1 INTRODUCTION

The ability of primary sensory afferent neurons to rapidly convert mechanical stimuli into electrical signals is a fundamental process that underlies mechanosensation. Almost all somatic sensory afferents respond to mechanical stimuli and this sensitivity is a property of their peripheral terminal structures. There is very little understanding of the molecular mechanisms that underlie this form of transduction in mammals. To date, the best characterized molecular model of mechanotransduction is the body touch receptor neuron of the *Caenorhabditis elegans* where direct electrophysiological evidence showed that mechanotransduction is mediated by a complex of proteins previously identified in genetic screens for impaired touch sensation (O'Hagan and Chalfie, 2005). Whether orthologues of these proteins are involved in mammalian somatic mechanosensation remains unclear because direct evidence for candidate molecules is still lacking (Gillespie and Walker, 2001; Lewin and Moshourab, 2004).

This thesis studies the consequences of deleting specific genes using classical gene targeting techniques for the transduction properties of single cutaneous sensory neurons in the mouse. These genes are mammalian orthologues of the identified *C. elegans* genes involved in mechanosensory transduction. Therefore, we will briefly present the key aspects of mechanotransduction in mechanosensory neurons, then present the model of mechanotransduction in *C. elegans* and the mammalian candidate molecules that might mediate mechanosensation. This is followed by a review of the different mechanosensitive sensory neurons that innervate the skin.

1.1 Mechanisms of mechanotransduction in sensory neurons

It is widely assumed that mechanosensitive neurons, such as the dorsal root ganglion neurons (DRG), possess specialized non-voltage dependent ion channels that are directly gated by mechanical stimuli. A mechanical stimulus activates a number of these channels and causes an inward ionic flux that results in a graded membrane depolarization called the receptor potential (Catton, 1970; Gillespie and Walker, 2001; Hu et al., 2006). Classical neurophysiological studies of mechanosensitive receptor potentials have been carried out using electrophysiological techniques in invertebrate preparations such as the spider slit receptor (French et al., 2002),

crayfish muscle stretch receptor (Eyzaguirre and Kuffler, 1955), and in the body touch receptor of the nematode worm (O'Hagan et al., 2005). In vertebrates, Loewenstein used the single axon within the cat Pacinian corpuscle whose anatomy and size allowed extracellular recordings of receptor potentials (Loewenstein and Skalak, 1966). From these studies it was proposed that mechanotransduction occurs in 3 steps: (1) a physical stimulus is coupled to the receptor on the cell membrane; (2) which transduces it into a graded receptor potential; (3) that is encoded into action potentials initiated near the transduction zone (French, 1992), (Figure 1).

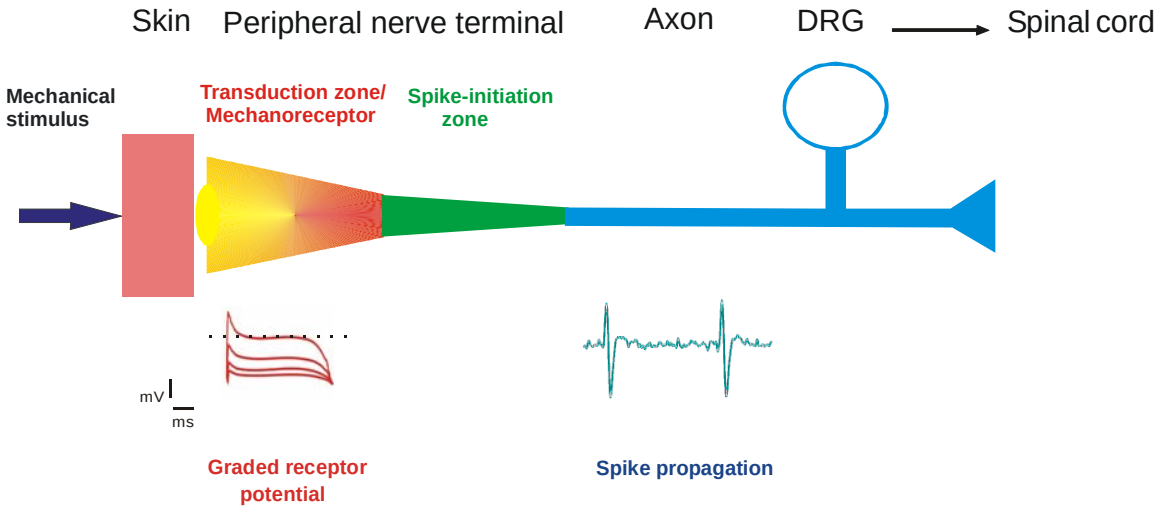

Figure 1: Schematic diagram of mechanotransduction process.

When a mechanical stimulus is applied onto the skin it produces a deformation that is converted into a receptor potential. If the receptor potential exceeds the threshold, an action potential is generated and propagates along the axon towards the spinal cord. DRG: dorsal root ganglion.

To date, there are no direct data available on the mechanosensitive receptor potential for other nerve terminals of the common cutaneous mechanoreceptors due to physical and anatomical limitations. Nevertheless, mechanical forces must directly open mechanically-gated ion channels in order to depolarize and excite the sensory neuron. To prove the existence of such a mechanism is still a challenge for two major reasons. First, mechanosensitive terminals are sparse, tiny structures (with sizes in micrometer scale except for the Pacinian corpuscle) scattered in non-sensory tissues preventing the biochemical purification of their components (Kernan and Zuker, 1995). For example, biophysical evidence of mechanosensory transduction in hair cells of the inner ear reveals less than a 100 mechanically gated conductances per cell (Shepherd et al., 1989). Second, the sensory terminals are far from the cell body and are embedded in other tissue which make electrophysiological recordings of their receptor potentials using patch pipettes

impossible (Garcia-Anoveros and Corey, 1997). To avoid this difficulty several models for the study of mechanotransduction were proposed.

Using the whole cell patch clamp technique, several groups were able to record mechanosensitive currents from acutely isolated rat or mouse sensory neurons grown in culture by mechanically stimulating their cell soma (McCarter et al., 1999; Drew et al., 2002; Hu et al., 2006). Two types of currents were observed in response to a step displacement mechanical stimulation; namely, a slowly inactivating and a rapidly inactivating inward current. Biophysical characterization of these currents is consistent with a non-selective cation channel that is inhibited by high calcium concentrations. However, the identity of the ion channel remained controversial because different research groups obtained contradictory results. For instance, Drew et al. found no effect of amiloride on mechanosensitive currents in mouse sensory neurons while McCarter found that benzamil, an amiloride analog, could selectively block slowly inactivating currents in the rat sensory neurons (McCarter et al., 1999; Drew et al., 2002). Amiloride is a known blocker of acid-sensing ion channels (ASIC), and blockade of mechanically-gated currents may support a role for ASICs as candidate mechanosensitive ion channels.

A major issue is whether these mechanically gated currents observed in cultured adult sensory neurons could explain what takes place in the peripheral nerve terminals. In other words, are the mechanosensitive ion channels in the nerve terminals also expressed on the cell soma or neurite of acutely cultured dorsal root ganglion neurons, and are they expressed in their native form? The answer to this question remains to be addressed, as there is no direct evidence showing that mechanosensitive ion channels in the soma are directly related to mechanosensitivity of the nerve terminal (Hu et al., 2006).

1.1.1 Molecular model of mechanotransduction

There are generally two types of models describing how mechanosensitive ion channels are gated by a mechanical stimulus. The simplest model describes a system of just a channel protein that is gated by tension within the plasma membrane. When the mechanical stimulus stretches the plasma membrane the channel takes an open conformational state and a flux of ions is allowed in. An example is the MscL (mechanosensitive channel of large conductance) ion channel of *Escherichia Coli* (Sukharev et al., 1994; Blount et al., 1999), which opens in response to bacterial

swelling and plays a role in turgor regulation (Levina et al., 1999). Stretch-sensitive channels have been found in eukaryotic sensory cells as well; for example, TREK (Patel et al., 1998), TRAAK (Maingret et al., 1999), *shaker* potassium channels (Gu et al., 2001), and NMDA-gated channels (Paoletti and Ascher, 1994). It is probable that single-celled organisms might depend on stretch-sensitive ion channels to detect or interact with the physical aspect of the surrounding environment. The question arises as to what molecules are involved in detecting mechanical strains in invertebrates and vertebrate sensory cells?

A more complex model is based on a mechanotransduction model of vertebrate hair cells of the inner ear (Pickles et al., 1984; Howard et al., 1988). In hair cells, the transduction channels located at the tips of the stereocilia are thought to be gated by the changes in the tension of the extracellular tip links directly attached to the channel, much like a gating spring (Gillespie and Walker, 2001; Lewin and Moshourab, 2004). The model describes a multimolecular complex that include a membrane ion channel that is tethered by extracellular matrix proteins and intracellular cytoskeletal proteins by linkage proteins. The hypothesis is that ion channels could detect mechanical stimuli by virtue of their attachment to cytoplasmic and extracellular elements (Ernstrom and Chalfie, 2002). One corollary of this hypothesis is that mechanotransduction ion channels when expressed alone may not necessarily be directly gated by membrane curvature.

1.1.2 Molecules of Mechanotransduction in invertebrates

Two major genetic screens have been carried out for mechanotransduction genes in *Caenorhabditis elegans* and in *Drosophila melanogaster* (Kernan et al., 1994; Ernstrom and Chalfie, 2002). In both cases mutant animals with deficient mechanosensory behaviors were isolated and the mutated genes were identified and cloned. In Drosophila the screen identified *remp* (*re*duced *m*echanoreceptor *p*otential) and *nomp* (*no m*echanoreceptor *p*otential) mutants. This resulted in identification of two mutant genes: nompA, which is an extracellular protein; and, nompC, which encodes an ion channel belonging to TRP (Transient Receptor Potential) ion channel family. Other genetic studies have also implicated other TRP channels to be necessary for mechanosensory function in nociceptor-like cells, namely *osm9* in *C. elegans* and *painless* in Drosophila larvae (Tracey et al., 2003; Tobin and Bargmann, 2004).

Mutagenesis screens for touch insensitivity in *C. elegans* worms identified 417 mutations assigned to 18 genes designated as *mec* (Mechanosensory abnormal) (Chalfie and Au, 1989; Ernstrom and Chalfie, 2002). The *mec* genes are randomly distributed on all 6 *C. elegans* chromosomes and are required for various aspects of the developmental program and function of touch cells. These genes are classified into three groups: (1) those required for generation of touch cells (such as *lin-32* and *unc-86*) (Duggan et al., 1998); (2) specification of touch cell fate (*mec-3*) (Way and Chalfie, 1988); and (3) *mec* genes affecting the function but not morphology (Tavernarakis and Driscoll, 1997). The latter group is composed of genes that are proposed to constitute the molecular transduction apparatus responsible for the sensory modality (O'Hagan and Chalfie, 2005). In summary, research on the body touch receptor in *C. elegans* focused on DEG/ENaC ion channels while genetic screens of other mechanosensory systems in *Drosophila* revealed major roles for the TRP channels in mechanosensation.

1.1.2.1 Mechanotransduction model of C. elegans

A loss-of-function mutation in any of the *mec* genes is sufficient to eliminate touch sensitivity with no ultrastructural deficits in the body touch receptor (Chalfie and Au, 1989). Surprisingly, a gain-of-function mutation in *mec-4* (dominant) and a *deg-1* (u38) caused a selective neurodegeneration leading to death of the touch cells (Chalfie and Au, 1989; Chalfie and Wolinsky, 1990; Driscoll and Chalfie, 1991). Nearly all of *mec* genes are cloned and code for extracellular matrix proteins (MEC-1, MEC-5, and MEC-9); cytoskeletal proteins (MEC-7, MEC-12); and ion channel subunits (MEC-2, MEC-4, MEC-10, MEC-6, DEG-1) (Gu et al., 1996; O'Hagan and Chalfie, 2005). MEC-4, MEC-10 and DEG-1 belong to the DEG/ENaC superfamily of ion channels. These proteins are proposed to form a mechanotransduction complex with the proposed ion channel subunits at its core (Tavernarakis and Driscoll, 1997; Ernstrom and Chalfie, 2002), (Figure 2).

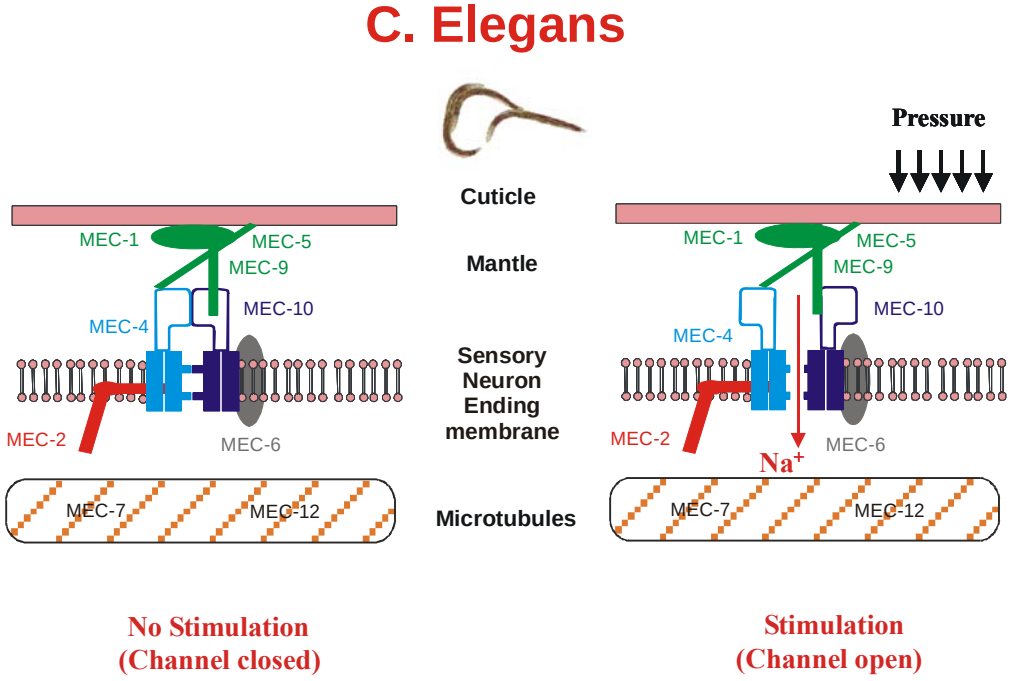

Figure 2: Schematic diagram of the proposed mechanotransduction complex in C. elegans body touch receptor.

At the center is an ion channel that is composed of MEC-4, MEC-6, and MEC-10. The ion channel interacts with MEC-2. MEC-7 and 12 are microtubules which are essential for normal mechanosensation. MEC-1, 5, and 9 are extracellular proteins.

1.1.2.2 Mechanotransduction core complex

The products of the four genes (MEC-2, MEC-4, MEC-6, and MEC-10) that code for membrane proteins are coexpressed and colocalized in punctate pattern in the body touch neurons. These proteins have been shown to immunoprecipitate one another *in vitro* (Gu et al., 1996; Chelur et al., 2002; Goodman et al., 2002; Zhang et al., 2004). The MEC-4 and MEC-10 form the pore of the channel complex and are required for touch-evoked increases in intracellular calcium (Suzuki et al., 2003a); whereas MEC-2, a protein related to stomatin, and MEC-6, a protein related to paraoxonases, associate and modulate the mechanosensitive ion channel activity. For instance, both MEC-2 and MEC-6 amplify MEC-4/MEC-10 currents in *X. laevis* oocytes to ~ 40 and ~30 folds, respectively (Chelur, Ernstrom et al. 2002; Goodman, Ernstrom et al. 2002). Direct evidence of the involvement of these proteins in mechanotransduction came from studying touch-evoked membrane currents using in vivo whole-cell patch clamp recording of the touch neurons in *C. elegans* (O'Hagan et al., 2005).

In the slit-worm preparation (Lockery and Goodman, 1998) the body touch neurons were sealed to patch pipette and mechanical stimuli were applied using a flexible calibrated glass probe one-length constant from cell body (O'Hagan et al., 2005). External forces as small as 100 nanoNewton evoked a rapidly activating mechanoreceptor current (MRC) that adapted while force was maintained. Increasing stimulus amplitude decreased latencies of activation (from 5 to ~ 0.5 milliseconds) and increased current amplitude. The data show that the generation of the MRC is a very rapid process and thus is likely to represent the first step in the transduction process. This implies that physical stimuli directly gate mechanosensitive ion channels. Interestingly, the MRCs were sodium-dependent and were blocked by amiloride *in vivo*. Moreover, null mutations of MEC-2, MEC-4, MEC10, and MEC-6 abolished MRCs. When more subtle mutations to MEC-4 and MEC-10 were introduced, MRCs exhibited a ~3.5 fold decrease in current amplitude without affecting ion selectivity or single ion conductances. Other mutations of MEC-4/MEC-10 altered specifically ion selectivity. In conclusion, these experiments demonstrated that MEC-4 is responsible for converting external force into an ionic current in touch receptor neurons.

1.1.3 Sensory Mechanotransduction in vertebrates

So far the primary candidate molecules for a role in mammalian mechanotransduction are members of the TRP and DEG/ENaC families of ion channels. The DEG/ENaC family includes members identified in *C. elegans*, *D. melanogaster*, snails, and mammals which are grouped in 5 major subfamilies (Figure 3A, 5^{th} subfamily is omitted from diagram). DEG/ENaC subunits range from approximately 500-1000 amino acids in length and contain two transmembrane domains (Figure 3B). The channel subunits are situated in the membrane such that the C- and N-terminal project to the inside of the cell and a single large loop projects extracellularly. The subunit stoichiometry has been controversial but models support either 4 or 9 subunits. We have seen that there is strong evidence supporting a function of DEG/ENaC in invertebrate mechanosensory system (for review see Kellenberger and Schild, 2002). The relationship between this system and those in operation in mammalian mechanosensation systems remains unclear at the molecular and cellular level. In mammals there are 9 identified DEG/ENaC that form two major subfamilies: the epithelial sodium ion channels (ENaC) and the acid-sensing ion channels (ASIC). An important question is whether a role in mechanotransduction is a conserved feature of other members of the DEG/ENaC family.

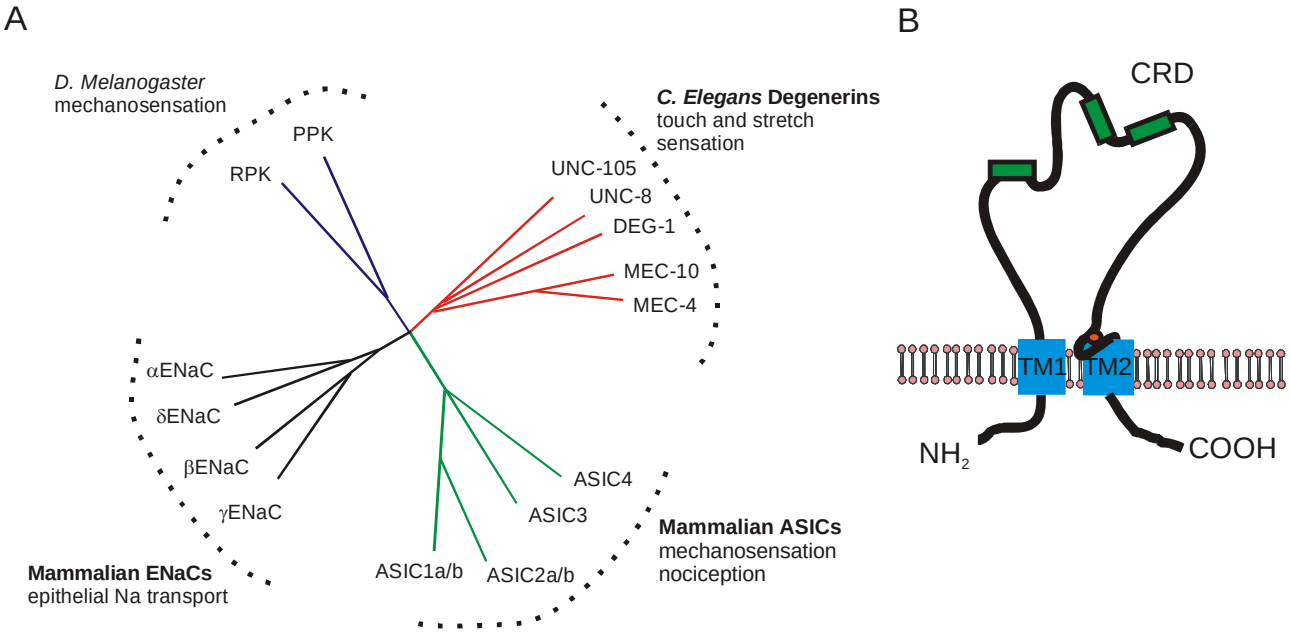

Figure 3: Common features of DEG/ENaC family

(A) Phylogenetic tree of the DEG/ENaC family of ion channels. (B) structure of the DEG/ENaC subunit, channel pore region (TM), a conserved Cys-rich domain CRD (green). A short loop in the pre-TM2 region participates in the channel pore. Amino acid mutations at this region (orange) result in a hyperactive channel.

1.1.3.1 Candidate mammalian mechanosensitive ion channels

The rat alpha subunit of epithelial sodium channel (ENaCα) was identified by expression cloning from *Xenopus laevis* (Canessa et al., 1993). The non-voltage gated sodium channel is known to play a role in sodium reabsorption in the distal tubules of kidneys, to be blocked by amiloride, and to be regulated by aldosterone. The homologous ENaCβ, γ, and δ subunits were later identified by functional complementation (Canessa et al., 1994). These channels are well known to form heteromultimeric complexes. In contrast to ion channels that appeared at an early stage in evolution such as potassium, chloride, or water channels, DEG/ENaC ion channel genes are present only in animals with specialized organ functions (Mano and Driscoll, 1999; Kellenberger and Schild, 2002). ENaC channels have been cloned from species such as human (McDonald et al., 1995), chicken (Goldstein et al., 1997), and bovine (Fuller et al., 1995). They are expressed in different non-sensory tissues like the apical surface of distal tubules of the kidneys and distal colon, lungs, and skin of amphibians and mammals (for review see (Kellenberger and Schild, 2002). Moreover, ENaC transcripts are also found in specialized sensory tissue such as cochlea (Couloigner et al., 2001), baroreceptors (Drummond et al., 1998), and tongue fungiform papillae

(Lindemann, 1996). Thus these proteins might have diverse functions in these tissues ranging from their well-studied role in sodium regulation and hypertension (Rossier et al., 2002), probable role blood pressure sensing, and mediation of taste transduction. Although immunostaining experiments showed that some ENaC subunits are present in several sensory structures such as Merkel cell complexes and Meissner corpuscles of the rat footpad, and the trigeminal nerve endings, a role in mechanosensation has not been directly demonstrated (Drummond et al., 2000; Fricke et al., 2000). Much of the attention has been given to ASIC as the potential mechanotransducer ion channels because of their relatively selective expression in primary sensory afferent neurons (Waldmann and Lazdunski, 1998).

1.1.3.2 Acid-sensing ion channels

It was natural that a search for a mammalian counterpart of the *degenerin* and *mec* genes specifically expressed in neuronal tissue with a role in mechanosensation was made by several groups. A mammalian neuronal degenerin was first cloned from human and rat brain and was named MDEG (Waldmann et al., 1996), or BNC1 (for brain sodium channel 1), (Price et al., 1996), or later ASIC2, and had a 20-29% sequence homology with *C. elegans* degenerins. Three other similar genes were identified which shared 41 to 65% sequence homology with MDEG, or ASIC2, and consisted of around 550 amino acids. In addition to being sodium permeable and blocked by amiloride, acid-sensing ion channels were activated by a drop in pH. Since these channels are expressed in sensory neurons it was hypothesized that they play a role in acid-evoked nociception (Sutherland et al., 2001). Therefore they were designated as acid-sensing ion channels (ASIC) and constituted a new subfamily of DEG/ENaC (for review see (Waldmann and Lazdunski, 1998). In total, four *ASIC* genes (*ASIC1-4*) have been identified and three exist in different splice variants. ASIC subunits are able to form heteromultimeric channels, which is a common feature of DEG/ENaC (Bassilana et al., 1997; Xie et al., 2002). The evidence came from the apparent colocalization of different ASIC subunits in the same tissue (Alvarez de la Rosa et al., 2002); coimmunoprecipitation experiments in heterologous systems; and that coexpression modifies biophysical properties of proton-gated currents (Xie et al., 2002; Askwith et al., 2004).

Homology between *C. elegans* MEC channels and ASICs have led to the hypothesis that ASICs function within a mechanotransduction complex in mammals (Welsh et al., 2002). The role of ASICs in mammalian mechanotransduction comes from electrophysiological *in vitro* recordings

from sensory afferents, or the *in vitro* recording from acutely isolated cultured DRG neurons from *ASIC* mutant mice. Using the former technique, Price et al. found that only the sensitivity of low-threshold rapidly adapting mechanoreceptors (RAM) was substantially reduced in *ASIC2* single mutant mice, while the response of other mechanoreceptors subtypes was not affected (Price et al., 2000). Both *ASIC2* splice variants, *ASIC2a* and *ASIC2b*, were deleted in these mutant mice. ASIC2 is expressed by a substantial proportion of sensory afferents with large diameters; moreover, antibodies to ASIC2 could label terminals associated with Merkel cells, Meissner cells, hair follicles, and some free nerve endings (Garcia-Anoveros et al., 2001; Alvarez de la Rosa et al., 2002). The ASIC2a proteins are capable of forming homomultimeric proton-gated channels when expressed in heterologous expression systems, whereas its splice variant ASIC2b apparently does not (Lingueglia et al., 1997). Even though ASIC2a ion channels are gated by low pH (Waldmann et al., 1996), the receptor endings of low threshold mechanoreceptors that express these channels are not excited by low pH solutions (Steen et al., 1992). One striking aspect of the *ASIC2* mutant phenotype is that only low threshold mechanoreceptors, preferentially RAM, are affected in the mutant although many more sensory neurons undoubtedly express the channel (Price et al., 2000; Garcia-Anoveros et al., 2001). The same experiments were carried out on *ASIC3* mutant mice this time revealing an increased mechanosensitivity of RAM to moving stimuli (Price et al., 2001). In addition, the mechanosensitivity of A-mechanonociceptors was significantly reduced. The *ASIC1* mutants did not exhibit any deficits in sensory afferent mechanosensitivity (Wemmie et al., 2002); however, a probable role in visceral sensation has been suggested (Page et al., 2004). Since at least 4 different ASIC subunits are expressed in the DRG neurons (ASIC1-4) (Waldmann and Lazdunski, 1998; Alvarez de la Rosa et al., 2002) that can form heteromeric channels, it appears likely that loss of one subunit can be compensated for by the other subunits leading to different kinetic properties of the ion channel complex (Benson et al., 2002). Table 1 shows briefly the features of ASIC ion channels.

Recently, some groups have tried to investigate in detail mechanically gated currents of acutely isolated DRG neurons (McCarter et al., 1999; Drew et al., 2002). In a study on *ASIC2*, *ASIC3*, and *ASIC2/ASIC3* mutant mice, Drew et al. found no differences in mechanically gated currents in DRG neurons between the different genotypes (Drew et al., 2004).

Table 1: Features of ASICs

Protein	Alternative names	Expression pattern	pH sensitivity in Heterologous systems	In vivo pH sensitivity	Physiological function
ASIC1a	BNaC2α/ASICa	Brain, spinal cord, DRG	~5.5-6.0	6.9	LTP, synaptic plasticity, visceral nociception
ASIC1b	BNaC2β/ASICb	DRG		?	
ASIC2a	BNaC1α/BNC/ MDEG1	Brain,DRG, tongue	~4.5-5.5	6.5	Mechanosensation, ? Taste
ASIC2b	BNaC1β/ MDEG2		Not gated by H^+		
ASIC3	DRASIC	DRG, testis, lung	Fast ~4.5-6.7 Sustained 3.7	Fast ~6.6 Sustained 3.7	Mechanosensation, acid-evoked nociception
ASIC4	SPASIC	Pituitary, Brain	Not gated by H^+	?	?

Adapted and modified from Bianchi et al (Bianchi and Driscoll, 2002).

1.1.3.3 Stomatin-like proteins

Stomatin (band 7.2b) was initially discovered to be a major integral membrane protein in erythrocytes and is the prototypical stomatin-domain protein (Wang et al., 1991; Stewart et al., 1992). It is widely expressed in mouse neuronal tissue, including DRG neurons, and non-neuronal cells (Mannsfeldt et al., 1999; Fricke et al., 2003). Stomatin has a hairpin-like structure in the membrane and evidence from non-neuronal cells suggest that they can interact with cytoskeletal components, namely actin filaments (Stewart et al., 1992; Snyers et al., 1997). On the basis of the amino acid sequence, stomatin remains the closest vertebrate stomatin-domain to MEC-2 protein with 65% identity, and 85% similarity in the core region (Huang et al., 1995). MEC-2 (481 aa) is longer at the C- and N-terminus than stomatin (287 aa/ ~ 30 KDa in weight) and is absolutely required for normal body touch receptor function in *C. elegans* (Goodman et al., 2002). Furthermore, C-terminal region of MEC-2 mediates interaction with MEC-4 ion channel subunit. In contrast, stomatin is not absolutely required for sensory neuron mechanotransduction since mechanoreceptor mechanosensitivity remained intact in *stomatin* mutant mice (Mannsfeldt, Lewin, unpublished data). Another interesting identified mammalian homologue of stomatin is stomatin-like protein 3 (SLP3). *SLP3* codes for a protein with similar sequence identity to MEC-2 protein (Goldstein et al., 2003). Selective expression in olfactory epithelium and sensory

afferent neurons has suggested roles in olfaction and mechanosensation. Recently, SLP3 is shown to be required for normal mechanosensation in mice. In *SLP3* mutant mice, 35% of skin mechanoreceptors did not respond to mechanical stimuli (Wetzel et al., 2006). Therefore, it is proposed that stomatin-like proteins might interact with the cytoskeleton and the putative mechanosensitive ion channel of the DEG/ENaC family of ion channels in mammalian sensory neurons.

1.2 Physiological properties of specialized mechanoreceptors

The mammalian primary afferent neuron can be regarded as an anatomical, signaling, genetic and metabolic unit. These neurons belong to a subclass of bipolar cells with a so-called pseudo-unipolar morphology. The cell body resides in the DRG of the spinal cord where one major process splits to form two processes. Both function as axons, one going peripherally to the skin and muscle, and the other centrally to the spinal cord. Each DRG neuron with its peripheral afferent fiber and receptive terminals in peripheral tissue, and its central axon and synaptic contacts represents an afferent unit. DRG neurons differ in a variety of ways and can be distinguished by the morphology of their peripheral terminals, diameter of their axons and presence of myelin sheath, in addition to morphology and biochemistry of the cell body and central patterns of termination (Figure 4). Almost all somatic sensory afferents respond to mechanical stimuli and this sensitivity is a property of its peripheral terminal structure.

New facets have been added to the study of afferent mechanisms of somatic/cutaneous sensation by the development of different techniques which include: single unit recordings from *in vitro* preparations and microneurography, channel analysis in the membranes of dorsal root ganglion neurons using patch clamp techniques, and methods in molecular biology and genetics. Extracellular recording from single sensory neurons innervating the skin has been the main electrophysiological method used to study the receptive properties of cutaneous mechanoreceptors (Birder and Perl, 1994). Recordings were initially made from cutaneous nerves of monkeys, rabbits and cats mainly in an ex vivo preparation. In the 1980s Reeh described a technique to make such extracellular recordings from single afferent units from an in vitro preparation of rat saphenous nerve (Reeh, 1986).

This technique will be referred to from now on as the *in vitro* skin nerve preparation. In the 1990s Lewin and Koltzenburg adapted this technique to the study of mouse and chick sensory neurons (Koltzenburg et al., 1997). The *in vitro* preparation offered the advantage of pharmacological manipulation of isolated sensory receptive fields, well-controlled mechanical stimulation, and in the case of mouse preparation the study of transduction properties of single neurons in genetically manipulated mice (Airaksinen et al., 1996). Another important aspect is that the *in vitro* properties of low threshold mechanoreceptors and mechanonociceptors in mice are generally comparable to *in vivo* recordings in other species like human, monkey, and cat (Burgess and Perl, 1967; Perl, 1968; Lewin and McMahon, 1991; Vallbo et al., 1995; Koltzenburg et al., 1997; Johnson, 2001). Classically, cutaneous mechanoreceptors can be classified by their conduction velocities into three main groups: large diameter myelinated axons of Aβ type with fast conduction velocities; small diameter unmyelinated axons of C type with slow conduction velocities; and thinly myelinated axons of the Aδ type with intermediate conduction velocities (see table 2 for a complete list of different mechanoreceptors in hairy skin). Another important feature that functionally characterizes mechanoreceptor subgroups is the pattern of firing in response to a ramp and hold mechanical stimulus (Figure 5).

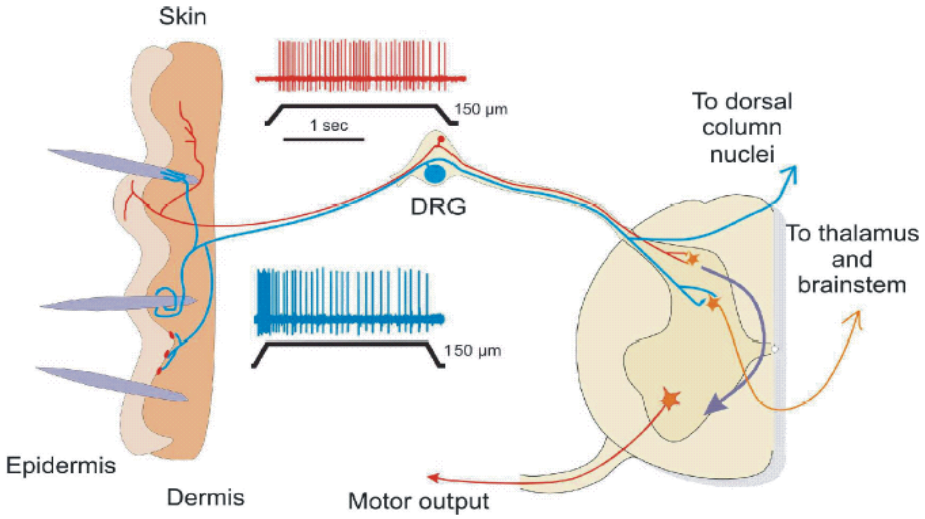

Figure 4: Anatomical and functional organization of mechanosensory pathways.

Two sensory neurons are illustrated, one a low threshold mechanoreceptor (blue) and the other a nociceptor (red). Low threshold mechanoreceptors can innervate the hair follicles in the skin with lanceolate endings (top) or Ruffini endings (middle follicle). They can also innervate Merkel cells at the epidermal/dermal boundary (bottom) and these neurons have slowly-adapting mechanosensory responses (illustrated). Low threshold mechanoreceptors can send a collateral to the dorsal column nuclei and also tend to form direct monosynaptic connections to deep dorsal horn neurons. Mechanonociceptors can form free nerve endings in the dermis or epidermis and typically form synapses in the superficial dorsal horn. Nociceptive mechanosensory information is relayed to deeper dorsal horn neurons that in turn send information to the brain.

1.2.1 Aβ-mechanoreceptors

Aβ-mechanoreceptors have large-diameter, thickly-myelinated axons and are usually low-threshold receptors with large, light cell bodies in the dorsal root ganglion. They can be functionally divided into two broad types: (1) those responding exclusively to movement of the skin and not to static indentation called rapidly adapting mechanoreceptors (RAM); (2) and those that respond both to movement and static indentation and are called slowly adapting mechanoreceptors (SAM). Therefore, the functional difference between SAM and RAM is mainly their adaptation behavior to static indentation.

1.2.1.1 RAM

Moving distortions and movement of hair appendages of the skin most effectively excites the RAM. These receptors are insensitive to static skin deformation. The RAM can be classified into subtypes depending on the species studied and tissue innervated (hairy, or glabrous skin; dermal-epidermal structures, or subcutaneous structures, see Table 2). In hairy skin, RAMs are usually excited by the movement of the intermediate in length, relatively stiff guard-hairs (G-hairs). Histologically, G-hairs receive terminal nerve endings of myelinated Aβ-fibers around the base of the hair follicle (lanceolate endings) (Iggo and Andres, 1982). In glabrous skin two types of velocity detecting receptors are known: the Meissner corpuscles, and the Pacinian corpuscles. Pacinian corpuscles are the largest of the corpuscular receptors that are found in the dermis and are characterized by an elaborate inner core of stacks of numerous thin lamellae arranged in a bilaterally symmetrical manner (Quilliam and Sato, 1955). They are extremely sensitive to vibration, or very rapid changes in tissue distortion, i.e., acceleration. The tactile corpuscle of Meissner resides in the dermal-epidermal papillae of glabrous skin (Vallbo and Johansson, 1984). Microscopically, a Meissner corpuscle is a capsulated ovoid structure comprised of a varicose nerve ending axon spiraling around a stack of lamellar schwann cells (Cauna, 1956). Each of the axonal swelling is sandwiched between two schwann cells forming a column of schwann cell-axonal complexes. Similar schwann cell-axonal complexes are known to occur in lanceolate endings (Munger and Ide, 1988). These structures are characterized morphologically by lamellations in association with the RAM nerve ending (Iggo and Muir, 1969; Johnson, 2001).

1.2.1.2 SAM

The SAMs can be classified as SA-I mechanoreceptors which have endings within the Merkel cell complex at the dermal-epidermal border, and SA-II which innervate Ruffini structures and detect skin stretch. The SA-I mechanoreceptors are Merkel-neurite complexes that involve a specialized epidermal cell, called Merkel cell, that enfold the unmyelinated nerve endings of SA-I axons where specialized synapse-like junctional regions exist (Johnson et al., 2000). In fact, an individual SA axon branches and innervates an unknown number of Merkel cells over an area at the intermediate ridges of the epidermis. In hairy skin, these receptors often associate with large hairs called the tylotrichs of Straile (Iggo, 1976; Iggo and Andres, 1982). The SAMs respond to both the phasic and static components of a displacement stimulus. The static component of the discharge is irregular whereas the phasic or velocity-related component is considerably prominent (Burgess et al., 1968; Pubols, 1990). The SA-II mechanoreceptors are associated with Ruffini nerve endings located in the dermal layer (Chambers et al., 1972). These mechanoreceptors typically fire at regular intervals and the firing frequency increases with increasing displacement strength (Perl, 1968; Horch and Burgess, 1975; Johansson and Vallbo, 1979). Moreover, they might exhibit a background discharge, in the absence of any stimulus, which might be related to their sensitivity to skin stretch or tension.

Electrophysiological studies show that they might be involved in detection of joint movement (Macefield et al., 1990). The SA-II mechanoreceptors have a less prominent discharge response to the velocity dependent component of a stimulus when compared to SA-I mechanoreceptors.

1.2.2 Aδ-mechanoreceptors

Aδ-fibers have thin axons covered by a thin myelin sheath and therefore have slower conduction velocities than Aβ-fibers. The cutoff velocity between Aδ- and Aβ-fibers differs according to species; for example, in large mammals, like humans, it is up to 25 m/s while in mice it is 10 m/s (Koltzenburg et al., 1997). There are only two major subgroups of Aδ-mechanoreceptors: the low-threshold D-hair receptors, and A-mechanonociceptors (AM) or the high-threshold mechanoreceptors (HTMR).

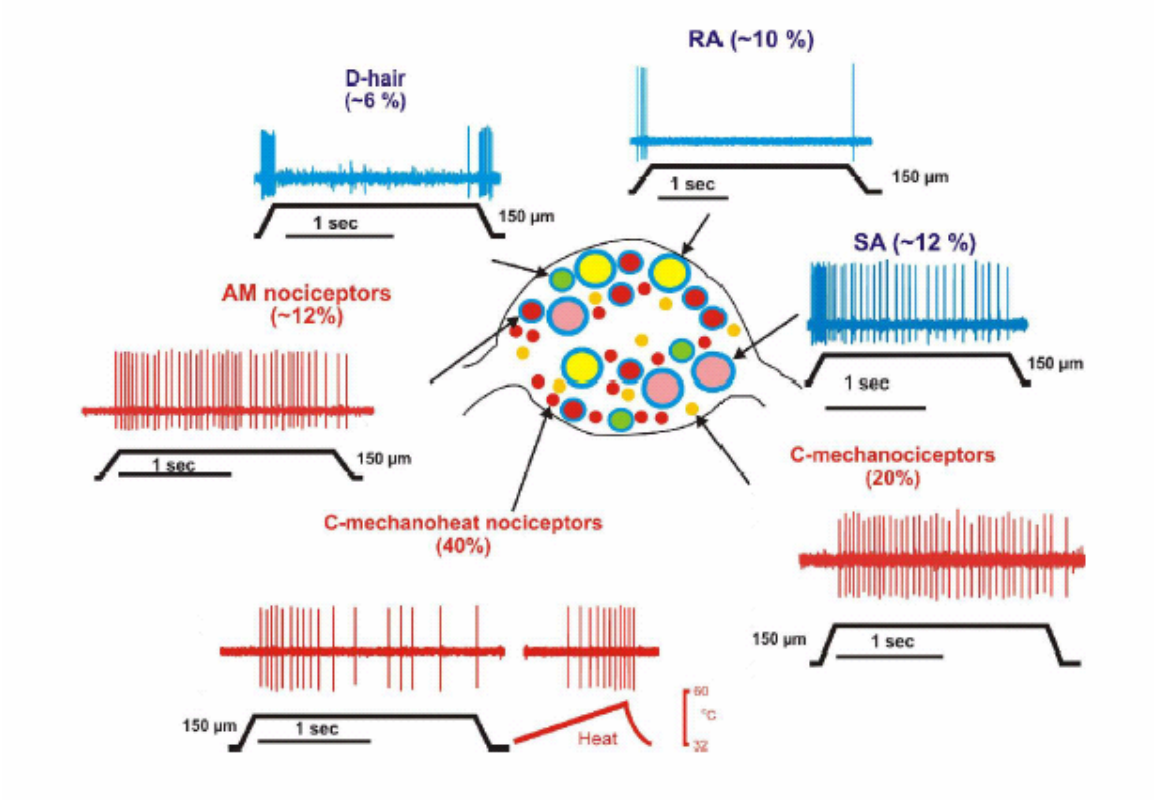

Figure 5: Typical response properties of mouse mechanoreceptors

Responses from the saphenous nerve to a standardized 2s ramp and hold indentation stimulus of 150µm are shown. In the centre a schematic diagram of the dorsal root ganglia depicts the approximate cell size and myelination state of the different mechanoreceptors (thick cell wall indicates myelinated neurons). The mechanoreceptors can be divided into two major groups, those depicted in blue are low threshold mechanoreceptors that all robustly respond to the ramp phase of the stimulus skin. Mechanoreceptors depicted in red are all nociceptive and respond primarily to the static phase of the stimulus. The approximate incidence (% of total cutaneous sensory neurons) is indicated next to its name. Note all action potential records are real recordings made using the mouse skin nerve preparation. The firing rates and patterns of discharge of the chosen examples are typical for the receptor type indicated. For example, AM neurons typically have higher rates of firing than do C-M or C-MH nociceptors.

1.2.2.1 D-hairs

D-hair receptors were first observed in cats by Iggo and Brown, and later in primates, rodents and humans. D-hairs are selectively stimulated by moving small sinus or down hairs in the skin, thus the name D-hair (Brown and Iggo, 1967). Although the morphological nature of these mechanoreceptors has not yet been shown, it seems likely to be associated with hairs (Stucky et al., 1998). D-hairs are distinguished by large receptive fields, a characteristic conduction velocity in the Aδ range (1-10 m/s), and von Frey thresholds up to 10 times lower than other low threshold mechanoreceptors. For example, D-Hairs have been shown to be excited by slow

deflections of hair or skin, pulsations produced by arterial pulse, and sudden cooling probably related to pilo-erection (Burgess et al., 1968). Quantitative measurements have established that these neurons are by far the most sensitive mechanoreceptors in the skin (Lewin and McMahon, 1991; Lewin et al., 1992; Koltzenburg et al., 1997; Woodbury et al., 2001; Dubreuil et al., 2004). Spikes can be elicited with movements as small as 1 μm and velocities as low as 1.5 μm/sec, which is about 100 times more sensitive than RA mechanoreceptors (Dubreuil et al., 2004).

1.2.2.2 A-mechanonociceptors

A-mechanonociceptors (AM) are sometimes known as Aδ-HTMR or Aδ-nociceptors. These receptors almost certainly have "free" nerve endings in the skin. Ultra-structural studies show that the individual fibers lose their myelin, and form terminal branches in the epidermis that are associated with Schwann cells or keratinocytes (Kruger et al., 1981). Rodent AM receptors typically have Aδ-fiber conduction velocities although receptors with clear characteristics of AM neurons are occasionally encountered with conduction velocities in the Aβ range (Ritter et al., 1993; Koerber and Woodbury, 2002; Lawson, 2002; Djouhri and Lawson, 2004). These receptors are often termed Aβ-nociceptors and have high mechanical thresholds and wide action potentials typical of nociceptors. There is no evidence to suggest electrophysiological and cytochemical differences between Aδ and Aβ-nociceptors (Djouhri and Lawson, 2004). Typically AM fibers respond prominently to the static indentation of the skin and do not respond with any phasic discharge to skin movement (Garell et al., 1996). AMs are effectively excited by strong, noxious mechanical stimulation of the skin, and the evoked response frequency is graded according to intensity. In the mouse, rat, cat, monkey and humans AM fibers can be sub-divided into those that have in addition a clear response to thermal stimuli: intense noxious heat or cold. The proportion of AMs with a heat response in rodents is typically around 20% (Caterina et al., 2000; Cain et al., 2001).

1.2.3 C-mechanonociceptors

Sherington postulated the existence of cells that are specialized to detect noxious events and introduced the term nociceptors to describe these cells. Years later myelinated Aδ-fiber, and unmyelinated C-fiber nociceptors that responded to noxious stimuli were demonstrated in the

skin (Burgess and Perl, 1967; Bessou and Perl, 1969). C-fiber axons lack a myelin sheath and consequently have very slow conduction velocities (in mice <1.2 m/sec). They make up the largest group of primary afferent neurons innervating the skin (60-70%). Early studies of C-fiber receptors focused on their mechanoreceptive properties although it was also clear at the same time that many C-fibers could also act as thermoreceptors (Iggo, 1960). In the late 1960s Perl and his colleagues first started to systematically study C-fiber receptors with a range of different stimuli including intense mechanical and thermal stimuli. The analysis revealed that most C-fiber nociceptors in the cat, rat and monkey are so called polymodal receptors that respond to both mechanical and thermal stimuli (Bessou and Perl, 1969; Lynn and Carpenter, 1982; Birder and Perl, 1994). In addition these fibers can be activated or sensitised to thermal stimuli by a wide range of exogenously applied algesic chemicals such as bradykinin, prostaglandins, and capsaicin. A substantial number of C-fiber nociceptors are also present that respond to mechanical but not thermal stimuli, and in the early 1980s Handwerker and colleagues started to use a simple and straightforward classification scheme to describe the sensory receptors according to the range of mechanical and thermal stimuli that activated them. Thus, polymodal C-fiber nociceptors became C-mechanoheat (C-MH), C-mechanocold (C-MC), or C-mechanoheatcold (C-MHC) (Fleischer et al., 1983; Kress et al., 1992; Lewin and Mendell, 1994). The remaining neurons lacking a response to noxious thermal stimuli can be classified as C-mechanonociceptors (C-M).

Two further groups of C-fibers exist in significant numbers. The first group consists of C-fiber low-threshold mechanoreceptors (C-LT), a group whose existence has been recognized for some time (Iggo, 1960). The proportion of low threshold C-fiber mechanoreceptors appears to vary strongly between species, high in cat and low in rodents, but they have also been shown to be present in significant numbers in humans (Wessberg et al., 2003), where they might mediate non-discriminative touch (Olausson et al., 2002). In contrast in the early 1980s Meyer and Campbell identified a second substantial group of C-fibers in monkeys, those displaying little or no mechanosensitivity under physiological conditions (Meyer et al., 1991). This class of nociceptors that were termed mechanically insensitive afferents (MIA) had also been identified in experimental studies in visceral and joint receptors and have sometimes been called “silent” or “sleeping” nociceptors (McMahon and Koltzenburg, 1990).

Recently developed microneurography techniques allow the detection and characterization of mechano and heat insensitive C-fibers in humans and these have been classified according to the

above-mentioned scheme as C-mechano insensitive, heat insensitive fibers (C-MiHi). They have been estimated to comprise around 15-25% of C fibers, and have been best characterized in humans and monkeys (Meyer et al., 1991; Davis et al., 1993; Schmidt et al., 1995; Weidner et al., 1999). They also appear to be present in rodent skin but most studies have found them to be present in much smaller numbers (~10%) than observed in higher mammals (Handwerker et al., 1991; Kress et al., 1992; Lewin and Mendell, 1994). Several distinct features allow their separation from other C-fibers. First, C-MiHi have higher electrical thresholds for activation than mechanosensitive C-fibers (Schmidt et al., 1997). Second, although they have comparable conduction velocities to other nociceptors, human C-MiHi exhibit stronger activity-dependent slowing. This is a phenomenon whereby low frequency suprathreshold electrical stimulation of the C-fiber axon or terminal ending induces a slowing of the apparent conduction velocity. Upon sensitization with algogens (eg. Capsaicin) some of these neurons can become responsive to tonic pressure and heat stimuli (Meyer et al., 1991; Kress et al., 1992; Schmidt et al., 1995). This latter phenomenon is of considerable interest as it strongly suggests that some sensory afferents possess a latent mechanotransducer that can be rapidly reactivated by algogenic compounds. This phenomenon may be of even greater importance in the case of primary afferents that innervate deep tissue including muscle, joint, and viscera (McMahon and Koltzenburg, 1990; Schaible and Grubb, 1993).

Table 2: Hairy skin mechanoreceptors

Mechanoreceptors types	Structure innervated	Vertebrate species	Mechanosensitive property
SA-I	Merkel discs	Mouse, rat, cat, human, monkey	Movement & Static Indentation
SA-II	Ruffini, Haarscheibe	Few mouse, rat, cat human, monkey	Stretch
FA-I	Pacinian Corpuscles.	Few mouse & rat, cat, human, monkey	Vibration-flutter
FA-II	Meissner corpuscles	Mouse, rat, cat, human, monkey	Movement
G1 Hair G2 hair	Hair-lanceolate	Cat, rabbit, rat, human	Rapid Hair movement Low velocity Distort.
Field ½		Cat, rabbit, rat	Skin movement
D-hair	Sinus Hair ?	mouse, rat, cat, human, monkey	Down/sinus Hair movement, very sensitive
AM	Aδ-HTMR. "Free nerve endings"	mouse, rat, cat, human, monkey	High threshold, Noxious Stimulus
AMH-AMC Type I & II	"Free nerve endings"	monkey, human, few in rodents	2 types with different thermal thresholds
AMi-H	"Free nerve endings"	monkeys, humans	Very high threshold
C-LT	"Free nerve endings"	mouse, rat, cat, rare human, monkey	Low mechanical threshold
C-M	"Free nerve endings"	mouse, rat, cat, human, monkey	High mechanical Threshold
C-MH/C-MC/C-MHC	"Free nerve endings"	mouse, rat, cat, human, monkey	High mechanical threshold
C-MiHi	"Free nerve endings"	mouse, rat, monkey, human	Very high threshold, non-mechanosensitive

Adapted from Lewin and Moshourab, 2004.

1.3 Aims and hypothesis

A major goal of our group is to identify new molecular components of the mechanotransduction apparatus in mammals. One approach is to use information from genetic analysis in *C. elegans* and test mammalian orthologues of genes that are involved in *C. elegans* mechanotransduction, namely *mec* genes. The gold standard of evidence is to show that deletion of a candidate gene impairs sensory mechanotransduction in knockout mice. Using this methodology, the group was successful in identifying ASIC2 and ASIC3 ion channel subunits, which are orthologues of MEC-4, as well as stomatin-like proteins, which are orthologues of MEC-2, as part of the mechanotransduction complex. The *mec* genes in *C.elegans* have been proposed to interact together in a mechanotransduction complex. One such interaction exists between MEC-4 and MEC-2 proteins. A key question of this project is whether stomatin interacts with and regulates the ASIC3 ion channel in a mechanosensory context. To address this question, a physical and functional interaction between ASIC3 and stomatin has to be demonstrated. First, coimmunoprecipitation experiments in a heterologous system were performed to detect a physical interaction. Second, mutant mice were generated where the *ASIC3* gene have been deleted together with the *stomatin* gene. Experimental analysis of the *ASIC3/stomatin* mutants was conducted using the *in vitro* skin nerve preparation to describe the mechanosensory phenotype. The main task was to measure changes in the transduction properties of mechanoreceptors to nociceptive and mechanical stimuli. In addition, mechanosensory transduction in wild-type and *ASIC3* single mutants was reevaluated.