4 Discussion

4.1 Search for a GPI-anchored protease

Experiments performed by Gary Lewin indicated that a GPI-anchored protein is involved in low threshold mechanotransduction (Lewin, 1999; society of neuroscience meeting 1999, vol. 32 abstract number 162.6). A few years ago, Vallet and colleagues reported on a serine protease, that activates the epithelial sodium channel ENaC (Vallet et al., 1997). This protein was called CAP-1, for channel activating protease, and its sequence analysis suggested that it is probably linked to the cell membrane via a GPI-anchor. Since ENaC is related to the neuronal ASIC channels like BNC1, we hypothesized that the mammalian CAP-1 homologue (Vuagniaux et al., 2000) or another related GPI-anchored protease modulates sensory mechanotransduction by interacting with ion channels like BNC1.

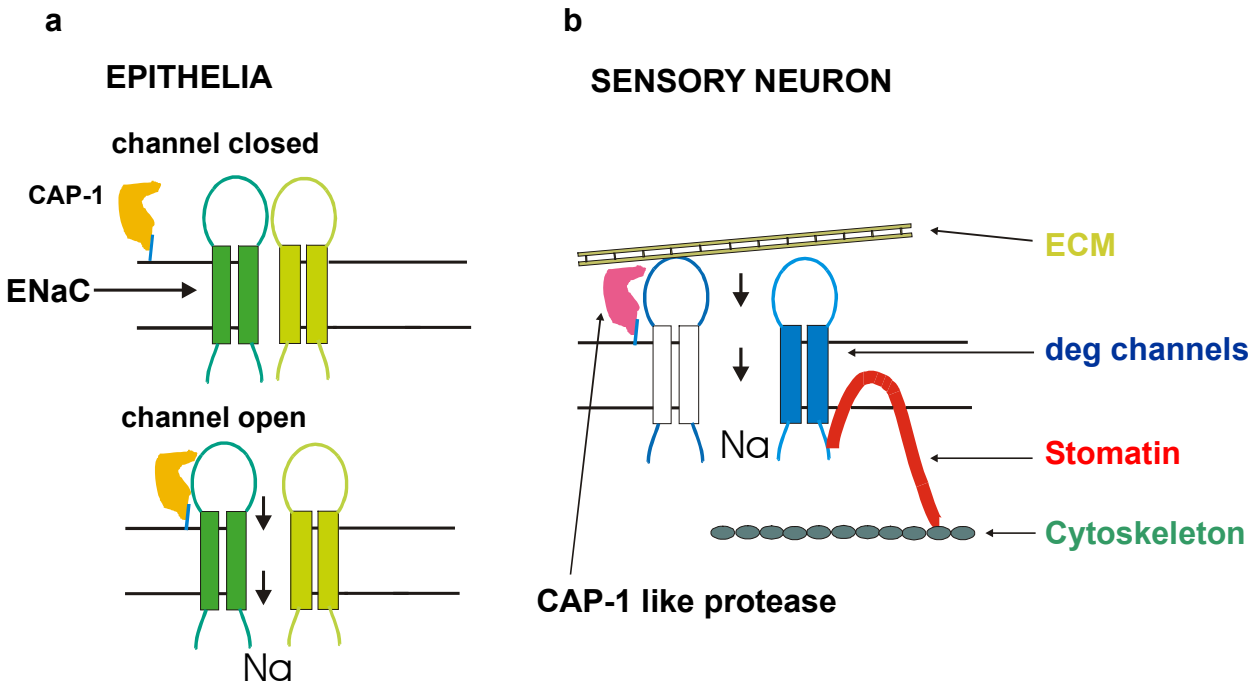

Figure 28: Panel a: Model of CAP-1 action in kidney epithelial cells. The GPI-anchored CAP-1 protein interacts with the extracellular part of ENaC and potentiates the inward sodium current (Vallet el al., 1997). Panel b: Hypothetical model of mechanotransduction in sensory neurons, in which a CAP-1 like protease interacts with DEG-channels and potentiates the receptor potential.

Meanwhile, three mouse orthologues of CAP-1 were cloned from a mouse kidney derived cell line (Vuagniaux, 2000, 2002). I carried out RT (reverese transcriptase)-PCR with specific

primers indicated that mCAP-1 (mouse CAP-1) is not expressed in mouse DRG (data not shown). We hypothesized that an unknown protease with similar properties to CAP-1 is expressed in DRG neurons. The first evidence for the presence of such a protease was obtained with protease assays. I could show the release of a protease from the sciatic nerve after GPI-anchor cleavage using the enzyme PIPLC. Furthermore binding experiments using the Biacore system showed that PIPLC treatment of sciatic nerve and skin releases proteins, that can bind to the mechanotransduction channel BNC1. Although these two observations were independent of each other, it is possible that the BNC1 interacting protein and the PIPLC released protease are identical. Since the protease assay was not suitable to assay protein activity during a purification procedure, the Biacore binding activity was used as an assay. Although a diverse array of chromatographic techniques were used, I was not able to purify the binding activity. It is possible that the protein is degraded, so that the activity was not localized in distinct chromatographic fractions. But it appeared more likely that the binding assay itself was not specific enough. BNC1 contains hydrophobic transmembrane domains that may have produced a strong unspecific binding background, so that a small fraction of specific binding could not be dissected out. However, the protein concentration of all samples was standardized prior to the binding test, and furthermore, tests for nonspecific binding using BSA in ten times higher concentration did not show any significant binding activity. In order to avoid nonspecific binding due to hydrophobic interactions, I constructed a mutated BNC1 ion channel that lacks the two transmembrane domains. Unfortunately I failed to purify this protein for immobilization on the Biacore chip. The purification attempts were halted at this stage. However, the finding, that GPI-anchored proteins may be involved in mechanotransduction, is of great interest and the identification of such a protein would increase our understanding of the mechanisms by which sensory mechanotransduction is regulated.

4.2 Search for BDNF-regulated mechanotransduction genes

BDNF null mutant mice display a mechanotransduction impairment in slowly adapting low threshold mechanoreceptors (Carroll et al., 1998). I was interested in finding genes responsible for this phenotype. I hypothesized that these genes are downregulated in BDNF null mutant mice and expressed in SA mechanoreceptors comprising about 50 % of large diameter neurons in the DRG. In order to detect expression differences, I used a combination of Affymetrix expression study and a DNA subtraction method.

Mice homozygote for the BDNF null mutation die 3-4 weeks postnatally and their DRG show a small loss of neurons at birth (Ernfors et al., 1994; Jones et al., 1994). The BDNF heterozygote mice display the same mechanotransduction deficit like the homozygotes, but are normal in all other contexts (Carroll et al., 1998). Genes that are downregulated in the heterozygote mice therefore should be good candidates for being specifically involved in SA low threshold mechanotransduction. The analysis of the Affymetrix experiment resulted in 142 genes that were consistently downregulated over three Affymetrix chip experiments. Analysis of the expression data revealed that reproducibility among the three independent experiments was relatively low. I assume that this problem is mainly due to variability in the dissection process of the DRG. Due to restricted number of mutant mice, I used around 5-7 mice per experiment. In order to decrease the variability among the experiments and to increase the chance to detect a real expression difference, one might consider using more animals per experiment. In the subtraction experiment, 284 genes were present in the subtracted library. The major advantage of the suppression subtractive hybridisation method against the Affymetrix experiment is the equal representation of low and high abundant genes. In order to identify genes present in the subtracted library, I hybridized the library onto Affymetrix chips. Although the analysis is then restricted to genes that efficiently hybridize to the oligonucleotides present on the array, this method speeds up the analysis considerbly. Furthermore, the two data sets generated by the regular Affymetrix experiment and the subtracted library can be analyzed conveniently for overlaps. By looking for such overlap we found that only two genes were common in both groups, the low overlap is probably mainly due to the difficult subtraction procedure. Comparing WT and heterozygote mice is problematic, because subtraction efficiency decreases the more similar the two subtracted pools are.

However, it is very interesting that those two genes that were downregulated in the Affymetrix expression profiling experiment and present in the subtracted library, a filamin-like protein and WAVE3 (Sossey-Alaoui, et al., 2002), are both actin-binding proteins. Recent results from our laboratory (personal communication from Carlos Martinez Salgado) indicate that the interaction between the actin scaffold and unconventional myosins is important for the mechanical adaptation property of sensory neurons. It will be interesting to test whether WAVE3 and the filamin-like protein are involved in the slow adaptation of SA mechanoreceptors.

Several genes that were downregulated consistently in the Affymetrix experiment and the subtraction experiment were cloned and their cellular expression pattern in DRG were examined using in situ hybridisation experiments. I looked for genes that are expressed in a subset of large diameter neurons and are downregulated in the BDNF heterozyogte mice. So far none of the

examined genes matched this criteria. In situ hybridisation experiments of the remaining candidate genes will tell whether this approach was succesful in finding a SA specific gene. However, we have to bare in mind, that the selection criteria (which are expression in large diameter neurons in in situ hybridisation experiment and downregulation in BDNF heterozygote mice) may be too stringent. The SA specific phenotype of BDNF heterozyogte mice could also be caused by genes that are widely expressed in the DRG and are downregulated only in the SA mechanoreceptors, because other receptor types do not express the BDNF receptor trkB. Downregulation of such genes would be detected in the Affymetrix expression profiling but not in in situ hybridisation experiments, since loss of around 10 % of total neurons would easily remain unnoticed. However, this explanation still leaves open the question, why D-hair mechanoreceptors expressing high levels of trkB (Stucky et al., 2002) are not affected in BDNF mutant mice. In addition, several other possible pitfalls have to be considered. The experiment was designed on the basis of the assumption that downregulated genes are very likely to be SA specific, since the SA defect is the only detectable phenotype. But this does not mean that any gene expression difference in the BDNF heterozygote mice in comparison to the WT is correlated with this phenotype. There are probably many genes downregulated in BDNF heterozygote mice without having a detectable phenotype and which came up in the screen. It is also possible that the whole approach was not suitable to find the genes responsible for the SA defect. In the original publication by Carroll and colleagues (Carroll et al., 1998) the Merkel cells, where the SA fibers terminate in the skin (Iggo and Muir, 1969), were examined for their anatomical integrity in the skin. Both were not affected in the BDNF mutant mice, and it was concluded that the SA defect must be a sensory neuron-intrinsic defect, probably due to a downregulation of neuronal mechanotransduction genes. But it cannot be excluded that BDNF deprivation leads to downregulation of trans-acting mechanotransduction genes expressed on Merkel cells. Such a gene expression defect may affect mechanotransduction without influencing the anatomy of the Merkel cells. Although studies using RT (reverse transcriptase)-PCR in Merkel cell complexes suggest that BDNF is not expressed by Merkel cells (Leung and Wong, 2000), Merkel cells appeared to express the BDNF receptor trkB (Ichikawa et al., 2001), so that they may in principle respond to BDNF. If this is the case, a different approach will be needed to identify the genes responsible for the SA defect, since RNA from Merkel cells is much more difficult to obtain. One feasible approach may be the use of a Merkel cell carcinoma derived cell line (Krasagakis et al., 2001; Leonard and Bell, 1997). Assuming that the carcinoma cell line has a similar phenotype to normal Merkel cells, one could compare the mRNA expression with and without BDNF treatment. The identification of such a trans-acting mechanotransduction gene

will definitely give us new insights into the complex interplay between different cell types for establishing mechanotransduction in the skin.

4.3 Search for D-hair mechanoreceptor specific genes

4.3.1 The T-type calcium channel CaV3.2 is specifically expressed in D-hair mechanoreceptors

Stucky and colleagues found that NT-4 knockout mice almost completely lack a specific subtype of mechanoreceptor. In contrast to the BDNF heterozygote mice, which display a functional defect without any cell loss, NT-4 null mutant mice lose a whole set of mechanoreceptive neurons, the D-hair mechanoreceptors (Stucky et al. 1998). D-hair mechanoreceptors are exceptionally sensitive mechanoreceptors (Burgess et al., 1968; Brown et al., 1967 a, b; Koltzenburg et al., 1997). Therefore I hypothesized that genes that are specific or enriched in this type of receptor may be good candidates for being involved in mechanotransduction. I made use of the latest gene array technology in combination with the suppression subtractive hybridization to identify genes that are downregulated in old NT-4 null mutant mice in comparison to the young mutant mice and to the wt mice respectively. Using this strategy I hoped to find genes that are specific for D-hair mechanoreceptors. Cellular expression studies using in situ hybridization showed that at least two of the downregulated genes, trkB and CaV3.2 were specifically or at least predominantly expressed in medium sized neurons and lost in old NT-4 mutants. They therefore exactly matched our criteria for D-hair mechanoreceptor specificity. TrkB is the cellular receptor for NT-4, so that a loss of NT-4 responsive D-hair mechanoreceptors would lead to a loss of trkB positive cells in the DRG. Indeed, when we compared the proportion of trkB positive neurons in DRG sections of mature wt mice and NT-4 knockout mice, we observed a substantial reduction (78%) in the number of trkB positive neurons. More interestingly, when we looked at the number of trkB positive neurons in 5-week-old NT-4 knockout mice and compared it to that from mature wild type mice, there was no discernable difference. Around 7% of the wt DRG neurons were clearly trkB positive in agreement with previous work (Mu et al., 1993). Our data would therefore suggest that a substantial proportion of trkB-positive neurons are D-hair receptors and that they disappear because of apoptosis triggered by the absence of neurotrophic support in the adult NT-4 knockout mouse. It is clear that other neurons eg. slowly-adapting (SA) mechanoreceptors which respond to BDNF must also express trkB receptors (Carroll et al., 1998; LeMaster et al., 1999). However, the expression level of trkB mRNA in SA mechanoreceptors was probably too low to

be detected in our in situ hybridisation protocol. This is in agreement with in situ hybridisation studies carried out by Wright and Snider, who found two sets of trkB expressing neurons in the DRG: around 10% of total DRG neurons, mainly medium sized neurons, express high levels of trkB mRNA, whereas a second set of neurons expressed trkB in low levels (Wright and Snider, 1995). It is therefore tempting to hypothesize that the medium sized neurons displaying high expression level of trkB mRNA are indeed D-hair mechanoreceptors and the neurons expressing lower trkB levels are SA mechanoreceptors. The variable expression level of trkB in DRG neurons also might explains the largely diverging cell counts of trkB positive DRG neurons discussed in the literature, ranging from 5-7 % (Mu et al., 1993; our results), over 25 % (Bergman et al., 1999) to almost 40 % (Karchewski et al., 1999).

The more interesting finding of my screen was that the T-type calcium channel CaV3.2, a low voltage activated calcium channel, was specifically and highly expressed in D-hair mechanoreceptors.

Calcium channels

From all ion channels mediating the entry and exit of ions in cells, calcium channels exert the most complex effect on the cell status, since calcium ions are not only charge carriers, but also function as the most ubiquitous second messenger in intracellular signalling processes (Berridge et al., 2000; Clapham et al., 1995). On the basis of channel activation, one can subdivide calcium channels into three different classes: ATPase driven or Na-coupled calcium channels can be considered as calcium pumps, “pumping” calcium ions out of the cell against their electrochemical gradient using ATP or the Na-gradient. Another class is comprised of all ligand activated or so called non-selective calcium channels like TRP (transient receptor potential) channels, which are activated by ligands in a broad sense, for example the TRP-channel VR1(vanilloid receptor 1) is activated by heat and capsaicin (Julius and Basbaum, 2001). Last but not least there is the large family of voltage-dependent calcium channels (VDCC), which are of particular interest in neuronal cells (Catterall et al., 2000).

Voltage-dependent calcium channels

The first recordings of calcium currents were carried out in cardiac myocytes (Reuter 1967, 1979). Since then many VDCC have been cloned and characterized and the members classified according to the biophysical properties of the isolated channels (Tsien, 1988; Catterall, 2000). So far there are ten isoforms isolated, not including the accessory subunits (ß-and gamma subunits) which influence the electrophysiological properties of the alpha main channels.

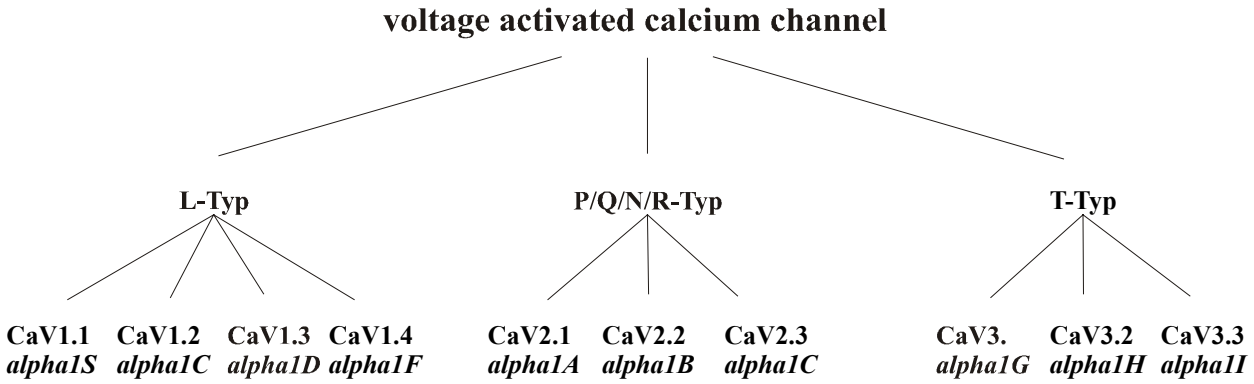

Figure 29: different classes of voltage activated calcium channels. *Italic* spelled names indicate old nomenclature

The L, N, P, Q and R-type channels comprise the family of high-voltage activated (HVA) channels, activated at strong depolarisations of the cell (Catterall et al., 2000, Perez-Reyes, 1995). In contrast, the recently cloned T-type calcium channels (Cribbs et al., 1998, Perez-Reyes et al., 1998, Huguenard, 1998)) are activated at low-voltages (LVA) (around -55 mV) and are therefore activated by moderate, subthreshold depolarisations. Whereas the high-voltage activated channels show large single channel conductance and slow voltage dependent inactivation, the low-threshold activated calcium channel exert small single-channel conductance, rapid inactivation (correspondingly T-type for transient-type kinetic) and slow deactivation (Lee et al, 1999 a). The striking activation property at low voltages led the researchers to many hypothesis regarding the function of the T-type calcium channels. Calcium influx via T-type calcium channels was proposed to partly explain behaviours like rebound burst firing (Llinas and Yarom, 1981) and rhythmic oscillations (Gutnick and Yarom, 1989; Von Krosigk et al., 1993). The three known members of the T-type calcium channels, CaV3.1 (alternatively α1G), CaV3.2 (α1H) and CaV3.3 (α1I) display very similar channel kinetics and also share high sequence homology (Perez-Reyes, 1999).

Our finding that the T-type calcium channel CaV3.2 was specifically and highly expressed in D-hair mechanoreceptors is in agreement with the data obtained by studies of other groups. Using radioactive in-situ hybridization, Talley and colleagues showed that CaV3.2 is expressed in medium sized neurons in the DRG (Talley et al., 1999) and Scroggs and Fox observed “giant” T-type calcium currents in medium sized neurons (Scroggs and Fox, 1992). Similar results were also obtained by Schroeder and colleagues, who determined the percentage of neurons with T-type calcium currents in the DRG as being around 5% (Schroeder et al., 1990). D-hair mechanoreceptors comprise about 5-7 % of DRG neurons (Stucky et al., 1998, Koltzenburg et

al., 1997). Assuming that T-type calcium currents are involved in nociception Talley and colleagues suggested that these medium sized DRG neurons are AM mechanoreceptors (Talley et al., 1999), but our data strongly suggests the real identity of these neurons is D-hair mechanoreceptors. Due to a lack of an appropriate antibody against this channel we could not show the expression of CaV3.2 protein in D-hair mechanoreceptors. However, the in situ hybridization study in combination with the identification of matching calcium currents in medium sized neurons are convincing arguments for the hypothesis that CaV3.2 is exclusively expressed in D-hair mechanoreceptors.

4.3.2 Mechanical sensitivity of D-hair mechanoreceptors is decreased after treatment with mibefradil

In the next step we asked whether CaV3.2 fulfills a critical role in D-hair mechanoreceptor function. To test this we made use of mibefradil, a potent antagonist of T-type calcium channels, which was developed by the pharmaceutical company Roche for treatment of hypertension and withdrawn from the market due to severe interactions with dihydropyrine compounds (Mullins et al., 1998). The EC50 of mibefradil on recombinant CaV3.2 ranged between 0,1 and 3 μM depending on the expression system (Martin et al, 2000). When we applied mibefradil in a concentration of 50 μM to the in vitro skin nerve preparation, we observed a total block of mechanosensitivity in D-hair mechanoreceptors but also in other receptor populations (data not shown). There are several possible explanations for this observation. The in situ hybridisation experiments we performed with specific CaV3.2 probes indicated, that CaV3.2 is specifically expressed in D-hair mechanoreceptors. With less stringent hybridisation conditions (hybridisation temperature lower than 54°C, higher probe concentration), many neurons in the DRG were positive for CaV3.2 mRNA, therefore we cannot exclude that CaV3.2 is expressed at a lower level in neurons other than D-hair mechanoreceptors. However, this is not likely, since the results of the in situ hybridisation studies from Talley and colleagues showed CaV3.2 staining only in a subset of medium sized DRG neurons (Talley et al, 1999). Therefore, the nonspecific effect of mibefradil probably results from the inihibition of other calcium channels. CaV3.1 and CaV3.3, the other two LVA (low voltage activated) calcium channels are also potently inhibited by mibefradil and are expressed in the DRG (Talley et al, 1999). I performed in situ hybridisation experiments with probes against these subtypes, but could not detect any expression (data not shown). In contrast Talley and colleagues found using radioactive probes weak CaV3.3 staining in medium sized neurons and weak CaV3.1 staining in all neurons. Total block of mechanosensitivity with 50μM mibefradil could thererefore result from the inhibition of

all LVA T-type calcium channels. However, DRG neurons also express HVA (high voltage activated) calcium channels such as L-type and N-type calcium channels. It is known that these HVA calcium channels are inhibited by higher doses of mibefradil (EC50 about 10x higher than for T-type calcium channels) and these channels are widely expressed in the DRG (Fox et al. 1987; Mintz et al. 1992). It is possible that they contribute to the electrical excitability of these neurons, perhaps with a direct involvement in mechanotransduction.

We quantitatively tested the effect of lower doses of mibefradil on mechanical sensitivity. We isolated the receptive field of D-hair mechanoreceptors or AM high threshold mechanoreceptors respectively with a metal ring and applied mibefradil into the ring. Using a mechanical stimulator we applied repetitive mechanical stimuli onto the receptive field and action potentials were recorded. In order to achieve a more specific effect we decreased the mibefradil dose to 15 µm in the isolation ring. The EC50 on recombinant channels is about 1 µM (Martin et al., 2000), but we assumed that the ring application requires some compensation for dilution and diffusion effects. Soon after application of mibefradil the number of action potentials decreased and reached a plateau of around 40 % of the buffer treated control. To test for reversibility, mibefradil was washed out for 15 minutes. The mechanosensitivity recovered partially, but no complete recovery was observed. Two factors may have prevented a complete recovery: i.) washout of mibefradil from complex tissues such as skin might take more time and ii.) mibefradil might sequester in the pore of closed channels since it is a pore blocker (Martin et al, 2000). Similar experiments were carried out with AM (Aδ-mechanoreceptor) high threshold mechanoreceptors. We first noticed that the variability of the responses from one AM to the other was very high, indicating heterogeneity in ion channel expression among the AMs. Although AMs show a slight reduction after mibefradil treatment, this effect was not significant. The blocking effect of mibefradil on AMs started with a delay of about 3 minutes after mibefradil application, whereas the effect on D-hair receptors was very fast (< 90 sec). We hypothesize that mibefradil blocks D-hair mechanosensitivity in a fast manner by blocking CaV3.2 and that the weak effect on AMs is mediated by a slow and unspecific block of other ion channels.

4.3.3 The electrical excitability of D-hairs is decreased after mibefradil treatment

CaV 3.2 forms a voltage-gated calcium channel with a threshold for activation just above the resting membrane potential (Cribbs et al., 1998). The blockade of mechanosensitivity after T-type calcium block might therefore arise as a result of reduced electrical excitability. The effect of mibefradil on electrical excitability was difficult to test directly at the receptor ending. We

therefore decided to measure spike frequency adaptation, a technique whereby a microelectrode is used to deliver trains of suprathreshold electrical stimuli of increasing frequency to the receptor ending. At low frequencies (< 10Hz) an action potential follows each electrical stimulus in both D-hair and AM receptors. As the frequency is increased, the percentage of failures steadily increased. When a large number of such recordings were made, it became clear that AM-fibers were less able to follow high frequency trains than are D-hair receptors. At a stimulation frequency of 20 Hz, around 50% of stimulations produced an action potential in AM receptors (median following frequency is 20 Hz), whereas the median following frequency in D-hair mechanoreceptors was 100 Hz. In the presence of just 3.3 μM of mibefradil, a leftward shift in spike frequency adaptation was observed for D-hair receptors, to a median frequency of 40 Hz, but no change was seen for AM fibers. I therefore hypothesized that mibefradil decreases the mechanical sensitivity by affecting the electrical excitability of D-hair mechanoreceptors. I will discuss this idea in more detail in the following chapters.

4.3.4 Model for a function of CaV3.2 in mechanotransduction

In order to propose a model for the function of CaV3.2 in mechanotransduction, the basic priniciple of mechanoelectrical transduction in sensory neurons should be recalled. The primary response of the cell to a mechanical stimulus is the receptor potential (or generator potential). A mechanical stimulus changes the tension in the cell membrane and mechanosensitive ion channels open. This cation influx leads to the local receptor potential (French, 1992). Receptor potentials exceeding a certain threshold potential trigger action potentials (Loewenstein, 1959; Loewenstein, 1971) which are propagated along the axon for further processing in the CNS. It is established since from early biophysical work that calcium influx is irrelevant for axonal action potential propagation (Hodgkin and Huxley, 1952; Hille, 1984). Accordingly, mibefradil did not affect mechanosensitivity when it was exclusively applied onto the nerve (data not shown). We can therefore exclude that CaV3.2 is involved in axonal action potential propagation. Furthermore, several properties of T-type calcium channels precludes them as candidate mechanosensitive ion channels, which contributes to the receptor potential. First they are strongly selective for calcium and it is known from sensory neurons and hair cells, that the mechanosensitive current is carried by a non selective cation channel (Loewenstein, 1959, 1971; French, 1992; Corey and Hudspeth, 1979). Secondly, CaV3.2 is voltage dependent which is not a necessary attribute of a mechanosensor. The spike frequency adaptation test moreover indicated that CaV3.2 serves to increase the excitability of the receptor, maybe by amplifying the receptor potential. We therefore suggest the following model:

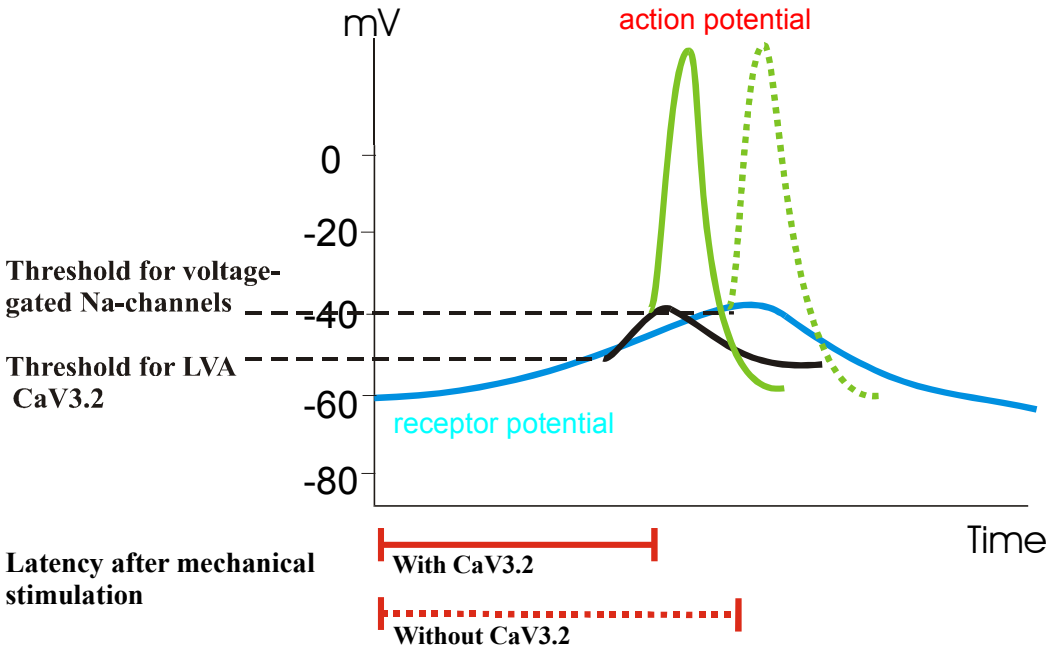

Figure 30: Model of the role of CaV3.2 in elevating the excitability of D-hair receptors

When a mechanical stimulus produces a receptor potential at the nerve terminal (French, 1992), the depolarizing membrane potential will then reach the threshold for activation of low voltage activated calcium channels, including CaV3.2. The channel would then open and accelerate the depolarisation, therefore the threshold for voltage gated Na-channels would be reached more rapidly and an action potential would be produced. If the CaV3.2 component is missing, the action potential would be initiated later if at all. CaV3.2 therefore may function as an amplifier of the receptor potential. In the mechanotransduction context, one can interprete the role of CaV3.2 as a factor to decrease the mechanical threshold for activation and therefore help to make the receptor ending more sensitive to mechanical stimuli. One way to test this hypothesis is to determine the mechanical threshold. The mechanical activation threshold, i.e. the minimal amount of force needed to elicit an action potential, is normally measured using von-Frey filaments producing a certain bending force when pressed onto the receptive field (von Frey, 1897). In our skin-nerve preparation, the surface tension of the buffer covering the skin precludes the use of von Frey filaments calibrated for lower forces than 0,4 mN. Since in vivo studies on D-hair mechanoreceptors suggest much lower mechanical activation thresholds (Burgess et al., 1968; Brown et al., 1967 a, b; Koltzenburg et al., 1997), we determined the mechanical latency as an indirect measurement of activation threshold. The mechanical latency was almost doubled in D-hair mechanoreceptors after mibefradil treatment, suggesting an increase in mechanical threshold. This result clearly demonstrates that mibefradil treatment of D-

hair mechanoreceptors increases its mechanical activation threshold. One can therefore propose that CaV3.2 expression lowers the threshold and therefore makes D-hair receptors more mechanically sensitive.

D-hair receptors are the most sensitive mechanoreceptors (Burgess et al., 1968; Brown et al., 1967 a, b; Koltzenburg et al., 1997). The ability of D-hair mechanoreceptors to respond to gentle mechanical stimuli could be based on cytostructural and/or electrical features. One could for example hypothesize that the mechanotransduction complex in D-hair mechanoreceptor is linked more efficiently or directly to the extracellular matrix, so that small movements of the skin cause a receptor potential. However, so far no data supporting this cytostructural explanation of D-hair mechanoreceptor sensitivity has been provided. Based on our results we rather prefer the electrical explanation: we propose that higher sensitivity can be achieved by lowering the threshold for action potential generation. Therefore, our results are the first concrete evidence for a mechanism that explains the sensitity of highly mechanoreceptive neurons on a molecular level. The functional properties of T-type calcium channel are well suited to fulfill such a function since they are activated at voltages near the resting membrane potential (Cribbs et al. 1998). Subthreshold depolarisations upon mechanical stimuli, which normally are not sufficient to trigger action potentials, would activate CaV3.2, which again leads to a stronger depolarisation, so that the membrane potential exceeds the threshold for action potential generation. A similar idea was already proposed for other sensory neuron types: It was suggested that the low threshold activation of T-type calcium currents may have controlling influence on threshold and shape of action potential in sensory cells (Schroeder et al. 1990, White et al. 1989). In addition Kawai and colleagues suggested that T-type calcium channels could help to enhance olfactory sensation by lowering the threshold for action potential generation (Kawai et al. 2001). Therefore, expression of different classes ion channels might explain how higher organisms generate a whole array of mechanoreceptors with different sensitivities: Highly sensitive mechanoreceptors like D-hair receptors would express LVA (low voltage activated) ion channel like CaV3.2, amplifying moderate receptor potentials to generate action potentials. Less sensitive mechanotranducers would lack LVA calcium channels, so that an action potential is only elicited upon a sufficiently strong deformation of the membrane. We assume that other factors such as the cytostructural architecture of the receptor or expression of modulating proteins also influence receptive properties. However, our data provides the first concrete evidence that expression of different ion channels could be the main aspect in tailoring receptive properties of mechanoreceptors.

4.3.5 Open questions

The calcium influx through T-type calcium channels will not only amplify membrane depolarisation, but will also influence intracellular signalling, since calcium is not only a charge carrier, but is also the most ubiquitous second messenger. The question is whether the role of calcium as an intracellular signal is critical for the receptor endings response to mechanical stimuli. One could address this issue by replacing calcium in the bath solution by barium. Barium passes through CaV3.2 and produces a larger current. If calcium was only needed for carrying charge into the cell, substitution by barium would not affect the D-hair mechanoreceptor function, in contrast: it would even increase the sensitivity, since barium produces larger currents. If calcium as a second messenger is also needed for proper mechanosensation, this treament would block D-hair receptor function. These experiments still remain to be carried out. Wolfe and colleagues showed that CamKIIγC (Calcium/calmodulin-dependent protein kinase II γC) is able to potentiate CaV3.2 activity, when it is coexpressed in a heterologous system (Wolfe et al., 2002). This implies that calcium might exert a positive feedback effect on CaV3.2 by its influence on CaMKIIγC. However since in situ hybridisation experiments with DIG-labelled RNA probes derived from CaMKIIγC showed expression in all DRG neurons (data not shown), I therefore suggest that CaMKIIγC is not specifically involved in D-hair receptor function.

Another interesting issue is how the group of BDNF and the NT-4 regulated genes overlap. When the list of the genes that were most consistently downregulated in these two experiments were compared (195 genes in NT4 experiment and 142 genes in the BDNF experiment), only two genes were common in both groups (alpha-tropomyosin, genebank accession number aa939674 and an unknown transcript with the accession number aa210488). This indicates that although NT-4 and BDNF both share the same TrkB receptor, the downstream pathways are indeed very different. This was expected, since it was shown in vivo that BDNF and NT-4 activate distinct genetic programs upon binding to trkB (Minichiello et al., 1998).

4.3.6 Further experiments

It is obvious to generate CaV3.2 null mutant mice to finally examine in detail how this channel contributes to D-hair receptor sensitivity, but it is still possible that the expression of CaV3.2 in other tissues including brain might preclude analysis of a sensory neuron phenotype. In addition, compensational effects of other ion channels may complicate the analysis. Another experiment would be to use the specific expression of CaV3.2 in D-hair mechanoreceptors to examine the development of these receptors. It is still not clear where D-hair mechanoreceptors terminate in

the skin of adult mice (Woodbury et al., 2001; Stucky et al., 1998; Millard and Woolf, 1988; Payne et al., 1991) and antibody experiments or experiments with mice where the CaV3.2 gene locus is replaced by a GFP-fused CaV3.2 construct may help to reveal the anatomical structure of D-hair mechanoreceptor endings in more detail.