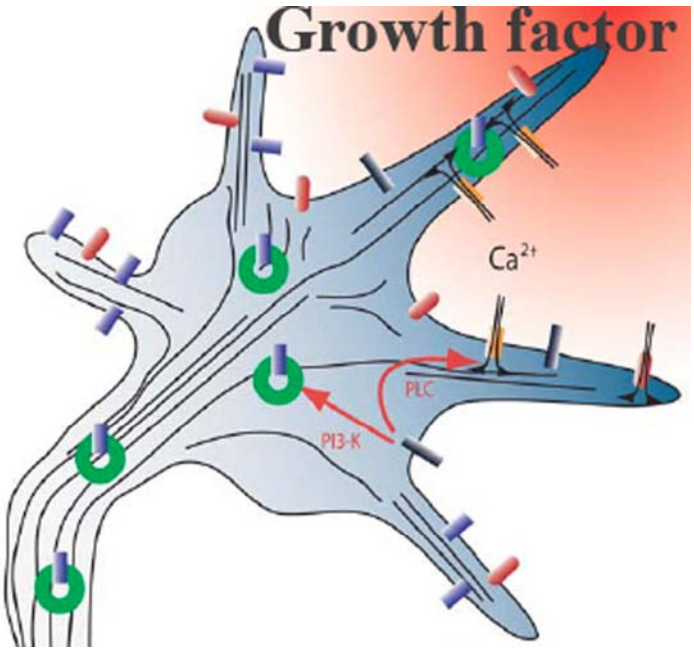


**Figure 3.18. TRP channels in growth-cone guidance.** Growth cones turning in a gradient of an attractive growth factor (like netrin-1, *red shading*) exhibit more elaborated filopodial structures in which growth-factor concentrations are higher. Resting TRP channels (*blue rectangles*) are activated by receptors for growth factors or other guidance cues (*black rectangles*) that signal through PLC. Open TRP channels (*orange rectangles*) permeate $Ca^{2+}$ directly and indirectly stimulate $Ca^{2+}$ influx through voltage-dependent $Ca^{2+}$ channels (Cav; *red ovals*) by membrane depolarization. Growth factor signalling through PI3-kinase (PI3-K) may also stimulate rapid insertion of TRP channels contained in vesicles (*green circles*) to augment TRP channel activity. $Ca^{2+}$ influx presumably establishes a gradient of increased $[Ca^{2+}]i$ (*blue shading*) across the growth cone that is instructive for turning. Adapted from Ramsey et al., 2006.

### 3.3.2. Over-expression of TRPV1: Effect on neuritogenesis and filopodial structure formation?

Previously, involvement of TRPV1 in the neurogenesis pathway has been speculated (Jin et al. 2004). It has been shown that the neurogenesis in TRPV1- knockout mice was

significantly reduced and defective when compared to the wild type mice. However the exact reason for such an effect of TRPV1 was not clear.

A massive number of neurite-like structures were observed in the majority of F11 cells over-expressing TRPV1 (data not shown). Interestingly, formation of many small filopodial structures at all neurites as well as at the cell body, and the presence of TRPV1 in these structures were observed. The identity of these structures was confirmed as a true filopodia and not as immature neurites by immunostaining with anti-tubulin antibody. Lack of anti-tubulin immunoreactivity in the majority of these small protrusions confirmed that these structures are indeed filopodial structures (figure 3.19).

Using TRPV1-GFP and live cell imaging, these filopodial structures were observed to be dynamic in nature (data not shown). Interestingly, HEK cells (Human Embryonic Kidney cell, a non-neuronal cell) also develop many filopodia-like structures upon over-expression of TRPV1 (figure 3.20). TRPV1 was also observed in these structures. Filopodial structure formation was also reported when TRPV2, a close homologue of TRPV1 was over-expressed in HEK cells (Hellwig et al. 2004). More interestingly, over-expression of TRPV1-ΔCt (a construct which consists of the N-terminus with the trans-membrane sequence of TRPV1, but lacks the entire C-terminal domain of TRPV1) also induces the formation of many filopodial structures (figure 3.21). This suggests that filopodial structure formation is independent of TRPV1-channel function (deletion of the entire C-terminus completely abolishes the channel function, Vlachova et al. 2003). This filopodial structure formation was even observed when only the N-terminus of TRPV1 was over-expressed.

Considering the filopodial structure formation as the consequence of a complex signalling event, which needs perfect recruitment and co-ordination between many regulatory and structural proteins, one can speculate that the N-terminal sequence of TRPV1 may

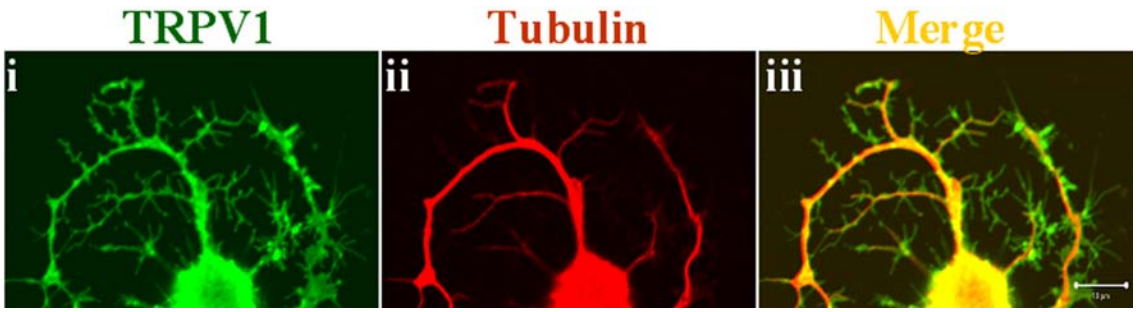


**Figure 3.19. Over-expression of TRPV1 in F11 cells results in the filopodial structure formation.** Confocal immunofluorescence images of an F11 cell transiently expressing TRPV1 is shown (i-iii). Cells were immunostained for TRPV1 (green, i) and tubulin (red, ii). Areas of co-localization appear as yellow (iii). Majority of the small protrusions reveal a lack of any anti-tubulin immunoreactivity. Scale bar 10μm.

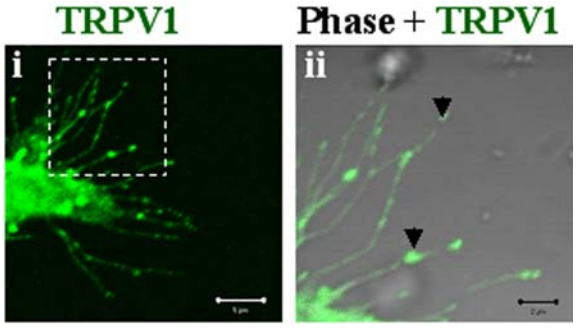


**Figure 3.20. Over-expression of TRPV1 in non-neuronal cells results in the filopodial structure formation.** TRPV1 was expressed in HEK cells by transient transfection and immunostained for TRPV1 (green). Confocal immunofluorescence images of a part of a HEK cell transiently expressing TRPV1 are shown (i). An enlarged area (indicated by white dashed line) of the same cell superimposed on phase-contrast image is shown in left (ii). Arrows indicate Filopodial structures. Scale bar 2μm.

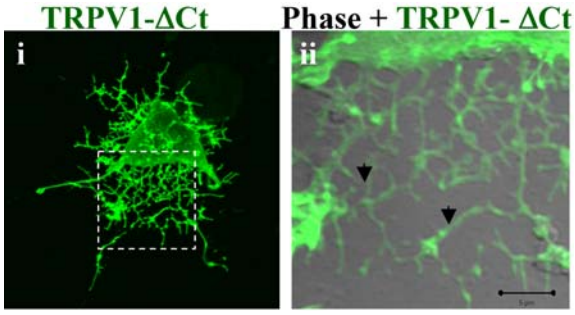


**Figure 3.21. Filopodial structure formation is independent of TRPV1 channel function.** TRPV1-ΔCt was over-expressed in F11 cells, and immunostained by anti-TRPV1 antibody (green). Confocal immunofluorescence image of a F11 cell transiently expressing TRPV1-ΔCt is shown (i). An enlarged area (indicated by white dashed line) of the same cell superimposed on phase-contrast image is shown in left (ii). Arrows indicate Filopodial structures. Scale bar 5μm.

interact with some of the signalling molecules involved in this process and modulate this signalling pathway. Importance of TRPV1 as one of the many important molecules involved in the pain pathway has been established to certain extent. It has been well established that filopodial structure formation is the initial step for neurite formation. Therefore, induction of filopodial structures and regulation of filopodial dynamics in a subset of DRG neurons by TRPV1 may well be a new important aspect in chronification of pain transmission.

### 3.3.3. TRPV1 and other TRP channels behave as synaptic proteins.

The importance of various $Ca^{2+}$ channels, such as the NMDA receptor, for synaptic functions is well established. Several studies report that TRPC channels are localized at growth cones and regulate growth-cone morphology or function (Montell C. 2004; Gomez T. 2005; Li et al. 2005; Wang et al. 2005; Shim et al. 2005; Bezzerides et al. 2004; Greka et al. 2003). In addition, many studies suggest that TRP channels can have synaptic localization and function, too. For example, TRP channels have been shown to interact with synaptic proteins (Singh et al. 2004). Additionally, TRP channels and proteins involved in synaptic

functions share a remarkable similarity in distribution and in dynamic behaviour. These proteins are observed to be transported by synaptic vesicles and also through “cytoplasmic transport packets”. These “cytoplasmic transport packets” are defined by the presence of at least one synaptic protein in such dynamic structure (Greka et al. 2003; Morenilla-Palao et al. 2004; Singh et al. 2004). They are present at filopodial structures and also at the areas that are involved in dendritic spine formation (Ahmari et al. 2000; Nakata et al. 1998; Marrs et al. 2001; Matus A. 2001; Sabo and McAllister. 2003; Okabe et al. 1999; Roos and Kelly. 2000).

In agreement with the properties of synaptic proteins, presence of TRPV1 has been detected in isolated synaptosomes indicating that TRPV1 is indeed present within synaptic structures (see figure 2.40). When over-expressed, presence of TRPV1 was observed at the filopodial structures and/or tip of such structures originating from cultured F11 cells (see figure 2.43, 3.19, 3.20, 3.21). Often these filopodial structures resemble with abnormal elongated dendritic spines (figure 2.40, 3.19, 3.20, 3.21). This observation is supported by reports by Toth et al., which describe the presence of TRPV1 at dendritic spines, also in different parts of brain (Toth et al 2005) and is in agreement with the synapse-formation hypothesis (Sabo and McAllister. 2003). Moreover presence of TRPV1 at both the pre- and post-synaptic membranes has been shown (Valtschanoff et al. 2001; Doyle et al. 2002). Additionally, rediolabelled RTX binding was detected in the many parts of brain, especially at the cortex and at the hypothalamus that are known to be important regions of brain involved in synapse formation (Acs et al. 1996; Szabo et al. 2002; Roberts et al. 2004). TRPV1 has also been reported to interact with synaptic vesicular proteins like syt IX and snapin (Morenilla-Palao et al. 2004). This is in agreement with the presence of TRPV1 at synaptic structures. More interestingly, loss of TRPV1 due to chromosome breakage was reported to be the cause of mental retardation in a group of patients (Walter et al. 2004). Mental retardation is characterized as a problem associated with neuronal networking. This is in full agreement with respect to the presence of TRPV1 at dendritic spines and synaptic structures, and also with the functional abilities of TRPV1 to regulate the neuronal-network formation. Taken together, all these results indicate that TRPV1 may have synaptic functions, too.