4 DISCUSSION

Our experimental data strongly suggest that ASIC3 is a necessary component in the process of mechanotransduction in mouse sensory neurons. The data also suggest that a weak functional interaction exists between ASIC3 and stomatin because absence of stomatin introduces a slight enhancement in the mechanosensory phenotype of the *ASIC3/Stomatin* double mutant mice. Several observations support this conclusion. First, stomatin is immunoprecitable with ASIC3 *in vitro* and this implies that both proteins physically interact. Second, ASIC3 and stomatin are both expressed in small and large diameter DRG neurons and ASIC3 immunoreactivity is detected in the peripheral nerve endings (Waldmann et al., 1997; Price et al., 2001; Alvarez de la Rosa et al., 2002). Third, recordings from sensory nerves in *ASIC3* single mutants and *ASIC3*/*stomatin* double mutants reveal altered mechanosensitivities in specific sensory modalities. However, these observations are indirect and do not exclude the involvement of a large array of other molecules in the mechanism of mechanotransduction in different mechanoreceptor types, e.g. SLP3, and TRPV4 (Suzuki et al., 2003b; Wetzel et al., 2006). Furthermore, a mechanosensory complex composed of an assembly of interacting molecules includes a DEG/ENaC class of ion channel and a stomatin-like protein is required for touch transduction in *C. elegans*. Since mechanotransduction is so varied and probably evolved independently in different cell types, evidence does not point towards a conserved molecular apparatus for mechanosensation. Different mechanosensory systems might employ common structural and organizational principles despite molecular heterogeneity.

4.1 Interaction of ASIC3 and stomatin-like proteins

4.1.1 ASIC3 and stomatin interact

Mouse stomatin mRNA is expressed in virtually all mouse DRG neurons using *in situ* hybridization techniques. Furthermore, stomatin proteins are localized in a punctate pattern on membranes of cell body and neurites in cultured DRG neurons in mice (Mannsfeldt et al., 1999). ASIC3 is selectively expressed to a substantial proportion of DRG neurons, and immunoreactivity is detectable in peripheral mechanosensory structures (Waldmann et al., 1997; Price et al., 2001; Alvarez de la Rosa et al., 2002). In addition, several lines of evidence suggest the possibility that a stomatin-like protein and DEG/ENaC ion channels interact. First, in the

nematode worm *C. elegans*, Goodman et al. have shown that MEC-2, a stomatin-like protein, physically interacts and regulates MEC-4/MEC-10 ion channels required for sensing light touch (Gu et al., 1996; Goodman et al., 2002). UNC-24, which has a stomatin-like region, co-localizes and coimmunoprecipitates with MEC-4 (Barnes et al., 1996; Zhang et al., 2004). UNC-1, another close homologue of stomatin, interacts with UNC-8, a Deg/ENaC (Rajaram et al., 1999). Second, in mammals, mouse stomatin and SLP3 physically interact with ASIC2a probably through the N-terminal region of stomatin and can modulate the pH-gated currents in HEK293 (Eilers et al, unpublished data). In a similar experiment, stomatin co-immunoprecipitates and co-localizes with ASIC2 and ASIC3 in heterologous cells (Price et al., 2004). Our observation that stomatin co-immunoprecipitates with ASIC3 ion channels *in vitro* is in agreement and extends previous findings. It indicates that ASIC3 and stomatin molecules are able to physically bind to each other, directly or indirectly, in a HEK293 heterologous system. However, evidence of a physical interaction in the peripheral terminals of sensory afferents is still lacking.

4.1.2 ASIC3 and SLP3 interact

SLP3 is predominantly expressed in neural cells, especially DRG neurons and neocortex. It has a high sequence homology to MEC-2 and stomatin. Furthermore, it has a very similar punctate membrane distribution when expressed in HEK 293 cells to that noted for stomatin (Wetzel et al., 2006). Here, we found that SLP3 can co-immunoprecipitate with ASIC3 ion channels in a HEK293 heterologous system. Interestingly, in addition to SLP3 band expected at 32 KDa two distinct bands are detected between 30 and 50 KDa in the immunoblots suggesting modified forms of SLP3.

The finding that there is a physical interaction between members of DEG/ENaC family of ion channels and a stomatin-like protein in nematode and mammalian cells prompted us to test the hypothesis that ASIC3 and stomatin are involved in sensory mechanotransduction in mammalian mechanoreceptors.

4.2 Effects of ASIC3 and stomatin on mechanosensitive afferents

To test directly for a role of ASIC3 and stomatin in mechanotransduction in mouse sensory neurons in vitro recordings of single units was conducted. In *C. elegans* loss of MEC-4 and/or

MEC-2 abolished completely mechanosensation to light touch in the six body touch neurons. What happens to sensory neuron mechanotransduction after *ASIC3* and *stomatin* genes are both deleted from mouse genome.

4.2.1 Fiber proportions

The saphenous nerve in the mouse consists of myelinated A and unmyelinated C afferent fibers. A-fibers can be functionally divided into four main groups, namely RAM and SAM in the Aβ group and D-hair and AM fibers in the Aδ group. The proportions of A-fibers classified into these four categories remain constant in the saphenous nerve in the wild-type, single, and double mutant genotypes. Moreover, the loss of *ASIC3* and/or *stomatin* did not produce differences in the proportions of C-M and C-MH. This indicates that (1) ASIC3 and stomatin do not influence mechanoreceptor identity, (2) ASIC3 and stomatin do not cause an apparent loss of any subset of mechanoreceptors. This is comparable to the findings in *C. elegans* where mutations in *mec-4* or *mec-2* genes resulted in no morphological changes in body touch receptors.

4.2.2 Conduction Velocity

Interestingly, the conduction velocity in C-mechanoreceptors is slower in double mutants. Conduction velocity can be influenced by age, temperature of preparation, and utilization time, which is time for an action potential to be generated after electrical stimulation of the axon. Conduction velocity of C-fibers increases little up to an age of 100-200 days and then remains constant in rats (Sato et al., 1985). Aging and temperature cannot explain this phenomenon because the mice used in the study are of comparable age and experiments are all done at 32°C. Another factor is that conduction velocity along afferents may slow more towards periphery and that should not be a factor in the experiments because all measurements are done between electrodes at receptive field and nerve distal to DRG (Waddell et al., 1989). The explanation could either be an incidental finding or that utilization time is prolonged in C-Mechanoreceptors in A3-/-S-/- double mutants.

4.2.3 Mechanical Thresholds

Loss of ASIC3 increases the mechanical threshold of AM and C-fibers without affecting mechanical thresholds of low threshold mechanoreceptors (LTM), namely RAM, SAM, and D-hairs. Price et al. showed an increase in mechanical threshold in AM fibers without detecting von Frey threshold changes in C-fibers (Price et al., 2001). In the same study, ASIC3 did not produce changes in paw withdrawal thresholds on the behavioral level. Surprisingly, loss of ASIC3 and stomatin increases the mechanical thresholds of AM and C-fibers to even a greater extent than in *ASIC3* single mutants. Thus loss of stomatin produces significant additional increase in mechanical thresholds in nociceptors (AM and C-fibers) alone. This could be due to a (1) decreased numbers of mechanotransducer complexes per nerve ending in nociceptors due to altered or decreased transport of mechanotransduction complexes to membrane, (2) and/or change in the mechanotransducer assembly so that a greater force is needed to gate mechanosensitive channels.

4.3 Effects of ASIC3 and Stomatin on Aβ-fibers

Aβ-fibers have either rapidly adapting (RA) or slowly adapting (SA) response properties to a constant mechanical stimulus. The RA Aβ-fibers are subdivided into three major categories in hairy skin, namely hair follicles, field receptors, and Pacinian corpuscles (Brown and Iggo, 1967; Burgess et al., 1968). Pacinian corpuscles are found in deep subcutaneous tissues of toes and feet in rodents are not encountered because they are not preserved in the in vitro skin nerve preparation (Lewin and McMahon, 1991). Because of the inside-out mounting of the skin, which prohibits selective stimulation of hairs, it is not possible to distinguish between field receptors, which do not respond to hair movements, and hair follicle receptors, which respond to hair movement. Furthermore, field receptors are described to be less sensitive than follicle receptors and they might be represented as RA with higher von Frey thresholds (Lynn and Carpenter, 1982). Because field receptors reside at the border between hairy and glabrous skin, which lies in the dissection line of the skin preparation, it is likely that few field receptors are recorded in this study. Therefore, the majority of RAM encountered are of the hair follicle type, and their responses resemble those of G-hair receptors described in rat and cat which respond to G-hair movements (Burgess et al., 1968; Baranowski and Lynn, 1985; Leem et al., 1993). The morphology of these receptors is likely to be the palisade (lanceolate) endings on hairs (Munger

and Ide, 1988). The majority of SAM encountered in this study probably belong to the SA-I group also found in hairy skin of cat and rat and known to innervate Merkel cell complexes of touch domes (Brown and Iggo, 1967; Iggo and Andres, 1982; Airaksinen et al., 1996; Koltzenburg et al., 1997). The SA-II mechanoreceptors known to have Ruffini endings are less frequently encountered in the mouse and rat preparation (Lewin and McMahon, 1991; Koltzenburg et al., 1997). Thus, the stimulus response properties of mainly SA-I and RA fibers of the G-hair type are studied.

Studies of the response functions of SAM and RAM are performed using controlled mechanical displacements rather than force control. The mechanical stimulus-response functions are obtained at the area of maximum sensitivity. RAMs are most effectively excited by moving distortions or change in displacement with time, i.e. they code velocity. RAM in single and double mutants show a significant two-fold increase in their firing frequency. This result was expected since previous work using displacement mechanical stimuli (not controlled for velocity) reveals increased RAM mechanosensitivity in ASIC3 -/- mice (Price et al., 2001). Both mutant genotypes have identical stimulus-response functions to varying velocities. Furthermore, both mutant genotypes have longer mechanical latencies (20-30 msec) to lower velocity mechanical stimuli. Interestingly, the only observable difference between *ASIC3* and *ASIC3*/*stomatin* mutant RAMs is an increase in mechanical latency of about 10-15 msec at relatively higher velocities between 700 and 1000 μm/sec. It seems that the derangement in mechanical latency to velocity coding in *ASIC3* single mutants is slightly enhanced by the additional loss of stomatin in RAMs. How can the increased sensitivity of RAMs be explained? RAM firing during the movement stimulus usually adapts rapidly and the magnitude of firing is influenced only by magnitude of velocity stimulus. It might be that the mechanically gated ion channels in RAM activate and deactivate slower with absence on ASIC3 and stomatin, explaining first, the delay in the initiation of the first action potentials (AP), and second, allowing longer receptor potential to be generated because of a longer open channel conformation that induces more APs. This is not the first evidence that an ASIC channel and a stomatin-like protein affect RAM function. Previous studies have implicated a role of ASIC2 in RAM function as well, however, detailed study of velocity sensitivity and mechanical latency was not done (Price et al., 2000). Moreover, a majority of RAMs in SLP3 mutant mice completely lose mechanosensitivity to mechanical stimuli (Wetzel et al., 2006). In conclusion, our data support a

role of ASIC3, existing in a heteromultimeric complex, and a limited role of stomatin-like proteins for normal RAM function.

In contrast to changes in RAM properties, SAM do not have significant alterations either in mechanosensitivity, except a modest decrease in *ASIC3*/*stomatin* mutants, or in mechanical latency. SA-I mechanoreceptors have two types of responses: a dynamic response to stimulus movement, and a static response to indentation with an irregular firing frequency. No alterations in either components are observed. However, an analysis of the rate of adaptation of SAMs approximated as a first order exponential decay of firing rate reveals a decrease in time constants (τ, time needed for the firing frequency to reach 36% of its initial value) in *ASIC3*/*stomatin* mutants. In other words, the rate of adaptation of SAM is steeper in *ASIC3*/*stomatin* mutants but has a modest effect on total firing rate without affecting the dynamic and static responses. An interesting question that needs further investigation is whether the adaptation is influenced by the mechanotransducer properties.

4.4 Effects of ASIC3 and Stomatin on Aδ-fibers

The only known mechanosensitive afferents that conduct in the Aδ range in rats, mice, and mammals are D-Hair receptors and AM nociceptors (Burgess and Perl, 1967; Leem et al., 1993; Vallbo et al., 1995; Koltzenburg et al., 1997). While, the peripheral terminals of AM fibers are non-specialized nerve endings at the dermal-epidermal borders, D-hairs might innervate hair follicles (Kruger et al., 1981; Munger and Ide, 1988). D-hairs are rapidly adapting mechanoreceptors with large receptive fields that are much more sensitive (~100 times) to moving stimuli (velocities as low as 1.5 μm/sec) (Dubreuil et al., 2004; Lewin and Moshourab, 2004). In this sub-population there are no significant detectable effects of the *ASIC3* and/or *stomatin* gene deletions on mechanosensitivity and latency, although the stimulus response function in *ASIC3*/*stomatin* mutants is significantly reduced. Consistently, a similar reduction in sensitivity is noted in D-Hair receptors to displacement stimuli in stomatin nulls (Mannsfeldt, thesis 1999, Eilers et al, unpublished). This might be an independent effect of stomatin on D-hairs. The effect of stomatin deletion on velocity sensitivity in D-hairs was not examined.

So far *ASIC3* has been the only gene associated with an impairment in Aδ and C-fiber mechanosensitivity (Price et al., 2001; Hu et al., 2006). Although deletion of stomatin increases

the von Frey thresholds of AMs, there is no detectable differences in stimulus-response patterns or latency, which is in this case prolonged in both single and double mutant mice.

It is known that some AM fibers with broader action potential and a characteristic response pattern have conduction velocities >10m/s (Djouhri and Lawson, 2004). No attempt has been made to distinguish between AM conducting in Aβ-range, known also as Aβ-nociceptors, and regular AM fibers.

4.5 Effects of ASIC3 and Stomatin on C-fibers

All the C-fibers studied were mechanosensitive. These fibers are further subdivided based on their response to noxious heat stimuli. Fibers that respond to suprathreshold mechanical stimuli and to noxious heat are called polymodal nociceptors, C-MH (Fleischer et al., 1983). A few C-fibers have von Frey thresholds less than 1.4 mN and therefore it is unknown whether they have a non-nociceptive function. C-fibers tended to fire less vigorously in response to suprathreshold stimuli than AM, a finding consistent with observations in rat, cat, and primates (including human) (Koltzenburg and Handwerker, 1994; Garell et al., 1996). In *ASIC3* and *ASIC3*/*stomatin* mutants C-fibers exhibited a significant reduction in mechanosensitivity, along with an increased von Frey mechanical threshold. This finding is not encountered in the previous investigation of C-fibers in *ASIC3* mutants. In the present experiments, mechanical displacements ranging from 8 up to 600 μm were applied, and reductions in mechanosensitivities occur as of 130 μm displacements, i.e., higher intensity mechanical stimuli. The effects on C-fiber reductions in mechanosensitivity are more pronounced in *ASIC3*/*stomatin* nulls, but not significantly different when compared to *ASIC3* nulls when the whole C-fiber population is analyzed. Surprisingly, C-MH of *ASIC3*/*stomatin* double mutants show a more dramatic significant decrease in mechanosensitivity only at higher, noxious mechanical stimulations compared to *ASIC3* single mutants. In this case, not only does the absence of stomatin produce a specific effect on a subset of C-fiber nociceptors also sensitive to noxious heat, but it exclusively affects mechanosensitivity to high intensity stimuli. There are two scenarios that might explain the reduction in sensitivity: either ASIC3 and stomatin play a direct role as part of a mechanotransducer; or they affect sensitivity via another mechanism, e.g., by affecting excitability. For instance, deletion of *ASIC3* and stomatin may alter excitability at nerve endings. So far, there is no evidence for a change in membrane properties in DRG neurons from *ASIC3,*

nor from *stomatin* mutants (Benson et al., 2002; Drew et al., 2004, Hu et al, unpublished data). However, Drew et al found no impairment in mechanically-gated currents in acutely isolated DRG neurons in *ASIC3* mutant mice. Whether such evidence represents a model of what occurs in the native peripheral terminals is not yet clear.

4.6 Nociception

The primary afferent nociceptor is, in most cases, central to the experience of pain. The majority of nociceptors belong to the Aδ and C fibers, and the rest have conduction velocities in the Aβ range. So basically nociceptors can have a wide range of conduction velocity. The majority of cutaneous nociceptors have „free nerve endings“ without a special end organ structure, and are embedded in the epidermal-dermal border. They typically respond to noxious stimuli (mechanical, thermal, or chemical) and therefore usually have high mechanical threshold. They do of course posses a high threshold mechanotransducer. If that should be the case then the question arises as to what is the molecular identity of the components of the high threshold mechanotransducer? Once nociceptors of one class are categorized according to molecular markers, the picture gets more complex and diversity rules. Many candidate molecules, mainly TRP and DEG/ENaC ion channel families, are proposed to play a role as mechanotransducer without solid direct evidence favoring a specific ion channel. Instead multiple types of mechanotransducers with nociceptive functions and employing different types of ion channels may be present in peripheral terminals of nociceptors.

ASIC3 is gated by extracellular protons and therefore may play a role in detecting tissue acidosis under inflammatory or ischemic conditions. Investigations implicating ASIC3 in acid-induced nociception, which includes cardiac ischemic pain, are supported by the following observations: (1) single fiber recordings in *ASIC3* nulls display a reduced sensitivity of C-fibers to peripherally applied acid; (2) ASIC3 responses to protons in heterologous cells mimics the acid-induced response of thoracic DRG neurons; (3) acutely isolated DRGs from *ASIC2*/*ASIC3* knockouts show alterations in response to protons (Price et al., 2001; Sutherland et al., 2001; Drew et al., 2004). In contrast, a study by Chen and colleagues reveals that *ASIC3* mutant mice showed an exaggerated behavioral pain response to acid injection when a high intensity, tail pinch but not low mechanical stimulus was applied. They concluded that ASIC3 plays an important role in modulating the perception of high intensity painful acidic stimuli, but is not essential for

generation of acid-induced sensation (Chen et al., 2002). It should be noted that only 20 to 30% of C-fiber peripheral terminals generate action potentials in response to a decrease in pH. Therefore, it might be that ASIC3 in heterologous systems and probably in acutely isolated DRG neurons has an exposed proton-binding site compared to an inactive, unexposed one in native state in peripheral terminals. Another speculation is that protons might be functioning as modulators of the ASIC channel function rather than in gating.

Another example where such discrepancies in observations between isolated cells and intact animals occurr is noxious thermal sensitivity and the effect of TRP-V1. In TRP-V1 knockout mice, somal heat responses are more impaired than single fiber responses in intact animals (Caterina et al., 2000). In fact, one recent report did not observe changes in noxious heat sensitivity in C-fibers in TRP-V1 and V2 null animals (Chen et al., 2002). Hence, in three cases, mechanical, thermal, and acid stimulation, observations in isolated sensory neurons failed to represent what happens in the nerve terminal in mutant animals. The nerve terminal then is structurally and functionally different from the soma. Much remains to be learnt regarding the molecular basis of mechanotransduction. When this is achieved it should be possible to determine the evolutionary relationships of multiple mechanosensory systems.

4.7 Conclusion

In summary, the analysis of mice lacking ASIC3 ion channels reveals that ASIC3 is necessary for normal low- and high- threshold mechanoreceptor function. Furthermore, the loss of ASIC3 and stomatin reveals a weak functional interaction. The diversity of the effects suggests that ASIC3 channel subunits and stomatin normally exist in a heteromultimeric complex whose composition varies among different types of cutaneous sensory neurons.

4.8 Future experiments

The effects of gene deletions on the physiology of mechanoreceptors are specific and diverse and do not point to a single type of mechanotransduction complex. Since we cannot record receptor potential from peripheral nerve terminal, the best available method is recording from single sensory neurons. To test whether a candidate gene is required for normal mechanosensitivity the

gold standard would be to show whether the absence or antagonism of a gene product leads to reduced or abolished mechanotransduction (Hu et al., 2006). What remains is a judicious selection of candidate genes and generation of knockout mice. From then on double knockouts could be easily generated and genetic interactions could be studied. Future experiments should center around two aspects. First, the effect of *ASIC3* and *SLP3* gene deletions on mechanosensation should be investigated because the two proteins physically interact and are required for mechanosensation in mice. Second, the effects of inflammation on the impaired nociceptor function in *ASIC3* mutants should be studied as well. Moreover, the C-mechanoinsensitive-heatinsensitive (C-MiHi), or the "silent" nociceptors, which constitute a considerable population of C-fibers in humans, have been gaining much attention due to their role in mediating inflammatory pain and hyperalgesia. A role of ASIC3 in this subpopulation of nociceptors under inflammation remains to be addressed.