4 DISCUSSION

At the molecular level the transduction of somatic sensation such as light touch and pinch is poorly understood. Physiological and genetic studies on the nematode *C. elegans* suggested that cutaneous mechanotransduction is mediated by a complex of several proteins (Chalfie and Au, 1989) (Chalfie and Sulston, 1981). In both invertebrates and vertebrates this mechanotransduction complex is localised to afferent endings of sensory neurones in the skin (reviewed by (Gillespie and Walker, 2001) (French, 1992) (Hu et al., 2006) (Lewin and Moshourab, 2004). It contains a mechanosensitive multimeric cation channel at its core and is connected to the extracellular matrix as well as to the cytoskeleton by accessory proteins that in *C. elegans* have been shown to function as regulatory elements of the ion channel itself (O'Hagan et al., 2005) (Chelur et al., 2002). The work presented here addresses the question of whether two mammalian proteins, stomatin-like protein 3 (SLP3), a stomatin-domain protein that is homologous to MEC-2 in *C. elegans* and paraoxonase, a MEC-6 homologue participate in a mechanotransduction complex in mammals.

The study was largely concerned with the investigation of a mutant mouse model, in which the *slp3* gene has been disrupted. Using electrophysiological recordings it was shown that one-third of myelinated mechanoreceptors in the skin do not function without SLP3. These fibres completely lack mechanosensitivity, as they do not form a functional receptive field (RF) in the skin. In contrast, polymodal nociceptors in the skin are not affected by loss of SLP3. It was also found that mechanically gated ion channels expressed by sensory neurones do not generate mechanosensitive currents in approximately 36% of cultivated primary DRG neurones obtained from SLP3$^{-/-}$ mice compared to <5% in wild-type mice. More importantly, mechanosensitivity could almost completely be restored when a mammalian *slp3* expression construct was transfected in cultivated primary neurones lacking SLP3 (Wetzel et al., Nature 2006 in press). In addition, we have developed a new, well-controlled behavioural assay for testing tactile acuity in rodents and could confirm the findings described

above by showing that SLP3$^{-/-}$ mice also detect tactile stimuli poorly as they have deficits in their ability to discriminate tactile cues. Generating a double mutant mouse line lacking *slp3* and *stomatin* genes products, it was found that these animals do not develop additional mechanosensitive deficits compared to *slp3* single mutants. In a smaller project using molecular biological and biochemical tools, it could be shown that one of the paraoxonase proteins, namely PON2, is expressed by sensory neurones of the DRG in mice. Protein expression studies localised the protein to the cell membrane where it might be attached to the outer side of the plasma membrane via a GPI anchor. This suggests that PON2 is a good candidate molecule for participating in sensory mechanotransduction in mammals. Further functional studies will be needed to test this idea more directly.

4.1 SLP3 is required for cutaneous mechanoreceptor function in mice

Using extracellular recordings from single sensory fibres in the skin, we found that thirty to forty percent of all myelinated cutaneous mechanoreceptors completely lack mechanosensitivity in SLP3$^{-/-}$ mice whereas non-myelinated C-fibres were not affected in these mice (Fig. 11). These fibres were referred to as mechano-insensitive fibres since although they responded to electrical stimulation mechanical stimulation produced no response. Since mechano-insensitive fibres did not exhibit a receptive field (RF) in the skin no further subclassification into rapidly-adapting (RAM) and slowly-adapting (SAM) fibres conducting in the Aβ-range or A-mechanonociceptors (AM) and D-hairs in the Aδ-range was possible. However, experiments in which a mechanical search stimulus was used to isolate single sensory afferents in the skin revealed that receptor proportions within the myelinated A-fibre population were similar in wild-type and SLP3$^{-/-}$ mice. Thus, the data indicate that there is no selective loss of either of the above mentioned mechanoreceptor classes (Fig 12). In other words, mechanically insensitive fibres were found more or less uniformly distributed amongst A-fibre subclasses in SLP3$^{-/-}$ mice.

Electron microscopic analyses revealed that there were no alterations of sensory neurone axons within the saphenous nerve in SLP3$^{-/-}$ mice. There is no evidence for

neuronal loss or degeneration caused by the absence of SLP3 since total axon numbers within the nerve are not different between the genotypes. Proportions of myelinated and non-myelinated neurones are similar in $SLP3^{-/-}$ and wild-type mice (Fig. 23) consistent with published data (Lewin and Moshourab, 2004; Stucky et al., 1999; Stucky et al., 2002). Furthermore, the absence of SLP3 does not cause hypo- or hypermyelination of individual axons within the saphenous nerve as reflected by similar g-ratios calculated for both genotypes (Fig. 23). The latter finding is consistent with the fact that conduction velocities analysed separately for each mechanoreceptor class were unchanged in $SLP3^{-/-}$ mice compared to wild-type littermates (Table 2) and this is in agreement with values published in the literature (Koltzenburg et al., 1997; Stucky et al., 1999). Comparing the average conduction velocity of mechano-insensitive A-fibres with that of the remaining mechanoreceptors, no alterations were found. In addition, mechano-insensitive fibres in $SLP3^{-/-}$ mice exhibit the same electrical thresholds as mechanosensitive receptors (Fig. 13) indicating that the mechano-insensitive phenotype in $SLP3^{-/-}$ mice is unlikely to result from any alterations in the electrical thresholds of mechano-insensitive fibres.

In addition, no substantial differences in sensory innervation density of the dermis or epidermis were observed in $SLP3^{-/-}$ mice by scoring an overall fluorescence intensity of skin sections labelled with antibodies directed against the pan-neuronal marker protein PGP9.5 (Reynolds and Fitzgerald, 1995) (Schofield et al., 1995) (Fig.24). Since no mechano-insensitive afferents were found in $SLP3^{-/-}$ mice amongst the non-myelinated nociceptors, which make up approximately 60 to 70% of all DRG neurones in mice (Lewin and Moshourab, 2004), one could argue that with this methodology innervation density differences in A-fibres in $SLP3^{-/-}$ mice could possibly be underestimated. Another methodological limitation of scoring overall fluorescence is the fact that PGP9.5 is a neurone-specific ubiquitin carboxy-terminal hydrolase distributed throughout a whole neuronal fibre, but functional mechanosensitivity is present exclusively in afferent endings of sensory neurones. Therefore, additional analyses were carried out on hair follicles that are innervated mostly by RAM fibres, which were severely affected by the loss of SLP3 (Fig. 15 and 16). Hair follicles, are often surrounded by palisades of lanceolate endings (Iggo

and Andres, 1982; Persson and Kristensson, 1979; Price et al., 2000) and circles of pilo-Ruffini endings (Halata, 1993) that occur either separately or together. However, 3D modelling of a series of confocal micrographs from hair follicles in SLP3$^{-/-}$ mice revealed no morphological disarrangements concerning how they are surrounded by lanceolate or pilo-Ruffini endings (Fig. 25). In addition, loss of SLP3 does not cause any quantitative differences in hair follicle innervation since the same numbers of hair follicles were innervated with both or only one of these ending types (Fig. 25). Thus, these data indicate that mechano-insensitive fibres found in SLP3$^{-/-}$ mice cannot be attributed to alteration, degradation or even loss of sensory neurones in the saphenous nerve or their afferent endings in the skin.

At the mRNA level a C-terminal transcript of *slp3* was amplified in SLP3$^{-/-}$ mice and a *met* start codon was found downstream of the amplified sequence within exon4 of the gene. It is possible that a so-called dominant negative effect of a putative truncated *slp3* gene product on the wild-type protein would result in a mechano-insensitive phenotype in SLP3$^{+/-}$ mice. However, the proportion of mechano-insensitive fibres observed in SLP3$^{+/-}$ mice was similar to that shown for wild-type animals (Fig. 20), indicating that our *slp3* mutant mouse model presumably does not produce a truncated gene product that would adversely affect the SLP3 wild-type protein or conceivably other stomatin-domain proteins like stomatin.

Several mutant mouse models for mechanotransduction candidate genes, such as *asic2*, *asic3* (Price et al., 2000; Price et al., 2001) and *stomatin* (Mannsfeldt, Thesis 1999, Martinez-Salgado, Benckendorff, Wetzel & Lewin MS in prep) have been investigated. ASIC2 and also ASIC3 can be detected in peripheral mechanosensory structures using specific antibodies. Previous studies on mechanoreceptor functions in ASIC2$^{-/-}$ and ASIC3$^{-/-}$ mice, in which the deleted proteins are proposed to form homo- or heteromultimeric mechanotransduction ion channels in the skin, have shown that the function of distinct mechanoreceptor classes in the skin of mice are impaired due to the loss of either of these proteins (Price et al., 2000) (Price et al., 2001). A mutant mouse model for the *mec-2* homologue *stomatin*, an accessory component that presumably regulates the mechanotransducing ion channel, also

revealed deficits in a single mechanoreceptor class, namely D-hairs (Mannsfeldt, Thesis 1999, Martinez-Salgado, Benckendorff, Wetzel & Lewin MS in prep).

In $SLP3^{-/-}$ mice, RAM fibres were most affected by the loss of SLP3. Approximately 45% of the RAM fibres were classified as so-called “tap” units as they could only be stimulated mechanically by harsh and rapid tapping of the RF (Fig. 15). “Tap” units were also found in the hairy skin of cats, but very rarely. They are considered to exhibit similar properties as pacinian corpuscles, which exhibit rapidly-adapting mechanical properties. “Tap” units in cats respond to vibratory stimuli at frequencies above 500/sec, but rather large amplitudes are required (Burgess, 1968). As shown by Burgess and colleagues mechanical stimulation of the “tap” units had to be manually delivered extraordinarily vigorously and rapidly, such that it was beyond the capability of the electromechanical stimulator. Although “tap” units recorded here, clearly exhibit properties of Aβ-fibres (CV_{tap}=15.8 ±0.8 m/s; $CV_{A\beta\text{-fibres}}$ ≥10 m/s) it is not possible to determine if they are RAM or SAM fibres. For technical reasons we are also not able to stimulate these “tap” units using a controlled stimulation device and therefore no stimulus response functions can be recorded from these fibres. However, since “tap” units respond exclusively to the movement (ramp phase), but not to the hold phase, of a mechanical stimulus, they share at least this physiological feature with RAM fibres. Therefore, in this study they were regarded as non-functional RAM fibres. Considering that the remaining RAM fibres did not show any deficits in their mechanosensitivity (Fig. 15), this supports the hypothesis that SLP3 is required for distinct RAM subclasses. ASIC2 and ASIC3 subunits are known to form homo- or heteromultimeric cation channels with distinct tissue distribution pattern and pH dependencies (for reviews see (Waldmann and Lazdunski, 1998) (Krishtal, 2003)). Thus, one could hypothesise that the composition of the channels may differ in distinct RAM subclasses and the accessory protein SLP3 might interact with one, but not the other, ion channel or channel components.

SAM fibres were also altered in $SLP3^{-/-}$ mice showing reduced mechanosensitivity upon displacement and velocity stimulation (Fig. 16). In addition, they have on average longer mechanical latencies to varying velocities indicating that the activation threshold for initiating action potentials is increased in SAM fibres due to the loss of SLP3.

Since mechanosensitivity in D-hair receptors was reduced by deletion of *stomatin* (Mannsfeldt, Thesis 1999, Martinez-Salgado, Benckendorff, Wetzel & Lewin MS in prep) we expected a similar or even stronger mechanosensory phenotype in this mechanoreceptor class also in SLP3$^{-/-}$ mice. However, no differences could be found for any of the mechanical parameters investigated for D-hairs in SLP3$^{-/-}$ mice (Fig. 17).

High threshold mechanoreceptors (HTM), such as A-mechanonociceptors (AM) and non-myelinated C-fibres were also not altered by the loss of SLP3 (Fig. 18 and 19). Thus, apart from the large proportion of "tap" units amongst RAM fibres in SLP3$^{-/-}$ mice we observed rather small physiological abnormalities within the remaining mechanosensitive myelinated A-fibres and no physiological differences in non-myelinated C-fibres resulting from lack of SLP3.

In this context, the most remarkable finding remains the large population of mechano-insensitive fibres, which does not seem to be restricted to one (or more) single mechanoreceptor class in SLP3$^{-/-}$ mice. The methodology we normally use to investigate mechanosensitive properties of sensory afferents in the skin is an *in vitro* preparation and a minor percentage of the mechano-insensitive fibres recorded here presumably arose from the dissection procedure of the hind limb skin. Mechanically insensitive units have also previously been described in the knee joint (Schaible and Schmidt, 1988) and the urinary bladder (Habler et al., 1990) in cats, where they are not responsive under physiological conditions, but become active in inflammation. Unresponsive cutaneous afferents have been identified amongst HTM conducting in both the Aδ- and C-fibre range in rats (Handwerker et al., 1991) (Lewin et al., 1992a), monkeys (Meyer et al., 1991) or humans (Lynn, 1991) and in the glabrous skin in mice (Cain et al., 2001). As also shown by Handwerker, upon repetitive application of noxious search stimuli, which always resulted in oedematous swelling of the skin, half of the C-fibres stimulated became responsive, either to mechanical, heat or even to both stimulations. This indicates that inflammatory processes may sensitise some of the unresponsive units (Handwerker et al., 1991). Unresponsive fibres conducting in the Aβ-range were also found in rats, although very rarely (2% of Aβ-fibres) and these fibres did not become responsive after repeated stimulation or by using very high mechanical stimulation strengths (Handwerker et al., 1991);

unresponsive Aβ-units however have not been reported in mice. The percentage of mechanically insensitive Aδ- or C-fibres reported in the literature strongly varies from 10 to 45% (Lynn, 1991; Lynn and Carpenter, 1982) (Handwerker et al., 1991) (Meyer et al., 1991) (Lewin and Mendell, 1994). On the one hand, experimenters often used different experimental approaches to study mechanically insensitive fibres on the other hand the term 'unresponsive' is of course also partly limited to the stimuli used. However, in wild-type mice, we found more mechano-insensitive C-fibres than those conducting in the Aδ- or Aβ-range, and these unresponsive C-fibres might represent so called silent nociceptors, which become active in inflammation and this may also be the case for mechano-insensitive C-fibres recorded from SLP3$^{-/-}$ mice.

Receptive properties of cutaneous sensory afferents have been analysed in a number of classical physiological studies (for review see Burgess, P.R. & Perl, E.R. (1973) (Iggo, 1977). From these studies, sensory receptors have been categorised into different receptor classes and individual classes were found to be similarly distributed in a number of mammalian species. In addition, mechanoreceptor classes found in mammals exhibit similar receptive properties in different species. To date the main methodology for studying cutaneous receptor properties has been to make teased fibre extracellular recordings from cutaneous nerves (Birder and Perl, 1994). Using the *in vitro* skin nerve preparation technique developed by Reeh (Reeh, 1986) (Reeh, 1988) and adapted by Koltzenburg and Lewin (Koltzenburg et al., 1997) for investigating receptive properties of myelinated and non-myelinated cutaneous afferents in the hairy skin in mice, afferents conducting in the A-fibre range can be divided into four major groups. In the Aβ- group one can distinguish between RAM and SAM, which are low-threshold mechanoreceptors (LTM) with either rapidly- or slowly-adapting properties, whereas the Aδ-group resembles D-hairs with low threshold and AM with high threshold activation properties (Koltzenburg et al., 1997). However, using this method, we are not able to distinguish between certain receptor subclasses (as described below) and here I will concentrate only on those receptors with myelinated sensory axons. Firstly, RAMs can be subdivided into three major groups Pacinian corpuscles, field receptors (F_1field, F_2field) and hair follicle receptors (G_1hair, G_2hair) (Brown, 1967) (Burgess, 1968) (Tuckett et al., 1978).

Secondly, there are two groups of SAM receptors, SA-I are associated with Merkel cell complexes at the epidermal dermal border and SA-II innervate Ruffini structures (Brown, 1967) (Iggo and Muir, 1969) (Koltzenburg et al., 1997) (Vallbo et al., 1995) (Cain et al., 2001). A-mechanonociceptors can be classified in A-mechanoreceptors (Aδ-HTMR) two other subclasses that can be excited by either heat (AMH) or cold (AMC) and those with very high activation thresholds (AMi-H) (Adriaensen et al., 1983) (Brown, 1967) (Koltzenburg et al., 1997) (Cain et al., 2001) (Burgess and Perl, 1967) (Caterina et al., 2000). D-hairs cannot further be subclassified.

Only very few RAM fibres are associated with Pacinian corpuscles, which are located deep in the subcutaneous tissue. Therefore, these receptors are not preserved in the skin nerve preparation we used. In addition, only few neurones with receptive properties of SA-II receptors seem to occur in mice (Koltzenburg et al., 1997) or they cannot be distinguished from SA-I (Airaksinen et al., 1996; Iggo and Andres, 1982; Iggo and Muir, 1969; Koltzenburg et al., 1997) by their physiological properties. It has to be mentioned here that selective stimulation of hairs or specific stimulations of touch domes accommodating Merkel discs is not possible in the *in vitro* skin nerve preparation. This is because the corium side of the skin is stimulated and that makes the receptor subclassification even more difficult. In the case of AM fibres we did not further subclassify these receptors by application of heat or cold stimuli for instance and subsequently we cannot distinguish between the subclasses of this receptor class. Thus, one hypothesis is that SLP3 is required for distinct mechanoreceptor subclasses. However, since no subclassification has been reported for D-hairs, mechano-insensitive D-hairs cannot be explained in that way.
The relative incidence of distinct cutaneous mechanoreceptors was 40% RAM and 60% SAM in the Aβ-range and 30% D-hairs and 70% AM in the Aδ-range in wild-type mice (Fig. 12) and this is in agreement with values published in the literature (Koltzenburg et al., 1997) (Lewin and Moshourab, 2004). If only one of the receptor classes, within the Aβ- and Aδ-group respectively, were affected by loss of SLP3, one would expect the following receptor distributions:

- mechano-insensitivity only for RAMs, but not for SAM fibres → receptor distribution would be 13% RAM fibres : 87% SAM fibres

- ● mechano-insensitivity only for SAMs, but not for RAM fibres → receptor distribution would be 58% RAM fibres : 42% SAM fibres
- ● mechano-insensitivity only for D-hairs, but not for AM fibres → receptor distribution would be 5% D-hairs : 95% AM fibres
- ● mechano-insensitivity only for AM fibres, but not for D-hairs → receptor distribution would be 41% D-hairs : 59% AM fibres

This theoretical calculation is of interest, because it clearly shows that mechanoreceptor distribution observed in SLP3$^{-/-}$ mice, i.e. 37% D-hairs and 63% AMs in the Aδ-group is consistent only with one of the examples. That would be the case if mechano-insensitivity within the Aδ-group were exclusively found in AM fibres.

Therefore, the hypothesis that SLP3 is required for distinct mechanoreceptor subclasses can be validated as followed:

(i) It is conceivably that D-hairs are not affected at all by loss of SLP3 and this correlates with our findings that receptive properties of mechanosensitive D-hairs were not changed in skin nerve preparation.

(ii) The loss of mechanosensitivity within the Aβ-group seems to result from certain mechanoreceptor subclasses amongst both RAMs and SAM fibres. (At least in case of RAM fibres there are three distinct mechanosensory features observed in SLP3$^{-/-}$ mice: mechano-insensitive, “tap” units, functionally completely normal fibres)

(iii) There might exist a molecular explanation underlying mechanoreceptor subclassification that cannot be assessed using classical electrophysiological and immunohistochemical techniques.

Studies using heterologous expression of different ASIC subunits, such as ASIC2a, ASIC2b and/or ASIC3 in a heterologous expression system indicate that ASIC2b alone does not from a functional proton-gated ion channel but can interact with ASIC2a or ASIC3 to form heteromeric proton-gated ion channels (Bassilana et al., 1997). ASIC2 and ASIC3 can physically interact with stomatin and SLP3 *in vitro,* whereas it has been shown for SLP3 that the protein does not interact with TRPV1 in heterologous expression system (Wetzel et al., Nature 2006 in press). Null mutations

introduced in several *asic* genes in mice result in distinct mechanosensory phenotypes. In ASIC2$^{-/-}$ mice RAM firing rates, and to a lesser extent, SAM mechanosensitivity were decreased compared to wild-type control (Price et al., 2000). In ASIC3$^{-/-}$ mice reduced firing frequencies were observed in AM fibres combined with increased activation thresholds, but RAM fibres showed higher firing rates upon mechanical stimulation (Price et al., 2001). However, other groups found no mechanosensory alterations in *asic2* mutated mice lacking transmembrane domain 2 (Roza et al., 2004). At least these data suggest that the transduction channel might be composed of an assembly of several ion channel subunits that might eventually also substitute for each other supporting the hypothesis that transduction channel composition may differ in distinct receptor classes and it might also be that SLP3 interacts with different subunits of the transducing ion channel.

4.2 SLP3 is required for the function of mechanically gated ion channels

By mechanical stimulation of newly grown neurites of primary sensory neurones obtained from DRGs of wild-type mice, which were cultivated on laminin substrate, Dr. Jing Hu in the lab showed that these neurones possess three types of mechanically gated currents (Hu and Lewin, 2006). The same stimulation protocol was applied on primary neurones obtained from SLP3$^{-/-}$ mice, but here approximately 36% percent of the neurones analysed did not possess any of these mechanically gated currents (Fig. 21). Interestingly, both primary neurones that normally display RA currents (regarded as neurones with Aβ-fibre axons in the skin) as well as those exhibiting SA currents (regarded as neurones with Aδ-fibre axons in the skin) were reduced similarly in SLP3$^{-/-}$ mice indicating that SLP3 presumably affects both fast- and slowly-inactivating ion channels. The remaining, mechanosensitive primary neurones, did not show any differences in their current kinetics or amplitude. Transfecting a recombinant SLP3-C-GFP tagged expression construct into *slp3* mutated primary sensory neurones successfully rescued the mechano-insensitive phenotype (Wetzel et al., Nature 2006 in press). The percentage of primary sensory neurones that lack mechanosensitivity in SLP3$^{-/-}$ mice correlates well with the proportion of mechano-insensitve afferent endings found in the *in vitro* skin nerve

preparation in (Fig. 11). Thus, the data strongly support the hypothesis that SLP3 is directly involved in the transduction of mechanical stimuli in mice. In addition, and in contrast to the SLP3-C-GFP fusion protein that placed SLP3 as expected in a punctuate expression pattern to the cell membrane, GFP alone when transfected into sensory neurones was found in the cytoplasm of the cell (Wetzel et al., Nature 2006 in press). It is often hypothesised that preferential "transduction zones" in the axonal or cell membrane, where local receptor potentials are generated may be formed from local complexes of mechanotransduction proteins.

Testing whether SLP3 can modulate ASIC channel properties Dr. Jing Hu found that sustained amiloride sensitive proton-gated currents were larger in primary DRG neurones of SLP3$^{-/-}$ mice than those observed in wild-type littermates. This is consistent with the finding that HEK 293 cells, endogenously expressing ASIC channels (Gunthorpe et al., 2001) showed higher amplitudes for proton-gated current compared to those transfected with recombinant *slp3* (Wetzel et al., Nature 2006 in press). No capsaicin sensitive currents were observed in the same primary DRG neurones, indicating that the proton-gated currents recorded did not result from TRPV1 ion channels. However, larger proton-gated current amplitudes in SLP3$^{-/-}$ mice were observed in both mechano-insensitive as well as mechanosensitive cells. Finally, these data suggest that SLP3 directly participates in the transduction of mechanical stimuli. It is possible that it interacts with mechanotransduction ion channels of the ASIC family also *in vivo* but there is currently no direct evidence for which mechanically gated ion channel is regulated by SLP3 in mice.

4.3 SLP3$^{-/-}$/Stomatin$^{-/-}$ mice show no additional mechanosensory deficits

SLP3 and stomatin, which share 65% overall identity at the amino acid level, remain the closest mammalian stomatin-domain containing homologues to MEC2 in *C.elegans*. Both proteins appear to possess a membrane insertion domain with a monotopic structure being exposed to the cytoplasmic side of the membrane as has been shown for stomatin (Hiebl-Dirschmied et al., 1991a) (Salzer et al., 1993). Stomatin can form oligomeric complexes (Snyers et al., 1998) and is likely to be

localised to the cell membrane in a punctuate expression pattern, sometimes considered as lipid rafts (Salzer and Prohaska, 2001) (Foster et al., 2003). Stomatin interacts with the cytoskeleton in erythrocytes (Stewart et al., 1992) and has been shown to co-localise with actin in epithelial cells (Snyers et al., 1997). Co-immunoprecipitation experiments, performed in heterologous expression system, revealed a physical interaction of stomatin and SLP3 via their C-terminal domain. There is also evidence that both proteins can physically interact with ASIC2 or ASIC3 (Moshourab, Thesis 2006; Martinez-Salgado, Benckendorff, Wetzel & Lewin MS in prep; Wetzel et al., Nature 2006 in press) (Benson et al., 2002), which are proposed to function as subunits of a mechanically gated ion channel in cutaneous sensory neurones (Price et al., 2000; Price et al., 2001). Similar to SLP3, stomatin modulates the pH gating of ASIC channels. It potently decreases the current amplitude when co-expressed with ASIC3 in CHO cells but increases the current amplitude when co-expressed with ASIC2a (Paul Heppenstall, personal communication). However, stomatin increases the rate of ASIC2 desensitisation in similar experiments (Price et al., 2004). Thus, we expected a strong mechanosensory phenotype in SLP3$^{-/-}$/Stomatin$^{-/-}$ mice generated in the lab.

However, analysis of mechanoreceptor function in SLP3$^{-/-}$/Stomatin$^{-/-}$ mice demonstrated no additional physiological effects on cutaneous mechanotransduction (Fig 26) compared to SLP3$^{-/-}$mice. Thus, the data indicate that mechano-insensitive fibres found in SLP3$^{-/-}$/Stomatin$^{-/-}$ mice presumably result only from *slp3* gene deletion, since proportions of mechano-insensitive fibres were similar in both genotypes. In addition, “tap” units also occurred in SLP3$^{-/-}$/Stomatin$^{-/-}$ mice but no proportional differences were found compared to SLP3$^{-/-}$ mice. In SLP3$^{-/-}$/Stomatin$^{-/-}$ mice, reduced mechanosensitivity was found in D-hairs (Fig. 27), but this has previously also been reported for D-hairs obtained from Stomatin$^{-/-}$ mice (Mannsfeldt Thesis 1999, Martinez-Salgado, Benckendorff, Wetzel & Lewin MS in prep). Apart from the mechano-insensitive phenotype, no additional mechanosensory deficits were observed in D-hairs from SLP3$^{-/-}$mice. This indicates that the D-hair effect in the double mutants can be attributed to the lack of stomatin. From these studies, one can conclude that if SLP3 and stomatin interact *in vivo* this interaction is not

necessary for the detection of mechanical stimuli by individual cutaneous mechanorecptor classes in mice.

Apart from SLP3 and stomatin, some other proteins have been shown to interact with ASIC channels. PICK1 physically interacts with the C-terminus of ASIC1 and ASIC2, but not ASIC3 via its PDZ domain as shown using yeast two-hybrid assays and it co-localises with ASIC1 at synapses in transfected hippocampal neurones (Hruska-Hageman et al., 2002). PICK1 contains a coiled-coil domain and such domains were reported for proteins that interact with actin filaments, microtubules or intermediate filaments (Alberts, B., 1994). Other putative interaction partners of ASIC channels are the multivalent PDZ domain-containing protein CIPP, which's mRNA is expressed at significant level in DRG neurones, interacting with the C-terminus of ASIC3 via its fourth PDZ domain (Anzai et al., 2002) and the Na^+/H^+ exchanger regulatory factor-1 (NHERF-1), which is co-expressed with ASIC3 in DRG neurones. NHERF-1, when co-expressed with ASIC3 in heterologous expression system, modifies the subcellular localisation of the channel that then co-localises with endogenous erzin an actin-binding protein providing evidence for a possible link of the channel to the cytoskeletal network (Deval et al., 2006). However, there is no direct evidence *in vivo* for a modulation of mechanically gated ion channels by these proteins. There are two other proteins interacting with ASIC channels in vitro, namely PDS 95 (Hruska-Hageman et al., 2004) and annexin II light chain p11 that physically interacts with the N-terminus of ASIC1a (Donier et al., 2005).

4.4 SLP3$^{-/-}$ mice have deficits in tactile discrimination

The extent of the mechano-insensitive phenotype observed in SLP3$^{-/-}$ mice prompted us to ask whether these deficits might lead to alterations in the animals' ability to detect and respond to tactile surface tasks.

To date, no suitable behavioural tests were available to investigate tactile discrimination in rodents. Tactile skills in rodents have so far mostly been studied on the mystacial vibrissae somatosensory system (Guic-Robles et al., 1989) (Hutson and Masterton, 1986) (Sutherland, N.S., 1971) (Vincent, S.B. 1912). Distinct types of

sensory afferents innervate mystacial vibrissae follicles in mice. The deeper regions of the vibrissae cavernous sinus (CS) contain a dense plexus of free nerve endings and the superficial part of this sinus displays a massive array of corpuscular endings. Innervation in the region of the ring sinus consists of Merkel endings and different morphological variances of lanceolate endings, but the region of the inner conical body had a circular plexus of free nerve endings (Maklad et al., 2004).

A tactile discrimination test described by Guic-Robles for rats, uses natural sandpaper cues serving as tactile surfaces. In these behavioural experiments rats learn to discriminate the smoother and the rougher surface, which are associated with reinforcing or non-reinforcing stimuli (Guic-Robles et al., 1989). However, in this behavioural assay, test subjects have to be habituated to the test system and need approximately 300 training trials, receiving 20 daily trials during discrimination training, prior to reaching a criterion of 85% (or more) correct responses. In addition, this test is at least very time-consuming in its application and cannot address vibrissae-independent tactile perception in rodents.

Therefore we developed and designed a tactile driven behavioural test using sandpaper or plastic grid cues. The latter possess grooves with distinct spatial frequencies. The test is performed in a featureless plastic box in a controlled environment accommodating a single test animal per experiment. Surface cues exhibiting tactile features are introduced to the mouse in complete darkness and the behaviour of the mouse in the vicinity of the tactile cue is constantly automatically monitored during the experimental trial. Since tactile surface cues in our test make up only approximately three percent of the whole exploratory area, the mice have to find, detect and then discriminate the surfaces from the surroundings. In contrast to the mystacial vibrissae–based roughness discrimination test (Guic-Robles et al., 1989), our assay for tactile acuity in mice was designed in such a manner that an experimental trial can be performed without any behavioural training phase needed for the test subjects. Another, general advantage of our behavioural assay is that the test is well controlled, i.e. data are collected independently of any measurements made by an experimenter, as it is the case for von Frey algesiometers routinely used for paw withdrawal threshold testing (in connection with the investigation of

neuropathic pain models). The test is quick to perform and gives reproducible results with small numbers of animals.

However, the sandpaper-based assay (Fig. 32) has the following disadvantages:

(i) The glues that are used to fix the sand on the paper in conjunction with the manufacturing procedure might contain volatile organic compounds perceived by mice. Therefore, one cannot exclude that mice also use their sense of smell to discriminate between these distinct surfaces tasks. At least in this context the test may not be a pure tactile discrimination test. Consequently, animals were tested on both the smooth as well as the rough side of the sandpaper.

(ii) Sandpaper cues differ in their surface properties from those of the rest of the floor plate. Our behavioural assay is designed to compare an animal's behaviour on tactile surfaces with its behaviour within the surrounding environment, therefore a negative or blank surface cue exhibiting the same tactile properties as the rest of the floor plate has to serve as control. Subsequently, three tactile cues (rough, smooth, blank) have to be taken into consideration. However, as shown in Fig. 32 in the case of three chosen positions they were statistically weighted not equivalent arranged in the box.

The grid-based assay (Fig. 34) represents a qualitative and methodological advancement of the sandpaper-based test.

(i) This test meets quantitative criteria.

(ii) Surface cues and the floor-plate are made of the same material (slightly roughened Plexiglas).

(iii) Two equivalent positions are symmetrically arranged along the middle axis of the floor plate accommodating the tactile surface cue at one position and the control insert exhibiting the same tactile property as the floor plate at the other position. This makes an additional negative experiment unnecessary. Reference and analysis values are calculated from the same animal. Thus, from the statistical point of view data are straightforward to evaluate.

Our studies on C57BL6/N wild-type mice clearly showed that the animals are able to discriminate structured tactile surfaces from the surrounding area (Fig. 33 and 35) and only approximately eight to twelve animals are necessary to generate reproducible test results. Testing SLP3$^{-/-}$ mice on the sandpaper-based as well as on the grid-based assay, the data demonstrate that the mutants indeed behave poorly to detect tactile cues, they were confronted with, than C57BL6/N mice. The data indicate that on average, SLP3$^{-/-}$ mice are not able to discriminate between the rough and the smooth side of the sandpaper. In addition, SLP3$^{-/-}$ mice did not discriminate finer grid spatial frequencies from the surrounding area. Thus, lack of SLP3 obviously results in tactile discrimination deficits in mice and this is consistent with the mechano-insensitive phenotype we observed in the skin and in primary sensory DRG neurones obtained from SLP3$^{-/-}$ mice.

Considering the finding that myelinated neurones or their afferents in the skin were selectively affected by loss of SLP3, it is interesting that SLP3$^{-/-}$ mice did not develop a mechanical allodynia following chronic constriction injury of the sciatic nerve (Wetzel et al., Nature 2006 in press). Thus, SLP3 might be a promising target for the treatment of neuropathic pain (Campbell and Meyer, 2006).

4.5 PON2 is a candidate mechanotransduction molecule.

The existence of an extracellular transduction pathway has been postulated for the *C. elegans* degenerins and its activation may be linked to the single pass membrane protein MEC-6. This protein is thought to serves as a regulatory subunit of the MEC-4/MEC-10 ion channel (Chelur et al., 2002) (O'Hagan et al., 2005) and is related to vertebrate paraoxonase/arylesterase a family that includes at least three members: PON1, PON2 and PON3 known to prevent lipid oxidation (Primo-Parmo et al., 1996).

The data presented here indicate that PON2 is a promising candidate molecule participating in mechanotransduction in mammals:

(i) It is expressed by all sensory neurones of the DRG regardless their somata size (Fig. 39).

(ii) PON2 is exclusively found in the cell membrane fraction when recombinant expressed in heterologous system (Fig. 40) whereas PON1 and PON3 are reported to be secretory proteins (Reddy et al., 2001).

(iii) PON2 is likely to be GPI-anchored to the extracellular side of the membrane (Fig. 41).

The epithelial sodium channel (ENaC) a family member of the DEG/ENaC superfamily plays a critical role in the control of sodium balance, blood pressure and blood volume (reviewed by (Rossier et al., 2002). The channel activating protease CAP-1 is a serine protease that increases channel activity when co-expressed with ENaC α, β and γ subunits in *X. leavis* oocytes. Mutating the GPI anchor consensus motif at the C-terminus of *cap-1* abolished the cell surface expression of the protein supporting the hypothesis of CAP-1 attachment to the cell membrane via a GPI anchor (Vallet et al., 2002). Since previous findings suggested that GPI-anchored proteins might also be involved in the function of LTM receptors in cutaneous mechanotransduction (Lewin, 1999; society of neuroscience meeting 1999, vol. 32), further experimental effort has to be made to prove whether PON2 indeed is involved in this process.

4.6 Conclusions

The data discussed here, strongly support an important role for SLP3 in mechanotransduction in mice.

(i) SLP3 is absolutely required for 30-40% of afferent sensory endings in the skin. A certain proportion of all myelinated fibres in the skin lost mechanosensitivity completely due to the loss of SLP3 and regardless of mechanoreceptor class analysed in the *in vitro* skin nerve preparation.

(ii) SLP3 participates in the transduction of mechanical stimuli in cultivated sensory neurones. The proportion of mechano-insensitive afferents in the skin in SLP3$^{-/-}$ mice is consistent with the proportion of primary DRG neurones, cultivated from these mice, which did not possess any mechanically gated ion currents.

(iii) SLP3 is important for the acuity of tactile perception. Deficits found in tactile discrimination in $SLP3^{-/-}$ mice support the impact of SLP3 in cutaneous mechanotransduction.