1 Introduction

1.1 Mechanotransduction

One major feature of living matter is its ability to sense the environment and to react in an appropriate manner. Various sensory organs enable us to detect light, chemical, thermal and mechanical stimuli. Whereas the molecular principles of vision, olfaction and taste perception are quite well studied, little is known about the process of mechanotransduction. Mechanotransduction in its broadest definition means all processes through which a cell senses mechanical deformation. It plays important physiological roles on the single cell level, like cell volume control, salt and fluid balance, turgor control in a variety of different organisms (Hamill amd Martinac, 2001, Gillespie and Walker, 2002). In higher organisms with a nervous system, it is in addition the basis for hearing, vestibular function, blood pressure regulation and the sensing of touch and pain. In my thesis I will focus on sensory mechanotransduction, the process which converts a mechanical stimulation of a mechanosensitive neuron into an electrical, nervous signal. The basic principles of mechano-electrical coupling in sensory systems was established in the sixites and early seventies (Catton, 1970): when a mechanical force is applied to the specialized terminals of mechanosensitive neurons, the tension in the cell membrane changes and mechanosensitive ion channels open. This cation influx leads to the so called 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. Despite this relatively simple principle we know little about the molecular components involved in sensory mechanotransduction in higher vertebrates. This is due in part to the long distance between the cell body and the mechanosensitive terminals making voltage clamp recordings from the cell body after mechanical stimulation of the nerve ending difficult. Whereas large scale screening methods like expression cloning was succesfully used to identify heat and cold sensing receptors (Julius and Basbaum, 2001), this methodology may not be so easily adapted to find mechanotransduction genes, mainly because the mechanotransduction process may require multiple proteins. In addition to cellular proteins like ion channels and a cytoskeletal scaffold, extrinsic components like extracellular matrix components may also be required. Consequently most of our knowledge about mechanotransduction stems from research using genetic screens with simpler organisms like *Caenorhabditis elegans* and *Drosophila melanogaster.*

1.2 Mechanotransduction in invertebrates

1.2.1 Mechanotransduction in *Caenorhabditis elegans*

C. elegans with its defined number of cells and determined cell fate was the ideal organism for a initial study of mechanotransduction. The nervous system of this nematode consists of 302 neurons of which 47 are predicted to be sensory neurons, and mechanical stimuli applied to the body wall are detected by 6 so-called touch cells, 4 located at the front and 2 at the rear of the animal that innervate the anterior and posterior half of the animal respectively (Kaplan, 1996). These neurons send out long processes directly underneath the hyperdermis that are ensheathed by a specialized extracellular matrix (ECM) called the mantle. They can be identified microscopically by their huge bundles of 15-protofilament microtubules which appear to be crosslinked with each other and whose distal ends terminate directly underneath the cell membrane (Chalfie and Thomson, 1979; Hodgkin, 1991; Chalfie and Jorgensen, 1998). A simple but elegant mutagenesis screen in *c. elegans* resulted in the identification of probably all genes involved in mechanotransduction (Chalfie and Wolinsky, 1990). Mutant worms showing an altered response to body touch with an eyelash were isolated and the corresponding genes were identified. From the 18 so called mec-genes, a few were involved in the development of the six mechanosensory neurons, but most of the identified genes seem to be part of the actual mechanotransduction complex: These genes coded for cytoskeletal proteins, extracellular proteins, ion channels and proteins that may link the different components together. Mec-7 and mec-12 encode beta- and alpha tubulins, representing the cytoskeletal part of the complex, and a mutation in these genes resulted in defect organization of the 15 protofilament microtubules constituting the hair bundle (Chalfie and Thomson, 1979; Savage et al., 1989; Fukushige et al., 1999). Mec-5 is a collagen-like protein and is believed to be part of the extracellular tether, whereas Mec-9 is a secreted protein, which may function as a link between the extracellular scaffold and the ion channel complex. The ion channel complex is linked to the intracellular cytoskeleton via Mec-2, which shows a high homology to mammalian stomatin, that originally was identified as an integral membrane protein in erythrocytes (Stewart et al., 1992; Snyers et al, 1997). But the most interesting finding of the screen was the identification of Mec-4 and Mec-10. These are thought to be subunits of a heteromultimeric ion channel (Chalfie et al., 1993; Huang and Chalfie, 1994; Hong and Driscoll, 1994). The predicted structure of these channels is very characteristic, with only two membrane spanning domains and both the C- and N-terminus facing the cytoplasma. The large extracellular loop, which constitutes about 50% of the protein

contains three cysteine-rich domains and multiple N-glycolysation sites (Lai et al., 1996; Mano and Driscoll, 1999; Hong et al., 2000).

Mec-4 and mec-10 were called Degenerins (DEG-channels), because a gain of function mutation in mec-4 resulted in the degeneration of the *C. elegans* sensory neurons, probably because of a constitutively opened channel (Driscoll and Chalfie, 1991). It is tempting to say that these DEG-channels constitute the mechanosensitive ion channel complex, but so far, it could not be shown directly that these channels are directly gated by mechanical stimulation. Expression in heterologous systems does not result in mechanosensitive currents, possibly because of the necessity of accessory components. Electrophysiological recordings from mutant sensory neurons are also still lacking. Nevertheless, the identification of these genes has so far revealed the most complete description of a mechanotransduction complex in any organism. The proposed model has influenced the approaches to study mechanotransduction in other organisms.

1.2.2 Mechanotransduction in *Drosophila melanogaster*

Another genetic screen in *Drosophila melanogaster* resulted in the identification of several so called remp (*re*duced *m*echanoreceptor *p*otential) and nomp (*no m*echanoreceptor *p*otential) mutants (Kernan et al., 1994; Kernan and Zuker, 1995). So far, two of the mutant genes have been identified: NompA, an extracellular matrix protein and nompC, that potentially encodes a TRP-like ion channel. NompA probably serves as an extracellular link for the transducing apparatus, whereas NompC is a good candidate for being the mechanosensitive ion channel (Walker et al., 2000). It is expressed in mechanosensory neurons and has ankyrin-repeats that may constitute a link to the intracelullar cytoskeleton. Flies with a loss-of-function mutation of NompC lose almost all transduction currents. But the possibility remains that the small remaining mechanotransduction current still present in nompC loss-of-function flies constitutes the real receptor potential and nompC is just an amplifier or modulator. It should be noted that nompC is a member of the TRP (transient receptor potential) channel familiy, whose various other members are involved in many sensory functions including vision, olfaction, taste and heat perception.

1.3 Mechanotransduction in vertebrates

With the evolution of higher organisms mechanotransduction became the basis of more and more diverse physiological functions. In vertebrates, it is the basis for the perception of sound, acceleration, pain and touch.

1.3.1 Auditory and vestibular system

Vertebrates perceive sound and acceleration through the inner ear. Sound, acceleration or changes of the head position cause the deflection of the mechanosensitive ciliae of hair cells, the so called hair bundles. Excitatory and inhibitory deflection of hair bundles directly opens or closes transduction channels and hyperpolarizes or depolarizes the cell, which in turn changes the neurotransmitter release from graded synapses on the basolateral surface of hair cells (Gillespie and Walker, 2002). The identity of the transduction channel is still unknown. One candidate was EnaC, a member of the Deg/EnaC-family which is expressed in the inner ear, but EnaC null mutants do not show any hearing loss (Rusch and Hummler, 1999). Electrophysiological recordings from hair bundles indicate that the transduction channel is a nonselective cation channel with high conductance and permeability for calcium and is blockable by amiloride and calcium channel antagonists (Kros et al., 1992). These properties narrow down the number of candidate channels, and currently the $P2X_2$ channel (Housley et al., 1999) or a member of the large TRP channel family are being pursued as candidates (Harteneck et al., 2000).

1.3.2 Cutaneous mechanotransduction

Our knowledge of cutaneous mechanotransduction, i.e. the perception of pain and touch through the skin, is quite rich regarding the physiology, but very poor in terms of the molecular mechanisms (Lewin and Stucky, 2000). Early anatomical studies revealed the existence of different kinds of touch receptors in the skin, the corresponding fibers and their connection to the spinal cord (Willis and Coggeshall, 1991; Brown, 1981). In mammals sensory inputs from the skin are received by a heterogenous group of sensory neurons with their cell bodies residing in the dorsal root ganglia (DRG). The DRG are segmentally arranged adjacent to the spinal cord between the vertebrae. These pseudounipolar neurons send out one axon that divides into two branches, one projecting out to the periphery and the other to the spinal cord, where they terminate in distinct regions depending on their modality. Sensory neurons in the DRG not only innervate the skin but also visceral organs, skeletal muscle and the joints. Also other ganglia like the trigeminal ganglion contain mechanosensitive neurons innervating specialized organs in the head such as the teeth and vibrissae of the whisker pads in rodents.

We perceive different sensations arising from the skin such as light touch, pressure, tickle, pinch etc, and all these result from mechanical stimulations of the skin that differ in their strength, duration and velocity. Different receptors are specialized in terms of their anatomical structure and their molecular repertoire to detect and code these stimuli into appropriate nerve signals. In

accordance to the variety of incoming stimuli, the DRG exhibits a large variety of different neuronal subtypes, with distinct, but sometimes overlapping receptive properties. The fact that they also respond to heat, cold and chemical stimuli and not only innervate skin, but also muscle, joints and visceral organs such as bladder and intestine, demonstrates the complexity of this neuronal population.

Receptor types

Some of the first psychophysical studies on tactile perception were performed by Mueller and later by von Frey, who found a mosaic of punctate fields in skin and suggested that these might be the receptive organs of the sensory neurons (Frey, 1897). A more detailed analysis of the different sensory fibers by Erlanger and Gasser (Erlanger und Gasser 1924; 1930; Gasser and Erlanger, 1927) classified these fibers by their conduction velocity, building the foundation for the classification used today in e.g. mouse: A-β fibers are thickly myelinated, conducting at a speed of over 10 m/sec. A-δ fibers are thinly myelinated, therefore propagating action potentials between 1-10 m/sec. C-fibers are unmyelinated with a conduction velocity of under 1 m/sec (Koltzenburg et al., 1997).

The A-fibers can be further subclassified according to their adaptation to mechanical stimulation. Slowly adapting neurons fire action potentials throughout the entire duration of a constant displacement of their receptive field, wheras rapidly adapting neurons are movement detectors firing action potentials only during the movement before and after the onset of a constant displacement. Thus the A-β fibers in mouse can be divided into two major groups: slowly (SA) and rapidly adapting (RA) mechanoreceptors. The A-δ fibers are classified into rapidly adapting D-hair receptors and slowly adapting A-mechanonociceptors (AM). C-fibers are mostly slowly adapting.

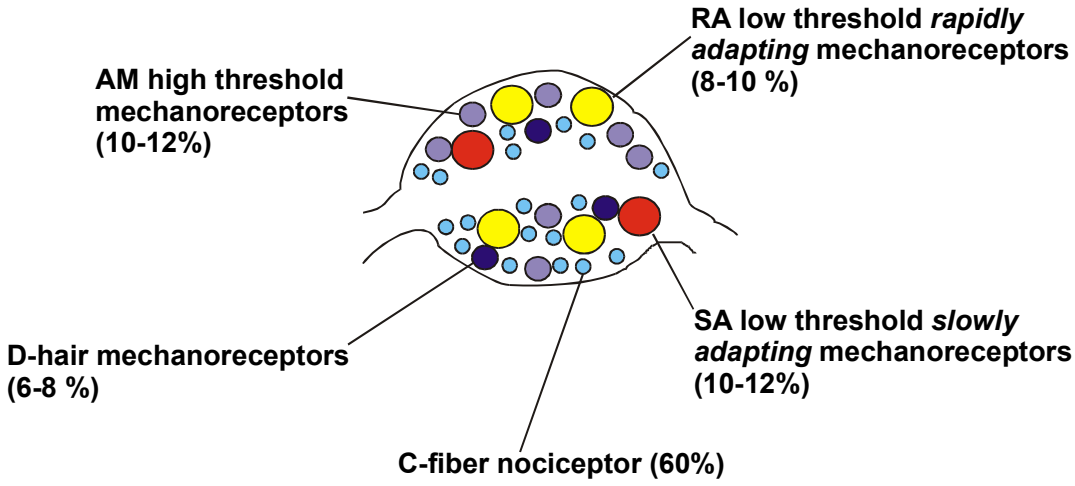

Figure 1: Scheme of different receptor types with their relative abundance in the DRG.

These neurons further differ in the specialized structures in the skin that they innervate. RA mechanoreceptors generally innervate Meissner corpuscle in glabrous and Pacinian corpuscle, two lamellar end organs, and hair follicles in hairy skin. SA mechanoreceptors terminate in Ruffini corpuscles in glabrous skin and on Merkel cells organized in touch domes and partially around hair follicles in hairy skin (Iggo and Muir, 1969). D-hair receptor endings were originally thought to solely encircle down hair follicles, hence the derivitation of the name (Woodbury et al., 2001; Stucky et al., 1998; Millard and Woolf, 1988; Payne et al., 1991). The myelinated afferent fibers of AMs have free nerve endings in the skin (Fleischer et al., 1983). C-fibers have „loose“ blunt unmyelinated endings (Perl, 1992).

The *in vitro* skin-nerve preparation, originally developed by Reeh in 1986, simplified the analysis of the properties of sensory neuron afferents innervating the skin. Koltzenburg and colleagues adapted this method to mouse in 1997. The purely cutaneous saphenous nerve, which is a side-branch of the *nervus femoralis* that originates from lumbar DRG L2-L4 (Swett and Woolf, 1985), and the skin innervated by it (a region from approximately the knee down to the toes of the animal) are excised and placed into an organ bath. After teasing the nerve in smaller fibers, one can directly record from the fiber upon mechanical stimulation of the corresponding receptive field. This enables the identification and characterization of single, defined afferents. Using von Frey hair monofilaments, the mechanical threshold, the minimal force required to activate a single unit, can be determined. Both RA and SA mechanoreceptors have von Frey thresholds (vFT) ranging from 0.4 mN to around 2.0 mN, i.e. being able to detect very low intensity stimuli. D-hair receptors, the most sensitive sensory neurons, always respond to stimuli of 0.4 mN and below (Burgess et al., 1968). The activation threshold of both AMs and C-fibers generally ranges from very low (2.0mN) to stimuli as high as 32mN and above, thus requiring a high amount of mechanical force to be activated.

C-fibers, like A-fibers, are a very heterogeneous population of neurons. They are called nociceptors due to their ability to detect stimuli that are potentially or actually tissue damaging (Sherrington, 1906). One can classify C fibers in accordance to their response to noxious heat. This divides them into C-mechanonociceptors (C-M), which are unable to respond to the heat stimulus, and polymodal C-mechanoheatreceptors (C-MH) able to respond to noxious heat by firing action potentials. A small population of C-fibers is able to respond to noxious cold stimulation. A proportion of the polymodal C-MH neurons is able to also encode chemical stimuli such as the drop of extracellular pH that can occur during injury and inflammation (Steen et al., 1992, 1993). Some C-fibers are insensitive to mechanical stimuli, but there is also a

fraction of C-fibers, that are even completely insensitive to mechanical and heat stimuli (Schmidt et al., 1995).

We can summarize, that combined psychophysical and neurophysiological research resulted in a quite complete description of the tactile perception. Despite this, our knowledge of the molecular components and mechanisms of vertebrate mechanotransduction is still poor.

1.3.3 A molecular model of mechanotransduction

The mutagenesis screen in *C. elegans* resulted in a very complete description of the components involved in the nematode mechanotransduction. Based on this mechanotransduction model a preliminary model has been suggested also for vertebrates. Despite the difference of complexity between the nematode and higher vertebrates like mouse and human, it was hoped that enough homologues genes with a conserved role in mechanotransduction would be found. Indeed, many of the *c. elegans* mec-genes turned out to have relatively close homologues in vertebrates (Lewin and Stucky, 2000).

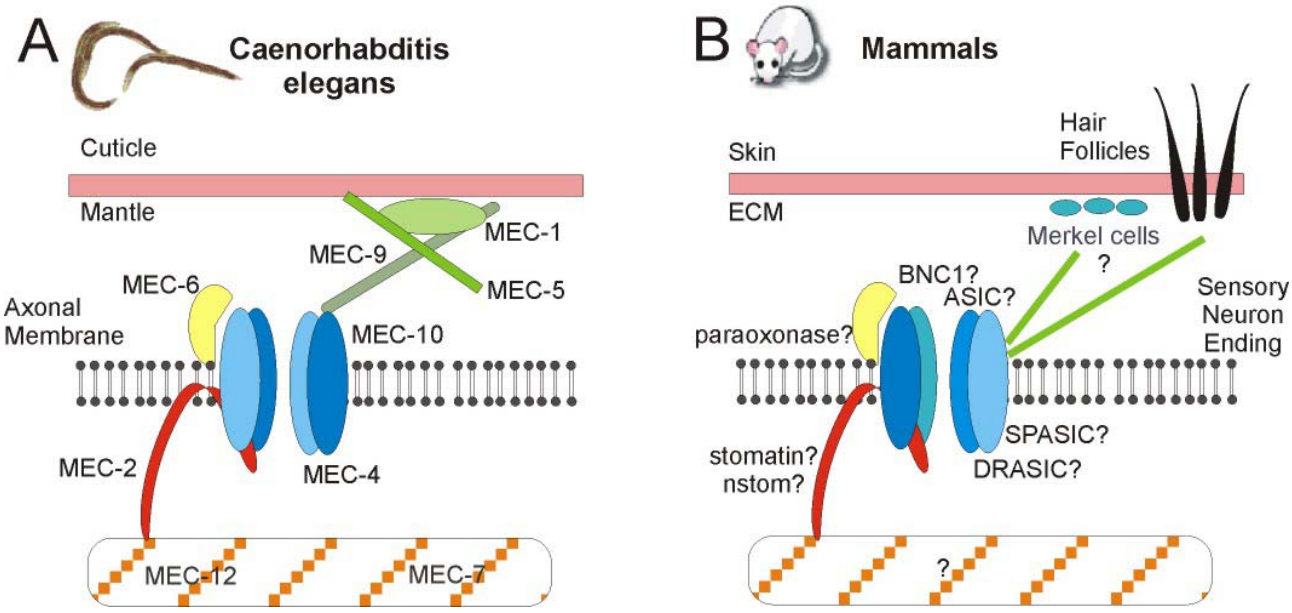

Figure 2: A: mechanotransduction model in *C. elegans* based on genes that were identified by the mutational screen (adapted from Tavernarakis and Driscoll, 1997). B: hypothetical mechanotransduction model in mammals, based on the *C. elegans* model.

1.3.3.1 Mammalian orthologues of MEC-4 and MEC-10

Soon after the identification of MEC-4 and MEC-10 as the putative mechanotransduction ion channels in *C. elegans* (Tavernarakis and Driscoll, 1997), Canessa and colleagues cloned the epithelial sodium channel EnaC (Canessa et al., 1993). Sequence analysis and comparison of the putative protein structure revealed the similarity between these two channel types. The sequence

homology on the amino acid level was only about 11-20 % between MEC-4, MEC-10 and EnaC (with higher homology in certain domains), but the predicted structure with two transmembrane domains, one large extracellular loop and both the C- and the N-terminus facing the cytoplasm was common in both ion channel types and suggested that these channels belong to the same ion channel super family, hence they were put together as the DEG/ENaC family (Jentsch, 1994).

ENaC is the prototypical channel in the DEG/ENaC family and is the best characterized (Garty et al., 1997). Although the existence of a sodium resorbing channel in epithelia was known for many years, the channel was cloned quite recently from rat distal colon (Canessa et al., 1993). Since then, EnaC has been cloned in many other species like human (McDonald et al., 1995), chicken (Goldstein et al., 1997), bovine (Fuller et al. 1995) and frog (Puoti et al., 1995). ENaC is expressed at the apical surface of tubule cells of the kidney, the distal colon, ducts of secretory glands, the respiratory airways and in amphibian skin (Duc et al., 1994; Renard et al., 1995). Furthermore, the sensory transduction of salty taste is also mediated by ENaC channels expressed in the fungiform papillae located in the dorsal epithelium of the anterior tongue (Lindemann, 1996). Three homologous subunits were identified: α, β and γENaC that together form a functional channel. αENaC as the pore-forming subunit is the only subunit that can form a functional ion channel on its own when expressed in a heterologous system. It is a non-voltage gated sodium channel that can be blocked by amiloride. The other subunits serve as auxiliary subunits (Canessa et al., 1993; Lingueglia et al., 1992; Canessa et al., 1994; Waldmann et al., 1995). Mutations in these ENaCs cause disorders like hypertension and Liddle's syndrome (Shimkets et al., 1994; Chang et al., 1996).

One obvious question to ask was whether ENaC was expressed in the sensory system and whether it is involved in mechanotransduction like mec-4 and mec-10 in *C. elegans*. Indeed all three subunits were shown to be expressed in trigeminal ganglion neurons and their endings (Fricke et al., 2000), but in dorsal root ganglia, only β and γENaC have so far been detected. Immunostainig showed localization in rat Merkel cell complexes, Meissner-like corpuscle and small laminated corpuscle in glabrous skin indicating a possible function in mechanosensation (Drummond et al., 2000). But since the main pore forming α subunit is not expressed, it was assumed that other channels are involved, and therefore several groups started looking for other neuronal members of the DEG/ENaC family. Soon after the the cloning of ENaC, a second mammalian subfamily of DEG/ENaCs was isolated and called mammalian degenerin or acid-sensing ion channel family (ASIC). So far, four closely related genes have been cloned, which are thought to be selectively expressed by neurons: The first one was BNC1 (brain sodium channel), also called as BnaC1, mdeg or ASIC2 (Price et al., 1996; Waldmann et al., 1996;

García-Anoveros et al., 1997; Lingueglia et al., 1997; Price et al., 2000). Two splice variants are known, BNC1a and BNC1b, differing in the N-terminus. Next are the two splice variants of the acid-sensing ion channel gene, ASICα, also called BNaC2, and ASICβ, which is also named ASIC1a and ASIC1b (García-Anoveros et al., 1997; Waldmann et al., 1997; Chen et al., 1998). The third member of this subfamily is the dorsal root acid-sensing ion channel (or DRASIC) (Waldmann et al., 1997a) and the final mammalian degenerin is spinal cord acid-sensing ion channel, short for SPASIC (Akopian et al., 2000). All these ion channels share a sequence homology of between 41-65 % to each other and consist of around 550 amino acids. Many these ion channels are able to build homomeric non-voltage gated ion channels with sensitivity to amiloride and open upon extracellular pH drop with an transient sodium current. Exceptions are BNC1b, ASICβ and SPASIC. Co-transfection studies using two different subunits, BNC1a and BNC1b, BNC1a and ASICα, BNC1a and DRASIC or BNC1b and DRASIC showed that they are able to form heteromeric ion channels with properties differing from the homomeric channel (Bassilana et al., 1997; Lingueglia et al., 1997, Babinski et al., 2000).

Due to homology to the *C. elegans* mec-genes and their expression in dorsal root ganglia these ASIC channels were considered good candidates for constituting the mechanotransduction current in mammals. Indeed, studies of our laboratory in collaboration with the Welsh group on the BNC1a null mutant mice revealed, that this ion channel is involved in mechanotransduction (Price et al., 2000). Although BNC1 null mutants display a clear defect in low threshold mechanotransduction, the effect was still minor so that it appeared likely that other channels also contribute to the mechanosensitivity.

1.3.3.2 Proteins that modulate Deg/ENaC channels

In the original *C. elegans* mechanotransduction model Mec-2 was thought to tether the Mec-4/Mec-10 ion channel complex to the cytoskeleton. Electrophysiological studies with cell lines coexpressing Mec-2 and Mec-4/Mec-10 have shown that Mec-2 not only interacts with Mec-4/10, but directly increases the activity of the "d" from of Mec-4 and Mec-10 more than 40 fold (Goodmann et al, 2002). Regulators have also been described for ENaC. In addition to the known pathways of hormonal regulation by aldosterone and vasopressin, recent studies have revealed more proteins that interact with ENaC and modulate its activity. Nedd4 is a ubiquitin ligase that is expressed by many organs including brain, lung, kidney and colon. Nedd4 binds to the carboxy-terminus of α-ENaC and γ-ENaC and ubiquitinates it, which leads to degradation of the channel (Staub et al. 1997). It was also proposed that ENaC interacts with CFTR, since in patients with cystic fibrosis caused by mutations in the cystic fibrosis transmembrane

conductance regulator (CFTR) gene (Riordan et al., 1989), sodium absorption is larger than in normal individuals (for review Kunzelmann et al. 2001). The mechanism of this interaction is not yet known. Also enigmatic is the mechanism by which proteases modulate ENaC activity. Garty and Palmer reported 20 years ago that proteases like trypsin can lead to a reduction of sodium transport in urinary bladder (Garty and Palmer, 1983; Garty and Palmer, 1997 review). These experiments were interpreted as a protease that cleaves the activity-relevant part of ENaC. However recently, Chraibi and colleagues showed that trypsin increases ENaC activity expressed in Xenopus oocytes. Soon after this first study, Vallet and colleagues cloned a serine protease from a xenopus kidney cell line, that interacts with ENaC and reversibly increases its activity by two-fold (Vallet et al, 1997). Interestingly this protease called CAP-1 (for channel activating protease) contains a signal sequence for a GPI (glycosylphosphatidylinositol)-anchor. A wide variety of proteins like enzymes, receptors, immune system proteins and recognition antigens are linked to the exterior surface of the plasma membrane via a GPI group (Low, 1989). GPI groups are appended to the C-terminus of proteins in the endoplasmatic reticulum minutes after their ribosomal synthesis. Proteins destined to receive such an anchor are synthesized with a C-terminal hydrophobic sequence which is removed while the anchor is added to the protein. GPI-anchored proteins can be cleaved from the plasma membrane using a phosphatidylinositol-specific phopholipase (PIPLC). The mechanism of the activation of ENaC by the GPI-anchored CAP-1 though is still unknown.

So far, no such interactions and regulations were shown for the neuronal members of the DEG/ENaC family like BNC1, Drasic, Asic and Spasic. But unpublished data from our lab propose that BNC1 is regulated by stomatin through a similar mechanism as has been suggested for Mec-2 and Mec-4/10 interaction (personal communication from Paul Heppenstall). One part of my thesis also was to test whether a CAP-1 like protease modulates the mechanotransduction channel BNC1 and therefore may also be involved in sensory neuron mechanotransduction.

1.3.3.3 Mammalian homologues of Mec-2

The closest relative of mec-2 in mammals stomatin (also called erythrocyte membrane protein 7.2b) was purified and cloned from human erythrocytes (Wang et al., 1991, Hiebl-Dierschmied et al. 1991, Stewart, 1992). Although expression studies by Anne Mannsfeldt in our lab showed that stomatin is expressed in mouse DRG (Mannsfeldt et al., 1999), examination of the stomatin null mutant mice did not reveal any defect in sensory mechanotransduction. Mannsfeldt also cloned another homologue called nstomatin-1 (neuronal stomatin 1) due to its specific neuronal expression. Our lab has generated a mutant mouse that is null for this new gene, these mice are

currently under investigation. The mouse genome also contains more stomatin homologues like hslp (human stomatin-like protein) (Seidel und Prohaska, 1998), slp3 (stomatin-like protein 3) (Goldstein et al., 2002) and nstom2 (neuronal stomatin 2) (personal communication from Paul Heppenstall, unpublished), which are also expressed by sensory neurons. It will be interesting to test whether these genes exert a role in mechanotranduction.

1.3.3.4 Extracellular proteins

The *C. elegans* screen revealed that apart from cytoskeletal, extracellular matrix and ion channel components secreted proteins also play a role in mechanotransduction (Du et al, 1996). One of these genes is mec-6, and it will be interesting to examine whether its closest relative in mammals, paraoxonase, is involved in mechanotransduction (Chalfie, personal communication).

1.4 Neurotrophins

1.4.1 General

Neurotrophins are a group of peptide growth factors whose expression is mainly restricted to the nervous system of vertebrates. With a molecular weight of about 13 kDa, they are relatively small, highly basic, and dimerize to build the active form. Neurotrophins are involved in a vast variety of functions. Many seem to be active in a very restricted time period during development, whereas others act throughout the whole livespan. In the nervous system many more neurons are generated than are finally required, as many as half of neurons die during development, most of them by apoptosis. The so called “neurotrophic factor hypothesis” states that at the time when neurons reach their target tissues, they compete with other neurons for a limiting amount of neurotrophins expressed by the target tissue. Only those with enough neurotrophic support would survive. This mechanism is a convincing model to explain the tailoring of the number of neurons in the nervous system (Davies, 1996). In many cases, more than one neurotrophin, and also other growth factors like EGF contribute to the development of a neuron, some in successive and also in an overlapping manner. For example spinal motor neurons are presently known to be influenced by 15 different factors (Oppenheim, 1996). Neurotrophins on the other hand function not only in a target derived manner, since also paracrine and autocrine mechaninsms have been described. This means neurons produce neurotrophins for themselves and their neuronal neighbours for a variety of physiological functions (Ernfors, 1994). The responsiveness of a cell to neurotrophins depends on expression of appropriate receptors. The

receptors are often receptor tyrosine kinases and the binding of a neurotrophin to its cellular receptor leads to complex cellular signaling events.

Neurotrophins consist of five, highly conserved members: NGF, BDNF, NT3, NT4/5 and NT6 (only expressed in fish) (Gotz et al., 1994). Two cellular receptors bind neurotrophins: first the low affinity p75 receptor which belongs to the tumor necrosis factor superfamily, and second the high affinity Trk receptors, the tropomyosin-like receptor kinase family (Barbacid, 1995; Friedman and Greene, 1999). The three Trk receptor subtypes TrkA, TrkB and TrkC are highly conserved and mainly differ only in their extracellular domains.

NGF was the first growth factor to be identified (Levi-Montalcini and Angeletti, 1968). It is absolutely required for the development of sympathetic ganglia and the nociceptive subset of sensory neurons. NGF is synthesized by its target tissue, and many of NGF function can be explained by this fact according to the neurotrophic hypothesis (Lewin and Barde, 1996). The character of NGF dependency differs from one neuronal type to the other. Whereas sympathetic neurons need NGF throughout the whole life span, sensory neurons probably only depend on NGF during development (Lewin and Mendell, 1993). NGF has not been shown to actively support proliferation, but is a protective factor that prevents cells from undergoing apoptosis. Furthermore, it also acts to stimulate cellular metabolism, leading to extensive outgrowth of axonal and dendritic processes and it was also shown to have a specifying role in determining neuronal phenotype (Lewin, 1996). In the CNS, NGF synthesis seems to be restricted to the hippocampus and neocortex in lower concentrations compared to the other neurotrophins (Huang and Reichardt, 2001). The main signalling pathway of NGF is mediated by the tropomyosin related kinase TrkA, a 140 kDa cell surface protein, which specifically binds NGF. The ligand/receptor complex is internalized and retrogradely transported to the soma (Reichardt and Farinas, 1997)

BDNF was identified on the basis of its ability to support sprouting of axons in peripheral sensory neurons, but was first isolated from brain (Barde et al., 1982; Leibrock et al., 1989). Sequence analysis revealed a high homology with NGF, but in comparison to NGF it supports the survival of other sensory neuron subtypes and parasympathetic neurons, but not sympathetic neurons. In the CNS, BDNF supports survival and process outgrowth of basal forebrain cholinergic neurons, retinal ganglion cells and some motor neurons. The most striking phenotype of BDNF null mutant mice, that die 2-3 weeks after birth, is the complete loss of vestibulocochlear neurons. The cellular receptor of BDNF is TrkB. In accordance with the "neurotrophic factor hypothesis" BDNF is secreted by target tissue, but it was also shown that

sensory neurons in DRG also express BDNF, suggesting an autocrine or paracrine mechanism of BDNF function in addition to a target-derived-factor mechanisms.

NT-3 is the most abundantly distributed neurotrophin and has a wider range of action than the others (Maissonpierre et al., 1990). In mature mice, it is expressed at high levels in cortex, hippocampus, thalamus, and cerebellum, and also acts on spinal motor neurons and cochlear neurons. In the periphery, NT-3 supports proprioceptive neurons, and consequently, these neurons are selectively lost in NT-3 null mutant mice (Farinas, 1994, 1996). From all neurotrophins, NT-3 plays the earliest role in development, for example in sustaining neural crest cells, which later become sympathetic neurons and sensory neurons. The cellular receptor of NT-3 is TrkC (Reichardt and Farinas, 1997).

NT-4 the last identified member of the neurotrophin family is not as well characterized as the others. The analysis of its function is complicated due to the fact that it uses the same cellular receptor as BDNF. But surprisingly, these two neurotrophins show only mild functional overlap. In vivo evidence suggest that NT-4 and BDNF activate distinct signalling pathways via the same receptor (Minichiello et al., 1998). Whereas both growth factors have a common supporting effect on sensory neurons and retinal ganglion cells, NT-4 null mutant mice in contrast to BDNF null mutants appear healthy. Detailed analysis of NT-4 and BDNF null mutant mice revealed a reduction in nodose and geniculate ganglion in NT-4 null mutants, which are not dependent on BDNF. The molecular basis for this apparent paradox is not fully understood (Conover et al, 1995, Conover and Yancopoulos, 1997).

In addition to the signalling through Trk receptors, neurotrophins also bind to the low-affinity neurotrophin receptor p75NTR, which is a member of the tumor necrosis factor receptor superfamily (Chao, 1994; Frade and Barde, 1998). Although in vitro studies have shown that p75NTR can potentiate the activation of TrkA (e.g. Verdi, 1994), it does not appear to potentiate activation of the other Trk receptors by their ligands in vitro, even though these also bind to p75NTR. In accordance with the potentiating effect of p75NTR, in vivo studies in p75NTR mutant mice revealed multiple classes of sensory neuron defects (Stucky and Koltzenburg, 1997; Bergmann et al, 1997; Kinkelin et al., 1999). But p75NTR also influences the survival effect of neurotrophins. This can occur in a indirect way via a interplay with Trk receptors. For example NT-3 appears to support the survival of sympathetic neurons in vivo more effectively in the absence than in presence of p75NTR (Brennan et al., 1999). Studies in Schwann cells and neurons have also shown that p75NTR has a direct role on neuronal survival, directly inducing neuronal death via apoptosis (Friedman, 2000; Carter and Lewin, 1996).

1.4.2 Role of neurotrophins in establishing DRG neuron phenotype

The impact of neurotrophins on DRG neurons is manifested through its survival supporting effects, but neurotrophins can also regulate the phenotypic specification of DRG neurons (Mendell, 1996; Lewin 1996).

NGF

Experiments using blocking antibodies (for reviews: Lewin and Mendell, 1993, 1994) and later with NGF null mutant mice, showed that in absence of NGF, nociceptors are completely lost due to their requirement for NGF during embryonic development. In adult animals, NGF was initially thought not to be required. But Lewin and colleagues showed that NGF influences the phenotypic specification of DRG neurons in postnatal mice. When they applied anti-NGF to postnatal mice in a sensitive time window between PD 4 and PD 11, AM nociceptors were converted to non-nociceptive D-hair mechanoreceptors (Lewin et al, 1992). This is an example of a phenotypic switch due to a lack of neurotrophic support (for review Lewin and Mendell, 1993).

NT-3

It is well established that NT-3 does not have an effect on the survival of nociceptive neurons. However, there is growing evidence that NT-3 might influence the phenotypic fate of nociceptive neurons. The effect of excessive amount of NT-3 on developing chick embryos was examined by implanting a NT-3 secreting cell line onto the chorioallantoic membrane (Ockel et al., 1996). When the properties of sensory neurons were examined afterwards using the skin-nerve preparation, one could observe that most of the neurons with unmyelinated axons, which are normally high threshold nociceptors, developed low threshold properties. It should be noted that nociceptive neurons only express the NGF receptor TrkA, so that a response of a nociceptor to NT-3 appears to be paradoxical. However experiments with cell lines and primary sensory neurons showed that NT-3 might also bind to TrkA (Clary and Reichardt 1994; Davies et al., 1995). It seems that the balance between NT-3 and NGF is important for the phenotypic determination of developing nociceptors and a shift to either side can lead to a shift of the phenotype. NT-3 is absolutely required for the establishement of proprioceptive neurons, muscle afferents and the specialized endings in the muscle as shown in NT-3 or TrkC null mutant mice. But it is unclear whether NT-3 fullfills a survival supporting function or rather is a differentiation factor for propioceptive neurons or both. Most cutaneous mechanoreceptors express TrkC and axon counts in the purely cutaneous saphenous nerve showed a 25% reduction in NT-3 heterozygote mice. More detailed analysis using skin nerve preparation revealed that this loss is due to a loss of 50% of their D-hair mechanoreceptors and 70% of SA

mechanoreceptors (Airaksinen et al 1996). It turned out that the development of these fibers seems to be normal until birth, but afferents together with the end organs are lost in the first few weeks after birth, indicating that this dependency starts postnatally in constrast to that of the nociceptors.

BDNF

It was shown that brain-derived neurotrophic factor is absolutely required for the development of vestibular afferents and a subset of cochleal afferents innervating outer hair cells (Ernfors et al. 1994a,b), but its effect on sensory neurons in the DRG is ambiguous. Studies on BDNF knockout mice reported neuronal losses of around 30 % of sensory neurons (Ernfors,1994 ; Jones, 1994), since there is no obvious loss of proprioception, it was examined whether a specific subtype of mechanosensory afferents were affected in BDNF heterozygotes. It turned out that the incidence of the four mechanoreceptive neuron types (SA, RA, D-hair and AM) was normal, but surprisingly, the threshold for mechanical stumulation of the SA mechanoreceptors was dramatically increased in BDNF heterozygote mice (Carroll et al. 1998). The axonal conduction velocity of sensory fibers and the morphology of Merkel cells in the touch dome were normal, indicating a direct functional involvement of BDNF in SA mechanotransduction, e.g in the transcriptional regulation of downstream mechanotransduction genes.

NT-4

The function of NT-4 in sensory neurons in the DRG was unclear for a long time. A first clue was delivered 1999 by the work of Stucky et al., who found that NT-4 was required for the survival of a subtype of mechanosensitive DRG neurons, the so called D-hair mechanoreceptors (Brown et al., 1967 a, b). Axonal counts and examination of afferent properties using the skin-nerve preparation in NT-4 knockout mice showed, that D-hairs almost completely disappeared in the mutant mice.

1.5 Aims and hypothesis

The main focus of our lab is the identification of new components of the mechanotransduction complex in mammals. One straight forward approach is to test orthologues of genes that are involved in *c. elegans* mechanotransduction. Members of our laboratory used this approach successfully to identify BNC1 as part of the mechanotransduction channel complex in mice and ongoing projects using gene targeting methods test the involvement of other orthologues of *C. elegans* mechanotransduction genes in vertebrate mechanotransduction (Price et al, 2000). When the first mechanotransduction proteins are identified, further interacting components can be isolated. In a subpart of my thesis I will search for a GPI-anchored protease that interacts with the mechanotransduction channel BNC1 and modifies its activity. However, the main project of my thesis takes a different approach to find mechanotransduction genes. Neurotrophins regulate the survival and functional differentiation of specific subtypes of sensory neurons including specific mechanoreceptors. By identifying genes that are regulated by neurotrophins we hoped to find candidate mechanotransduction genes. Since neurotrophins are unique for vertebrates we assume that this approach will be a reasonable method to identify mechanotransduction genes that are specific for vertebrates and therefore would not be found by just testing orthologues of *C. elegans* genes.

1.5.1 Part I: Search for a GPI-anchored protease that is involved in sensory mechanotransduction

1.5.1.1 Hypothesis

Recently, a serine protease, that had an activating effect on ENaC, was identified in *Xenopus* frogs (Vallet et al., 1997). This protein was called CAP-1, for channel activating protease and its sequence analysis suggested that it is a secreted protease, which is linked to the cell membrane via a GPI-anchor. The mechanism of the channel activation by CAP1 is still unknown. Since ENaC is related to the neuronal ASIC channels like BNC1, we hypothesize that the mammalian CAP-1 homologue (Vuagniaux et al., 2000) or another related protease interacts and modulates mechanotransduction channels like BNC1. Preliminary experiments performed by Gary Lewin showed that enzyme-mediated cleavage of GPI-anchors in the skin-nerve preparation reduces the mechanosensitivity of low threshold mechanoreceptors. This was the first finding that a GPI-anchored protein is involved in mechanotransduction and we therefore initiated an protein purification approach to identify this protein.

1.5.1.2 Aim

I will search for a GPI-anchored protein, probably a protease, that is expressed in sensory neurons and interacts with the mechanotransduction channel BNC1. The first problem for this protein purification approach is the lack of an appropriate assay for this unknown protein. On the basis of two assumptions, that first it might be a protease, and second, it interacts with BNC1, an assay will be developed to monitor this protein during the purification steps. The purification procedure will involve different kinds of chromatographic methods and protein sequencing.

1.5.2 Part II: Search for genes that are regulated by the neurotrophin BDNF

1.5.2.1 Hypothesis

Carroll and colleagues previously showed that the mechanosensitivity of slowly-adapting (SA) mechanoreceptors in mice heterozygote for a BDNF null mutation are severely impaired (Carroll et al., 1998). Using the in-vitro skin-nerve preparation it could be demonstrated that the threshold for activation with a mechanical stimulus was increased by two orders of magnitude. BDNF heterozygotes do not display any loss of neurons, we therefore hypothesized that BDNF regulates the expression of genes that are involved in mechanotransduction in these mechanoreceptors.

1.5.2.2 Aim

I will screen for genes that are downregulated in the BDNF heterozygote mouse in comparison to the WT in order to identify genes that are responsible for the reduction of mechanosensitivity in the BDNF heterozygote mice. In order to find downregulated genes in dorsal root ganglion neurons (DRG), I will combine expression analysis using oligonucleotide arrays with a suppression subtractive hybridization method. The DNA chips we will use probably contain the greatest part of the mouse cDNA and therefore allow a nearly whole genome screen. Use of the subtraction method enables us to confirm the gene array data and also to identify low abundant genes that are potentially not detected by the gene array screen. To further narrow down candidate genes we will examine the expression pattern of interesting genes in the DRG. Those genes with an pattern that suggests a function in mechanotransduction will be examined further using functional tests like the in-vitro skin nerve preparation.

1.5.3 Part III: Search for genes that are regulated by the neurotrophin NT-4

1.5.3.1 Hypothesis

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. Therefore we hypothesize that genes that are specific or enriched in this type of receptor may be good candidates for being involved in mechanotransduction.

1.5.3.2 Aim

In order to find D-hair specific genes, I will screen for genes that are downregulated in NT-4 null mutant mice as consequence of D-hair mechanoreceptor loss. The same methods as in Part II will be used (see part 1.5.2.2.).