3 RESULTS

3.1 Interaction of ASIC3 and stomatin-like proteins

3.1.1 ASIC3 and stomatin physically interact

ASIC3 and stomatin protein interaction was demonstrated with immunoprecipitation experiments. To produce recombinant ASIC3 ion channels and stomatin, HEK293 cells were doubly transfected with N-terminally Flag-tagged ASIC3 and a C-terminally Myc-tagged stomatin to generate a stable cell line expressing both proteins. A stable HEK293 cell line over-expressing ASIC3 alone was used as control. It is known that HEK293 cells endogenously produce ASIC1a (Gunthorpe, 2001), and also ASIC3 (Heppenstall and Lewin, communicated observation), while stomatin proteins are ubiquitously expressed in many cell types. The anti-Flag antibodies were used to obtain immunoprecipitates that contain Flag-tagged ASIC3 from total protein cell lysates, and the anti-Myc antibodies were used to obtain the immunoprecipitates that contain Myc-tagged stomatin. Western blotting was performed on the anti-Myc immunoprecipitates with anti-Flag antibodies to detect Flag-tagged ASIC3. A ~60KDa band in the ASIC3/Stomatin cell line lane could be detected, consistent with the predicted molecular weight of ASIC3 (Figure 7, output 1). Likewise, when the anti-Flag immunoprecipitates were immunoblotted with anti-Myc antibodies, a 31KDa band consistent with the predicted molecular weight of stomatin was detected in the ASIC3/Stomatin lane but not in the other lanes (Figure 7, output 2).

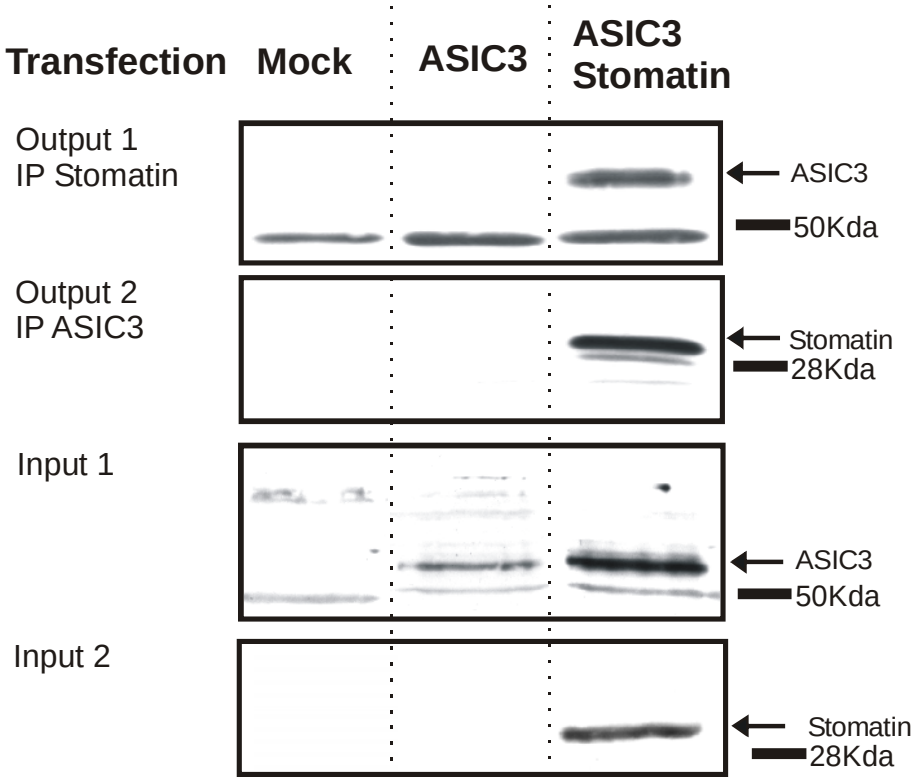

Figure 7: Association of stomatin with ASIC3.

HEK293 cells were transfected with a control empty plasmid (Mock), Flag–tagged ASIC3, and Flag-tagged ASIC3 with Myc-tagged stomatin to produce a stable ASIC3 (Input 1) and ASIC3/stomatin expressing cell lines (Input 2). When immunoprecipitates (IP) were treated with anti-Myc antibodies ASIC3 was detected using anti-Flag antibodies in the ASIC3/Stomatin stable cell line (Output 1). Similarly, stomatin was detected in the immunoblot of anti-Flag immunoprecipitates (IP) in ASIC3/stomatin stable cell line (Output 2).

3.1.2 ASIC3 and stomatin-like protein 3 physically interact

The same procedure was employed to detect an interaction between ASIC3 and stomatin-like protein 3 (SLP3). HEK293 cells stably expressing Flag-tagged ASIC3 (Figure 8) were transfected with either a control empty plasmid (Mock) or a Myc-tagged SLP3. Western blot analysis of the transfected HEK293 cells after 48 to 72 hours using anti-Myc antibodies revealed a modified SLP3 band at ~34Kda (Figure 8, Input 2).

Lysates from the transiently transfected mock and ASIC3/SLP3 cells were immunoprecipitated with anti-Myc antibodies targeting SLP3. ASIC3 was only detected in the immunoblots when treated with anti-Flag antibodies in lysates from ASIC3/SLP3 cells (Figure 8. output 1). When

the cell lysates were precipitated with anti-Flag antibodies and the immunoprecipitates were immunoblotted with anti-Myc antibodies, SLP3 was only detected in the ASIC3/SLP3 lane. In addition, anti-Myc antibodies detected two other bands with differential intensity between 30KDa and 50KDa that are probably modified forms of SLP3.

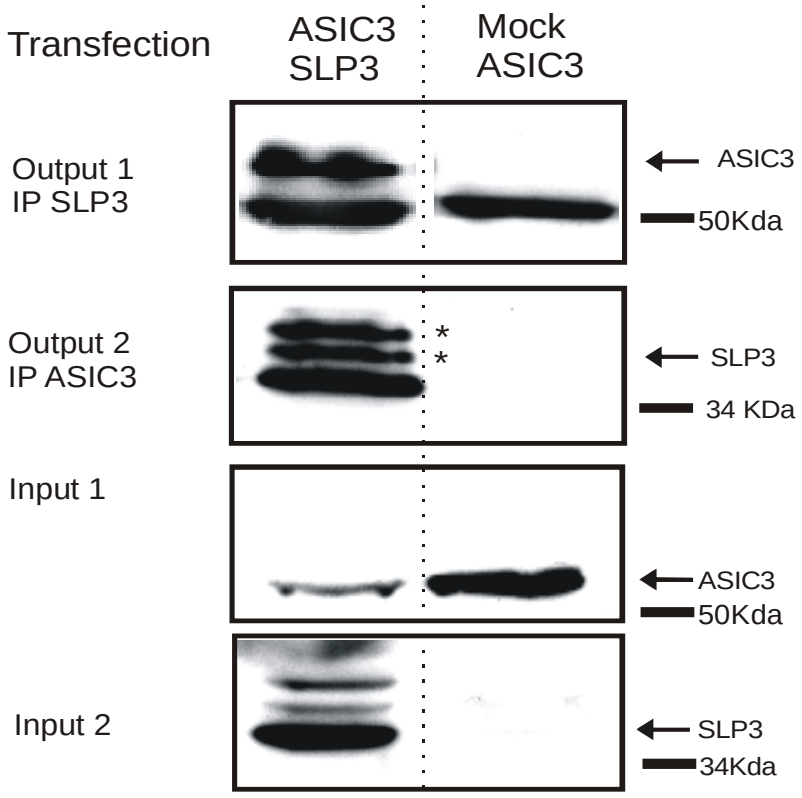

Figure 8: Interaction of ASIC3 with SLP3.

ASIC3 expressing cells were transfected with a control (mock) or a construct expressing SLP3 (Input 2). Anti-Myc immunoprecipitates were immunoblotted with anti-Flag, which detected a 60KDa band pertaining to ASIC3 proteins (output1). Western blots of anti-Flag immunoprecipitates treated with anti-Myc identified ~34KDa-sized band corresponding to co-precipitated SLP3 (output2). Note that the SLP3 band is modified (*).

3.2 Single fiber recordings using in vitro skin nerve preparation

The *in vitro* skin nerve preparation allows the study of mechanoreceptors and in particular the firing response of single sensory fibers in the mouse saphenous nerve to stimulation of their receptive fields. To test the possibility that ASIC3 ion channels and stomatin proteins play a role in mechanotransduction, responses to a ramp and hold mechanical displacement for each

mechanoreceptor type were generated in 3 different settings: 1) in normal genetically intact mice; 2) in the absence of the *ASIC3* gene; and 3) in the absence of both *ASIC3* and *stomatin* genes. Single fiber responses to mechanical events were thus recorded from the following mouse genotypes: C57Bl/6, *ASIC3* +/+ (A3+/+), and *ASIC3* +/+ *stomatin* +/+ (A3+/+S+/+) hybrid mice as controls; *ASIC3* -/- (A3-/-) single mutants; and *ASIC3* -/- *stomatin* -/- (A3-/-S-/-) double mutant mice.

3.2.1 Mouse saphenous nerve

From electron microscopy studies of the saphenous nerve from 3 adult C57Bl/6 mice, about 2500 axons of A and C types were found (639.78±32.99 myelinated A-fibers, and 2101.9±205.7 unmyelinated C-fibers). Therefore 70% of the nerve fibers in the saphenous nerve are C-fibers and the rest (30%) are thinly myelinated and thickly myelinated A-fibers.

The technique of recording from A-fibers in the *in vitro* skin nerve preparation is different from that of C-fibers so that the incidence of A- and C-fibers does not necessarily conform to the proportions found with electron microscopy. An important factor is the action potential amplitude, which is proportional to the area of the membrane recorded from. The greater the surface area of the membrane the greater will be the generated action potential amplitude and consequently the higher the probability of finding and recording from the single unit. The C-fibers generate smaller extracellular voltage changes because they have very much smaller axonal diameters than Aβ-fibers. Therefore the relative fiber proportions differ between the *in vitro* skin nerve preparation recordings and the axonal count made from electron micrographs.

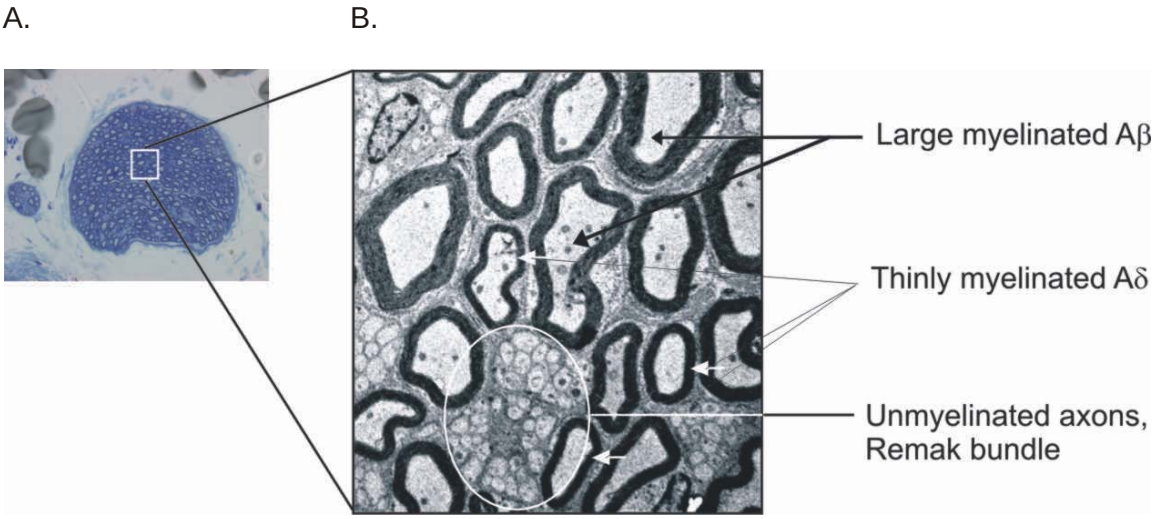

Figure 9: Electron microscopy cross-section of the sapheneous nerve.

In (A) a cross-section of the mouse saphenous nerve. In (B) a 40X40µm electron microscopy image of the mouse saphenous nerve shows large myelinated axons of Aβ-fibers (Black arrow); thin-myelinated axons of Aδ-fibers (white arrow); and a Remak bundle containing several unmyelinated axons (white circle) surrounding a schwann cell nucleus.

3.2.2 General properties of different types of afferent fibers

The saphenous nerves from adult mice with different genetic backgrounds were employed in the study as controls. The first group was a C57Bl/6 inbred strain. The second was a hybrid strain, a mixture between C57Bl/6 and sv129 inbred strain. These mice were carrying all wild-type alleles from *ASIC3* +/- and *stomatin* +/- parents. A total of 244 single afferents were identified from a total of 29 adult (>6 weeks) mice (10 C57bl/6, 10 *ASIC3* +/+ *stomatin* +/+ (A3+/+S+/+), and 9 *ASIC3* +/+ (A3+/+) mice. Comparison of the conduction velocities, fiber proportions, and stimulus response functions of myelinated and unmyelinated fibers reveals no difference between hybrids, i.e. A3+/+S+/+ and A3+/+ mice, and C57Bl/6. Therefore, the stimulus response functions that were obtained from 203 units, 77 Aβ, 67 Aδ, 62 C fibers, were grouped and analyzed together. The conduction velocity and stimulus response functions were used to categorize single afferent units into the conventional mechanoreceptor types: rapidly adapting (RAM) and slowly adapting (SAM) mechanoreceptors with Aβ conduction velocities (CV) greater than 10 m/s; D-hair and A-mechanoreceptors (AM) with Aδ CV between 1-10 m/s; and C fibers with CV less than 1 m/s.

A total of 130 mechanosensitive units were studied from the 9 adult *ASIC3*-/- (A3-/-) single mutants. Thirty-seven myelinated Aβ-fibers (19 SAM, and 18 RAM), 44 thinly myelinated Aδ-fibers (32 AM, and 13 D-hairs), and 49 unmyelinated C-fibers were identified. A total of 193 units were obtained from 21 adult *ASIC3*/*stomatin* double mutants (A3-/-S-/-). Stimulus response functions of 29 RAM, 31 SAM, 38 AM, 20 D-hairs, and 49 C fibers were recorded.

The relative proportions of the four distinct types of A-fibers in A3-/- single mutants, and A3-/-S-/- double mutants did not differ from those found in controls (Table 3). No apparent differential loss in a specific receptor type could be inferred from the relative incidence of the mechanoreceptor types in the mutant mice. In table 3, C-fibers were further divided according to heat sensitivity into C-mechanoheat sensitive fibers and C-mechanoreceptors designated as C-MH and C-M, respectively. The average CV of all C-fibers in A3-/-S-/- mutants was significantly slower than in control mice (T-test, $p = 0.029$), but not significantly different from A3-/- C-fiber CV (T-test, $p= 0.35$). The average CV of C-MH fibers was not significantly altered in A3-/- and A3-/-S-/- mutants. Moreover, the average conduction velocity of C-M in A3-/-S-/- mutants was significantly slower than the average CV of C-M in A3-/- (T-test $p=0.005$), and slower than the average CV of C-M in control mice without reaching statistical significance (T-test $p=0.056$).

3.2.3 von Frey thresholds

Mechanical thresholds as determined with von Frey hairs are helpful in distinguishing low threshold from high threshold fibers. The thresholds were examined using von Frey hairs of discrete strengths (0.4, 1.0, 1.4, 2.0, 3.3, 6.3, 13, 22.4, 32 milliNewtons (mN)); the smallest von Frey hair force sufficient to activate a unit was defined as the von Frey threshold (vFT) for that unit.

Table 3: Relative proportion and conduction velocities of mechanoreceptor in mouse saphenous nerve.

Aβ-Fibers	**Controls (n=84)**	**A3-/- (n=37)**	**A3-/-S-/- (n=69)**
RAM	n=42	n=18	n=34
%	50	49	49
CV (m/s)	13.59±0.45	14.10±0.69	13.51±0.52
SAM	n=42	n=19	n=35
%	50	51	51
CV (m/s)	14.11±0.38	15.43±0.62	14.72±0.58
Aδ-Fibers	**Controls (n=78)**	**A3-/- (n=44)**	**A3-/-S-/- (n=70)**
AM	n=54	n=32	n=46
%	71	73	65
CV (m/s)	6.27±0.43	6.02±0.50	6.69±0.59
D-hair	n=24	n=12	n=24
%	29	27	35
CV (m/s)	5.86±0.38	5.35±0.25	5.39±0.24
C-Fibers	**Controls** (n=82)	**A3-/-** (n=49)	**A3-/-S-/-** (n=54)
CV (m/s)	0.48±0.15*	0.44±0.10	0.42±0.11*
C-MH	n=35	n=32	n=21
%	64	75	57
CV (m/s)	0.41±0.08	0.41±0.09	0.40±0.09
C-M	n=20	n=11	n=16
%	36	25	43
CV (m/s)	0.50±0.15	0.51±0.06**	0.42±0.09**

The statistical significance of the proportion of fibers in a subset was tested by the χ^2 test, and differences in conduction velocity were tested by an unpaired t-test. The number of fibers is indicated. An (*) indicates a statistical significance ($p< 0.05$) in C fiber conduction velocities between controls and A3-/-S-/- mutants. An (**) indicates a statistical significance ($p< 0.05$) in C-M conduction velocities between A3-/- and A3-/-S-/- mutants.

von Frey thresholds for SAM, RAM, and D-hairs in A3-/- and A3-/-S-/- mutants were not different from controls. However, the median vFT of AM was significantly higher in A3-/- mutants compared to controls (Mann-Whitney test: p=0.000015; AM control n=59, median 3.3mN; AM A3-/- n=32, median 6.3 mN). Similarly, vFT of A3-/-S-/- AM fibers was significantly higher compared to control vFT (Mann-Whitney test: p=0.024; AM control n=54, median 3.3 mN; AM A3-/-S-/- n=46, median 6.3 mN), but not different from the vFT of AMs in A3-/- mutant (Mann-Whitney test: p=0.29; AM A3-/- n=32, median 6.3mN; AM A3-/-S-/- n=46, median 6.3 mN, Figure 10A).

The vFT of C-fibers in A3-/- was significantly higher than in control mice (Mann-Whitney test: p=0.011; C control n=82, median 4.8mN; C A3-/- n=49, median 6.3mN). Interestingly, the vFT of A3-/-S-/- mutant C-fibers was also significantly higher as compared to controls (Mann-Whitney test: p<0.00001; C-fiber control n=82 median 4.8mN; C-fiber A3-/-S-/- n=54, median 6.3 mN), and significantly higher than in A3-/- single mutants (Mann-Whitney test: p=0.018; C-fiber A3-/- n=49, median 6.3 mN; C-fiber A3-/-S-/- n=54, median 6.3 mN, Figure 10B).

Examining the vFT of C-MH and C-M fibers separately revealed the following. The A3-/-S-/- C-MH fibers exhibited higher vFTs compared to controls (Mann-Whitney test, p=0.000018; C-MH control n=35, median 3.3 mN; C-MH A3-/-S-/- n=21, median 6.3 mN), and also a higher threshold compared to A3-/- (Mann-Whitney test, p=0.03; C-MH A3-/- n=32, median 6.3 mN; C-MH A3-/-S-/- n=21, median 6.3 mN, Figure 10C). In the A3-/-S-/- C-M there was no difference in vFT compared to controls (Mann-Whitney test, p=0.07; C-M control n=20, C-M A3-/-S-/- n=16), or to the A3-/- mutants (Mann-Whitney test, p=0.09; C-M A3-/- n=11, C-M A3-/-S-/- n=16).

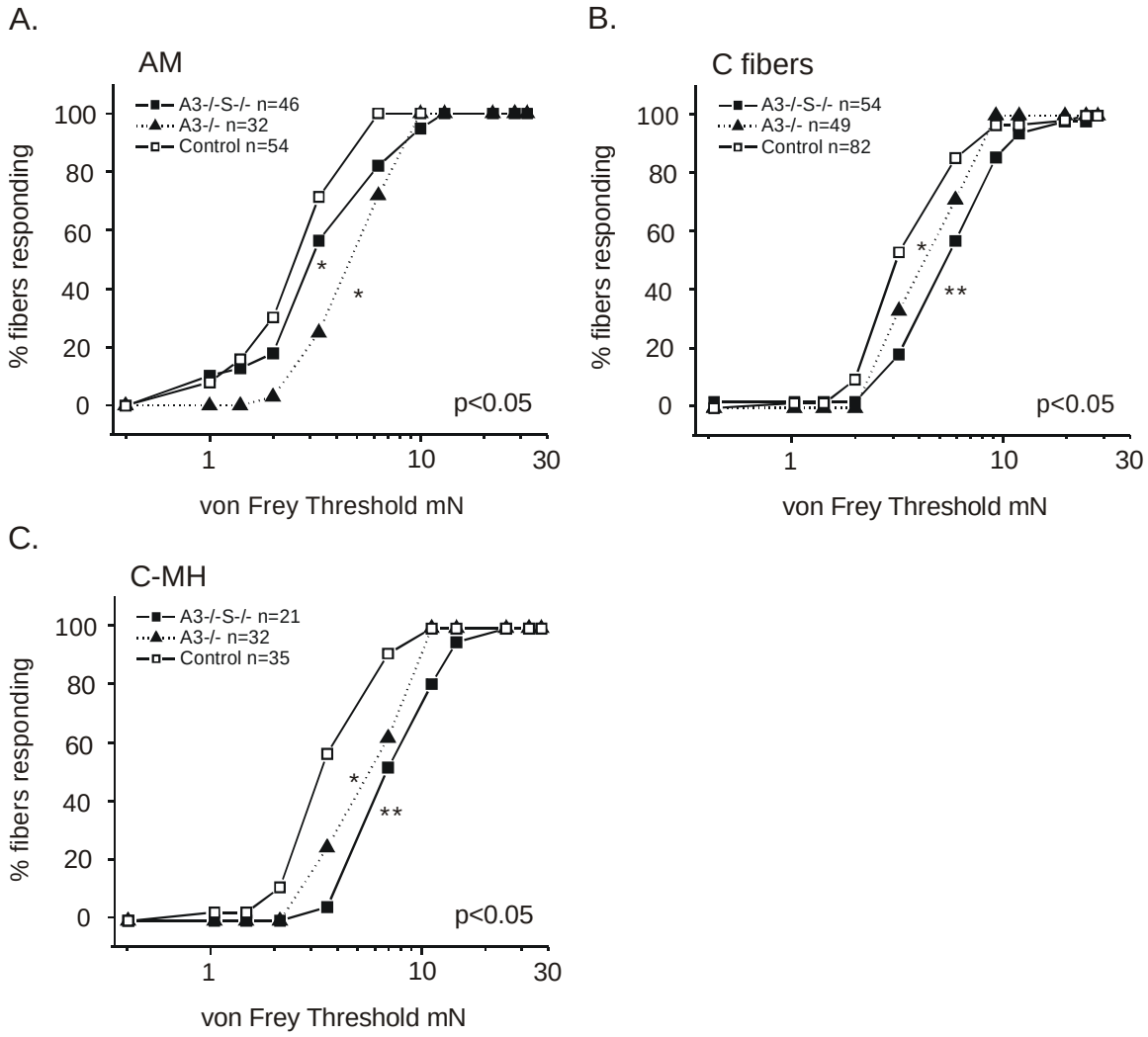

Figure 10: von Frey thresholds.

Cumulative distribution of von Frey thresholds (vFT) in A3-/- mutants (▲), A3-/-S-/- double mutants (■) and controls (□); A: AM fibers in A3-/- and A3-/-S-/- mice shows a significant rightward shift in vFT (* indicates a statistical significance ($p<0.05$) compared to controls); B: C-fibers A3-/-S-/- mice show significant rightward shift in vFT compared to controls and A3-/-. [(*) indicates a statistical significance between A3-/- and controls; (**) indicates statistical significance ($p<0.05$) between A3-/- and A3-/-S-/- C fibers, and a statistical significance ($p<0.005$) compared to control mice as well]. Similarly in (C) vFT of C-MH fibers in A3-/-S-/- were significantly higher compared to control and A3-/- mice.

3.3 Quantitative analysis of mechanoreceptor mechanosensitivity

3.3.1 Generation of stimulus-response function

For each single mechanoreceptor recorded, a series of mechanical displacements were applied to its receptive field. The responses were recorded and then analyzed offline by obtaining the average firing rate (spikes/sec) during a ten second mechanical stimulation for each displacement and deriving a stimulus response plot (Figure 11). Plots from all single recordings of a particular class of fibers were averaged together and the data for each displacement stimulus were calculated to produce a stimulus-response curve.

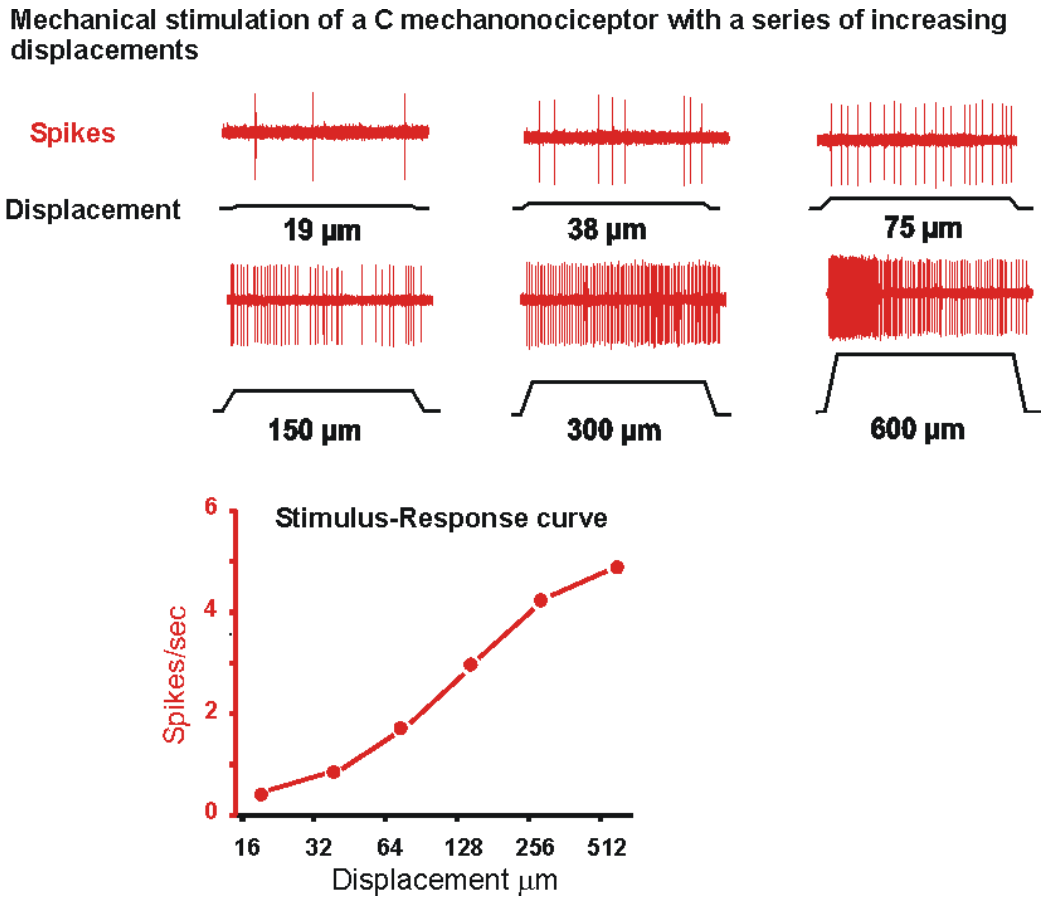

Figure 11: Generation of a stimulus-response curve.

Example of the response of a single nociceptor to a series of increasing mechanical displacements delivered using a computer-controlled motor. The average firing rate (spikes/sec) during the 10 sec stimulation is plotted against mechanical displacement to generate the stimulus-response curve.

3.3.2 Mechanical latency

In addition to the stimulus-response curve, which describes the average firing rate, information on the timing of the first spike and how it varies with displacement strengths and velocity of movement were also recorded and analyzed. The latency between probe movement and the first action potential will be called the "mechanical latency". The mechanical latency can be measured by taking the time from the start of stimulation to the first action potential minus the conduction time, which is the time it takes an action potential to travel from the initiation site along the axon to the recording electrode. The mechanical latency is therefore a rough estimation of the time it takes a mechanotransducer to convert a mechanical signal into an action potential, which is a parameter that varies between different mechanoreceptors (Figure 12). Low threshold mechanoreceptors tend to have mechanical latencies in the order of 10 ms, while high threshold mechanoreceptors and C fibers have longer latencies 5 to 10 times longer than those of low threshold mechanoreceptors.

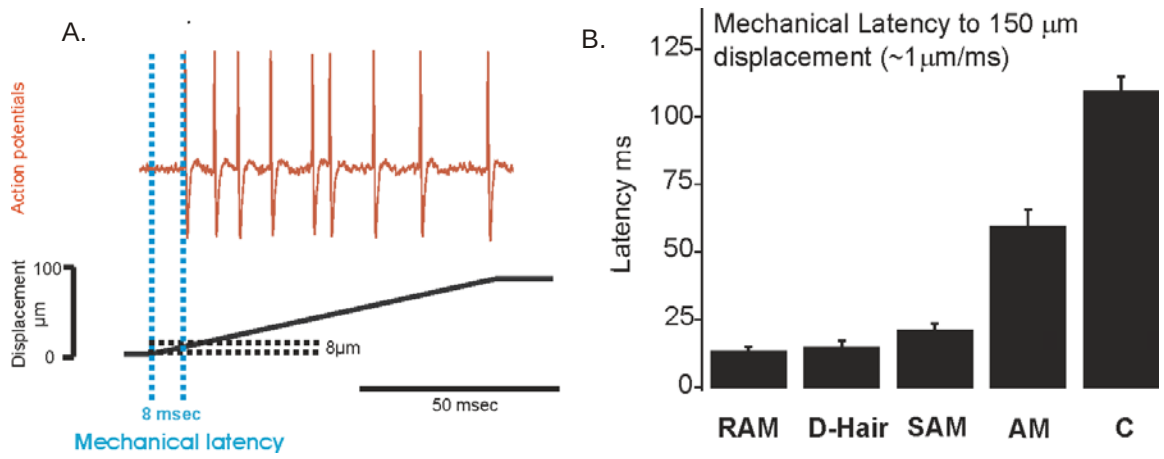

Figure 12: Mechanical latencies of cutaneous mechanoreceptors.

In (A) the mechanical latency is the time from start of probe movement to the first recorded spike. Graph (B) shows the average mechanical latencies (in milliseconds) for each mechanoreceptor type to a mechanical stimulus of 150 µm.

3.3.3 Mechanoreceptor sensitivity to moving stimuli

Low threshold D-hairs and RAM respond exclusively during the ramp phase of a mechanical stimulus. The mechanical stimulator could be adjusted so as to deliver mechanical stimuli of varying velocities ranging from 1.5 µm/s to ~3000 µm/s. Eleven D-hairs and 10 RAM were

studied from 4 C57bl6 mice. A differential sensitivity to moving stimuli was found between D-hairs and RAM in which D-hairs were sensitive to movements as slow as 1.5 μm/s (Figure 13).

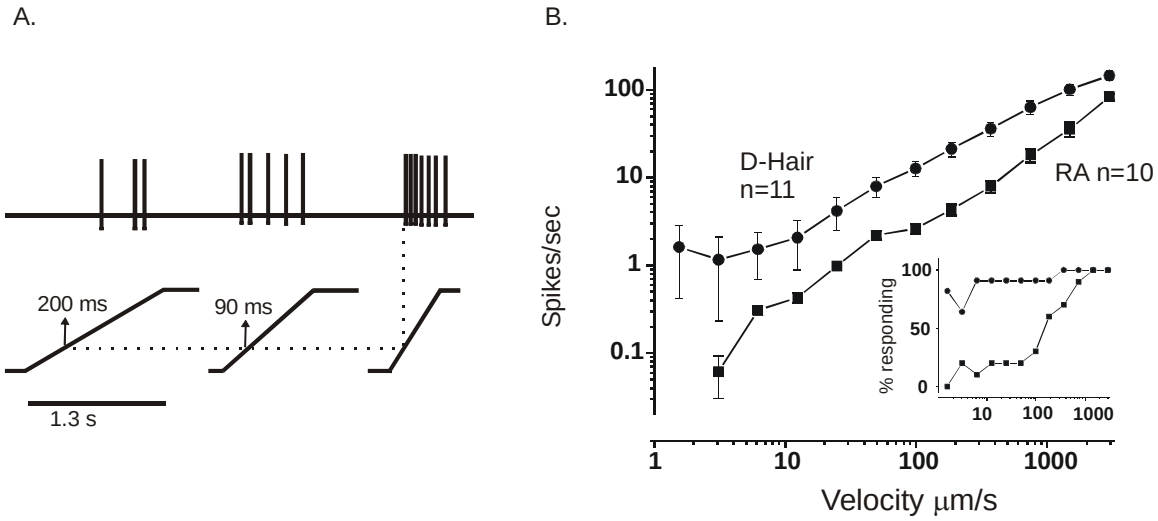

Figure 13: Velocity sensitivity of RA and D-hair mechanoreceptors.

(A) Describes the response of a fiber to 3 mechanical stimuli of different velocities. In (B) the mean firing rate and proportion of neurons responding (inset) to a series of velocity stimuli ranging from 1.5-3000 μm/s and 100 μm indentation is shown for D-hair (black filled circles) and RA mechanoreceptors (black filled squares).

3.4 Quantitative analysis of mechanosensitivity in ASIC3 mutants

3.4.1 Mechanosensitivity of Aβ-fibers

Although Price and colleagues using the skin nerve preparation already studied the mechanosensitivity of RAM in *ASIC3* mutant mice (Price et al, 2001), the mechanical stimuli used were applied manually. In this study a mechanical stimulator was used to deliver precise displacements between 10 μm and 600 μm with constant velocity, or constant amplitude and different velocities stimuli between 1.5 μm/sec and 2990 μm/sec. Since RAMs respond during the movement phase, a stimulation protocol was employed that delivers a series of constant 150 μm displacements of varying velocities (116 μm/s to 1000 μm/s). Twenty-one control RAM and 16 A3-/- mutant RAM were studied using the velocity stimulation protocol. A3-/- mutant RAM were around 2 times more sensitive than wild type RAM (repeated measures ANOVA $p<0.05$, Figure 14A). The mechanical latency was also altered; RAMs from A3-/- mice tended to initiate

action potentials with longer latency at slow velocities, but this approximated to controls at higher velocities (repeated measures ANOVA $F_{(7,245)}$=5.8040, p=0.000, Figure 14B).

There was no change in the firing frequency or mechanical latency of SAM in A3-/- mutants (repeated measures ANOVA p>0.05). Since SAM respond both during the dynamic and static phases of the stimulus, responses were separately examined. In this case, there were no changes in firing frequency during dynamic and static phases (repeated measures ANOVA, p>0.05; data not shown).

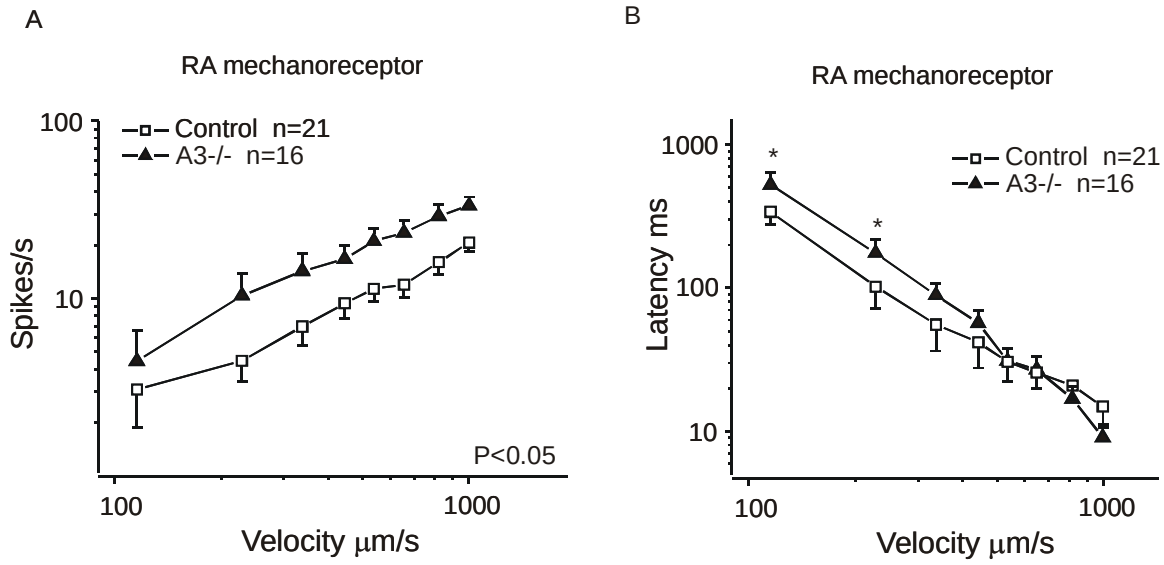

Figure 14: Velocity stimulus-response function and mechanical latency of RAM in A3-/- mutants.

In (A) the rate of firing is plotted against increasing velocity amplitude for RAM in wild type and A3 -/- mice; in (B) the mechanical latency is plotted against increasing velocity stimuli. A significant increase in mechanical latency (*) is noted at lower velocities, p<0.05 ANOVA.

3.4.2 Mechanosensitivity of Aδ-fibers

Thirty-two A-mechanonociceptors (AM) and 12 D-hairs from A3-/- mutant were studied. From the 32 AMs only 5 and 17 responded to the 10 μm and 20 μm displacements, respectively. AMs fire the most spikes during a 10-second ramp and hold mechanical stimulus and peak firing rates were found at around 300 μm displacement. The response was slightly diminished at 600μm displacement. It should be noted that the mechanical stimulation starts from the point at which the mechanoreceptor responds to the smallest mechanical displacement applied, and not from the mechanical threshold, which is a force parameter. Nevertheless, A3-/- mutant AM fibers

exhibited a significant rightward shift in the stimulus response functions seen best between displacements of 38.4µm and 307.2µm (repeated measures ANOVA, $F_{(7,213)}$=3.4199, p=0.01820, Figure 15A). Furthermore, the mechanical latency was increased in A3-/- AM (repeated measures ANOVA, $F_{(4, 284)}$=3.7185, p=0.00574, Figure 15B).

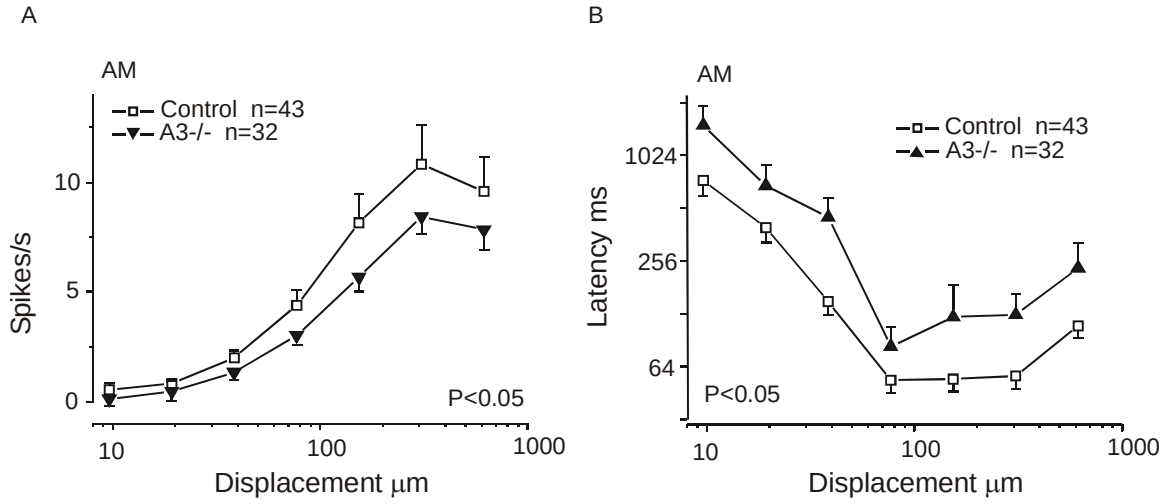

Figure 15: Stimulus-response function and mechanical latency of AM in A3-/- mutant mice.

A: mean responses of all AM in A3-/- and control to mechanical stimulation of their receptive fields (mechanical displacement 9-615µm). A significant decrease in mechanosensitivity is noted by a right shift of stimulus-response curve. B: the mechanical latency of AM in A3-/- is significantly increased. Bars indicate SEM.

All D-hairs responded to 0.4mN von Frey filaments and had relatively large receptive fields. Analysis of their stimulus-response functions to movement stimuli and mechanical latency curves revealed no significant statistical alteration compared to wild-types (data not shown).

3.4.3 Mechanosensitivity of C-fibers

From 49 identified C-fibers studied 32 responded to heat. Compared to control C-fibers, A3 -/- exhibited a higher threshold for activation as determined with von Frey hairs. This was paralleled by slight but significant rightward shift of the stimulus-response function (repeated measures ANOVA, $F_{(4, 412)}$=2.5823, p=0.03678, Figure 16A).

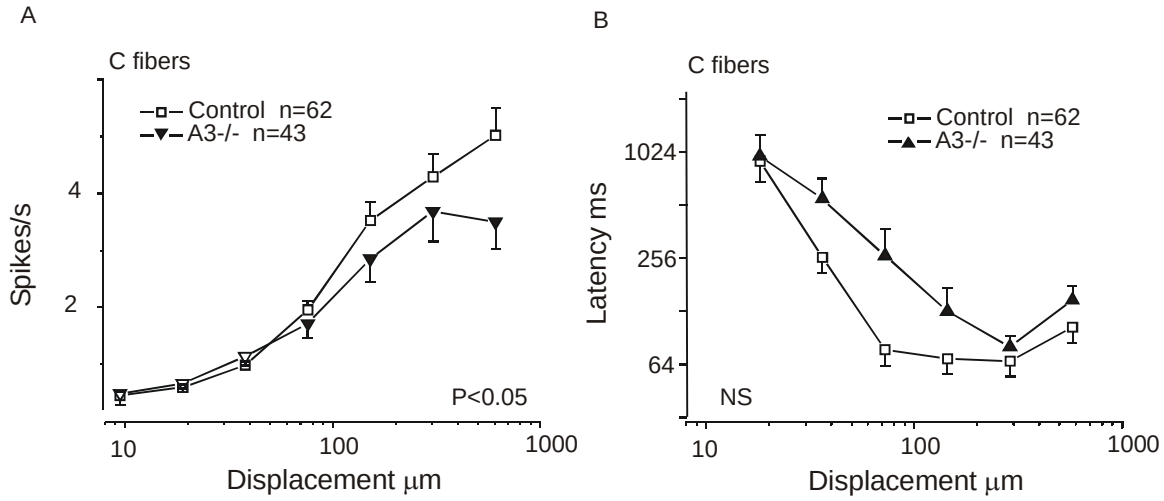

Figure 16: Stimulus-response function and mechanical latency of C-fibers in A3-/- mutant mice.

A: mean responses of all C-fibers in A3-/- and control to mechanical stimulation of their receptive fields (mechanical displacement 9-615μm, and inter-stimulus interval of 30 sec). B: the mechanical latency of C-fibers in A3-/- and controls. Bars indicate SEM.

Unlike other mechanoreceptor types which respond consistently to the lowest possible applied displacement, about half of the single units did not respond to displacements below 40 μm (Wild-type: 27 out of 62 units at 10 μm displacements, and 38 of 62 units at 20 μm; A3-/-: 6 out of 43 units at 10 μm, and 28 out of 43 units at 20 μm). The probe was lowered stepwise onto the single spot-like receptive field of the C-fiber unit using decreasing displacements to determine the smallest stimulus that generates at least one spike. A second identical stimulus applied to the unit did not result in a response in about half of the cases. Moreover, some of the C units fired spontaneously between the stimulation well after the stimulating probe was removed. This firing frequency averaged around 0.21±0.08 spikes/sec for wild types, and 0.22±0.05 spikes/sec for A3-/- mutants, this approximated to around 6 spikes in the 30-second inter-stimulus period.

Analyzing the C-mechanoheat (C-MH) and C-mechanonociceptor (C-M) mechanosensitivity separately and comparing it to control data showed nonsignificant decreases in both populations (repeated measures ANOVA, $p>0.05$). Mechanical latencies were not significantly altered in either C-M or C-MH in A3-/- mutants (repeated measures ANOVA, $p>0.05$, Figure 17 A and B).

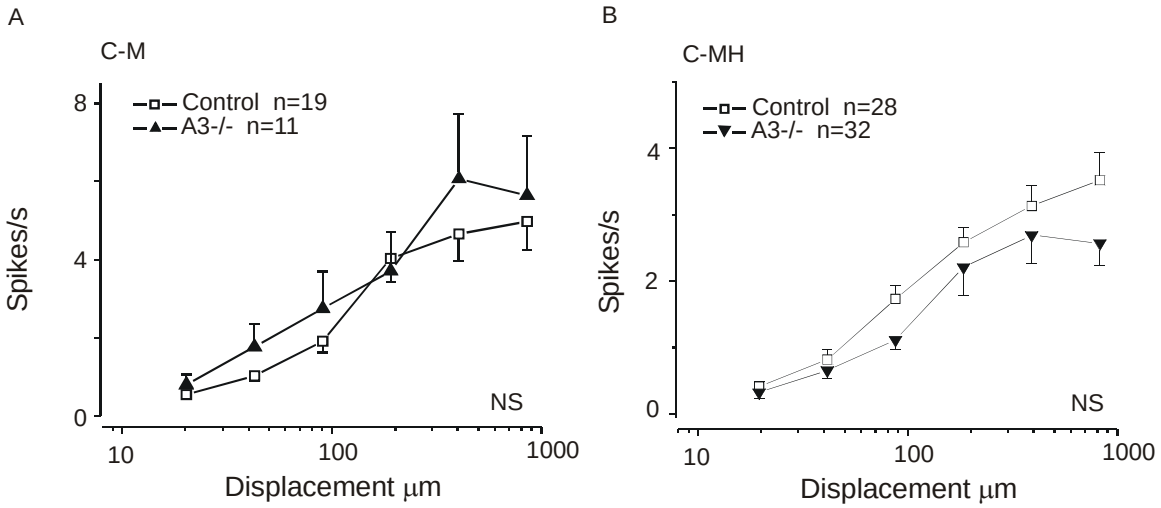

Figure 17: Mechanosensitivities of C-M and C-MH in A3-/- mutants.

Average intensity responses of C-M (A) and C-MH (B) nociceptors to mechanical displacements (16-615μm).

3.5 Quantitative analysis of mechanosensitivity in ASIC3-/-Stomatin-/- double mutants

The *ASIC3/Stomatin* mutant mice that were generated are viable and reproduce normally. We have seen that ASIC3 ion channel subunits and stomatin proteins physically interact in a heterologous expression system in vitro. Furthermore, the loss of *ASIC3* produced an altered mechanosensory phenotype in mice. Two former colleagues extensively studied the s*tomatin* null mutant mice and have observed normal mechanosensitivity except for D-hair mechanoreceptors, which were less mechanosensitive (Mannsfeldt and Martinez-Salgado, unpublished data).

3.5.1 Mechanosensitivity of Aβ- fibers

RAM velocity-response functions produced at a constant displacement of 150 μm and velocities varying between 116 mm/s and 1024 mm/s show an increase in mechanosensitivity compared to controls (repeated measures ANOVA; $F_{(7, 238)}=4.3420$, p=0.00015, Figure 18A). However this behavior was already seen in the A3-/- mice, a comparison between the single and double mutant mice revealed that the increase in mechanosensitivity to movement stimuli was of similar magnitude (Figure 18C). There were subtle differences in the mechanical latency. At lower velocities the latencies of single and double mutants were similar. However, as the velocity increased the mechanical latency of single mutants approximated control mice mechanical latency while the double mutant latency continued to be significantly larger than both controls

and single mutants (repeated measures ANOVA $F_{(7, 140)}=2.3209$, p=0.02861 compared to controls, Figure 18B; p=0.018 at 150µm and 1.0 µm/s stimulus compared to single mutants, Figure 18D). This was a consistent observation in double mutant RAMs describing a delay in spike initiation to both movement and displacement stimuli associated with an increase in firing rate.

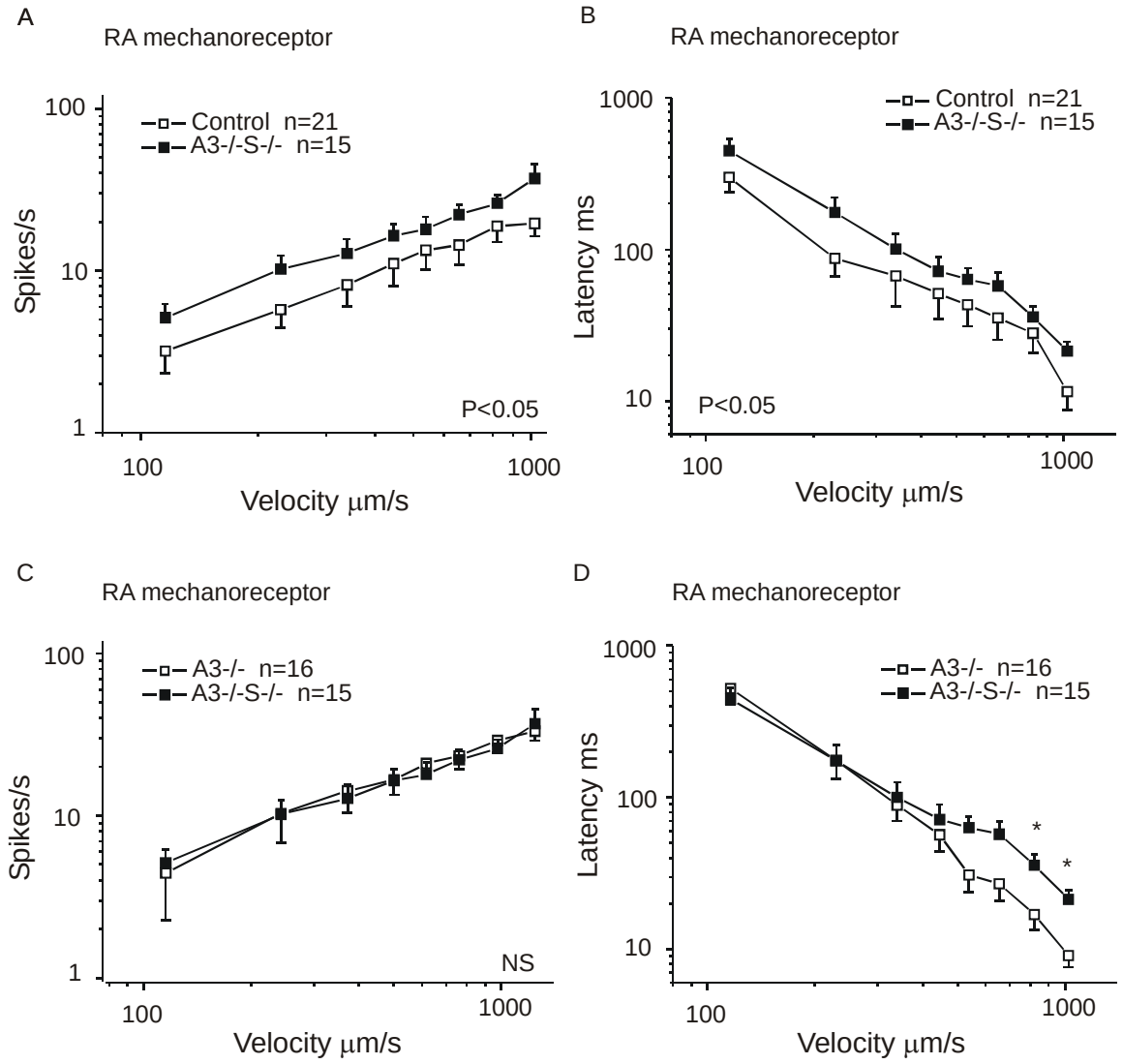

Figure 18: Mechanosensitivity and mechanical latency of RAM to movement stimuli in A3-/-S-/- mutants.

Mean responses to mechanical stimulation of constant intensity (150µm) and varying velocity (110 -1000µm/sec) of RAM (A and C) with their respective mechanical latency plots (B and D). Bars indicate SEM. * indicates p<0.05.

SAMs had normal mechanosensitivities and normal mechanical latencies when compared to both single mutants and controls. The dynamic and static responses were intact. However, on further examination, a non-significant decrease in the firing rate was evident in both A3-/- and A3-/-S-/-

mutants. The SAMs adapt slowly during the 10-second stimulus, and in most cases the firing decreased to less than 50% after several seconds and in many cases ends before the stimulation ends. The decrease in firing with time, or ”adaptation rate”, was plotted and fitted with an exponential curve of first order $y = e^{-x/\tau}$, where “τ” describes the time the firing rate decreases to 63% of its original value. At 150 µm displacement the τ values for SAM in control, A3-/-, and A3-/-S-/- mutants were 1.6, 1.1, and 0.9 seconds respectively (Figure 19).

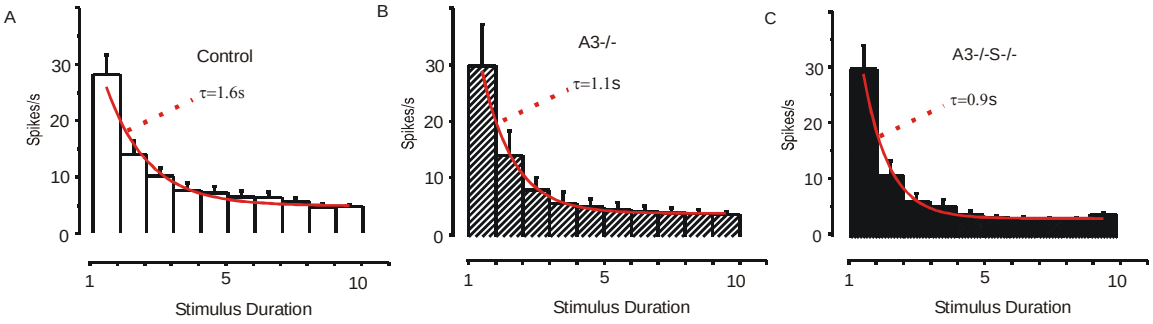

Figure 19: SAM adaptation rate

Average firing rate in 1 second-bins to a 150 µm displacement of 10-seconds duration for SAM of control mice (A), A3-/- mice (B), and A3-/-S-/- mice (C). The firing rate is fitted to an exponential decay function of the first order, predicted τ values are shown for each fitted plot (red).

3.5.2 Mechanosensitivity of Aδ-fibers

Forty-six AMs were identified in double mutant mice and stimulus response functions were obtained from 39 units. As in A3-/- mutants, the stimulus-response functions of AM fibers indicated a significant decrease in mechanosensitivity (repeated measures ANOVA $F_{(4, 312)}=2.8500$, p=0.02407, figure 20A), but no significant difference in mechanosensitivity when compared to A3-/- mutant AM (ANOVA, p>0.05, Figure 20B). The mechanical latencies of A3-/- single and A3-/-S-/- double mutant AM receptors were slightly increased relative to control latencies but this did not reach statistical significance.

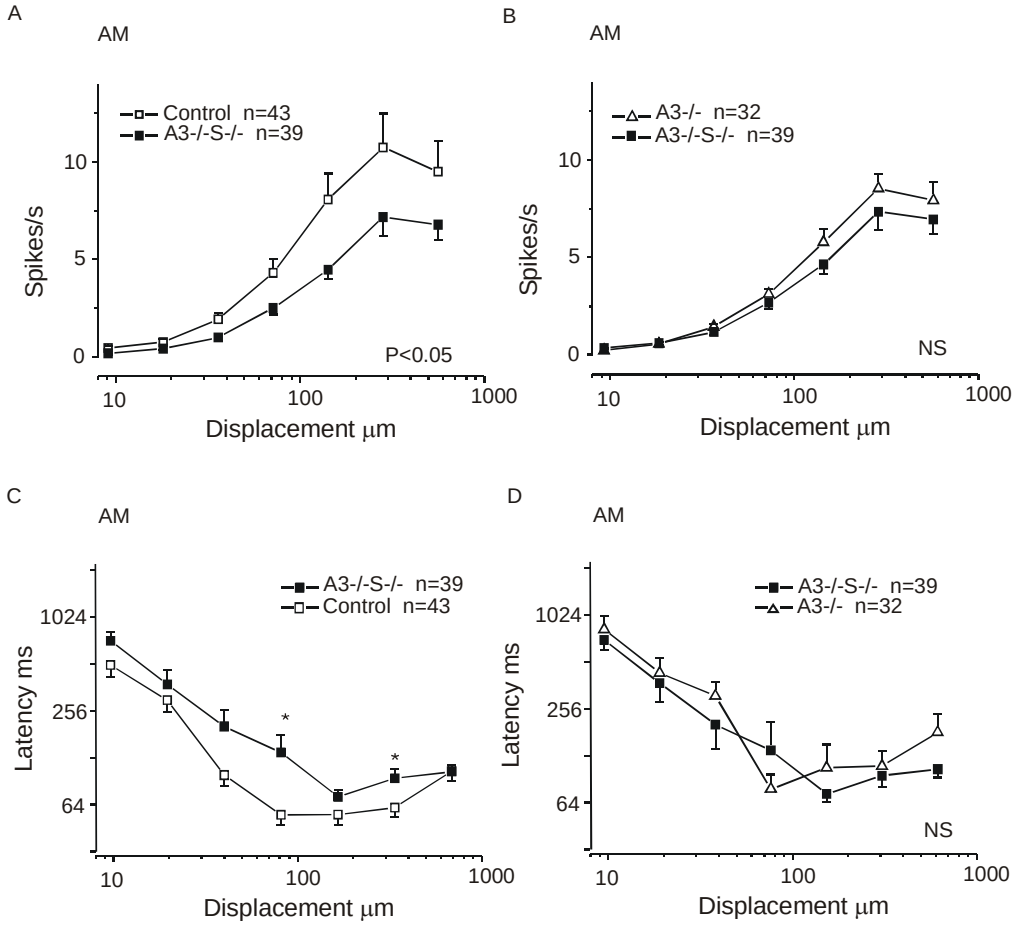

Figure 20: Mechanosensitivity of AM mechanonociceptors of A3-/-S-/- mutants.

Average responses of AM in A3-/-S-/- mutants compared to AM in controls (A) and A3-/- mutants (B). the respective mechanical latency plots in (C) and (D). Bars indicate SEM. NS: non-significant.

D-hair mechanosensitivity to movement stimuli was decreased but did not reach statistical significance (Figure 21).

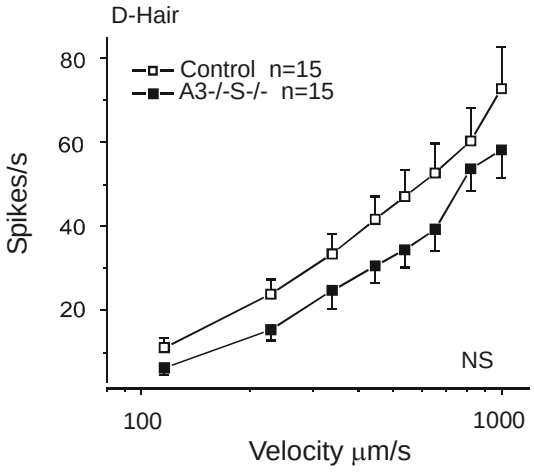

Figure 21: D-hair velocity stimulus-response function in A3-/-S-/- mutants.

Average response of D-hairs to stimuli of increasing velocities (110-1000 μm/sec) of 2-second duration and constant intensity (150μm). Bars indicate SEM.

3.5.3 Mechanosensitivity of C-fibers

A large sample of C-fibers (n=49) from A3-/-S-/- double mutants was studied. From 49 single C-fiber units, 37 were tested for noxious heat sensitivity (21 single units responded) and were classified as C-MH. Nineteen and 28 fibers responded to mechanical displacements of 10 and 20 μm, respectively. Analyzing the whole C-fiber population, a significant decrease in mechanosensitivity of A3-/-S-/- C-fibers (repeated measures ANOVA $F_{(4, 428)}$=3.4150, p=0.00916, Figure 22) was observed, however this decrease was not statistically different from the decrease observed in A3-/- mutants. The mechanical latency was increased with respect to controls, however between displacements of 80 to 600 μm mechanical latencies were not significantly different.

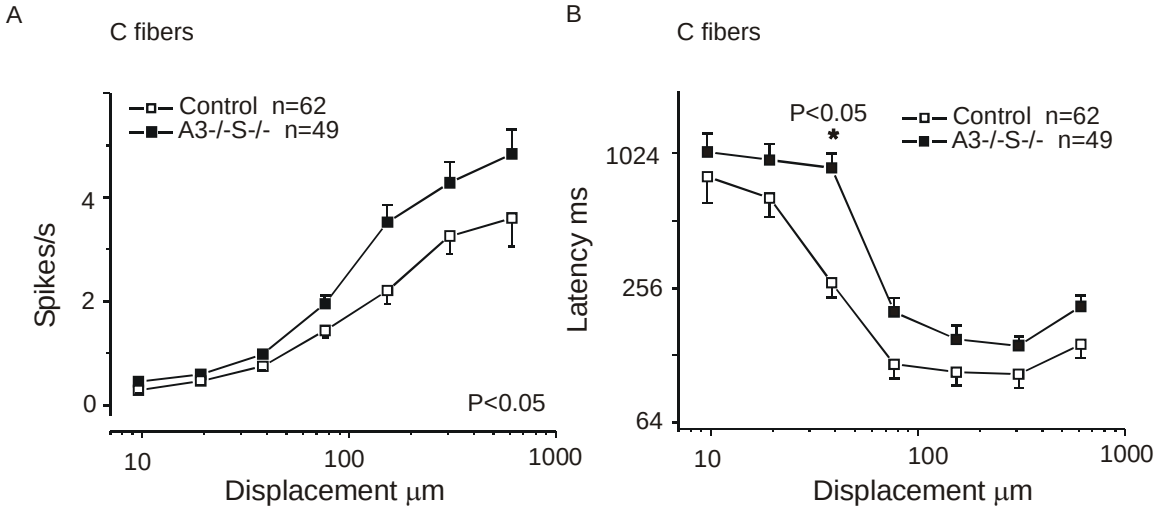

Figure 22: Stimulus-response and mechanical latency plots of C-fibers in A3-/-S-/- mutants.

Average response of all C-fibers for A3-/-S-/- compared to control (A) to mechanical stimuli (9-615μm) of 10-second duration to the receptive fields of each unit using the stepping motor. In (B) the average mechanical latency to mechanical stimulation is plotted, a significant difference ($p<0.05$) is indicated by an (*) at 38μm displacement stimulus. Bars indicate SEM.

Because a significantly higher mechanical threshold was observed in C-fibers in A3-/-S-/- mutants compared to A3-/- mutants, the stimulus-response functions of the C-MH and C-M were analyzed separately. The A3-/-S-/- CMHs were significantly less mechanosensitive than control C-MH (repeated measures ANOVA $F_{(4, 184)}$=3.9222, p=0.00443); however, this decrease

was not significantly different compared to A3-/- mutant C-MH (repeated measures ANOVA $F_{(4, 204)}$=1.6415, p=0.16517, Figure 22). However, at mechanical displacements of 600 μm C-MH fibers from A3-/-S-/- mice showed a significant decrease in firing rates compared to C-MH fibers from A3-/- (A3-/-S-/- C-MH 2.04±0.37 spikes/sec, n=23; A3-/- C-MH 3.19±0.49 spikes/sec, n=33; p<0.05). C-M mechanosensitivities for double mutants were decreased without reaching significance to either control or A3-/- mice.

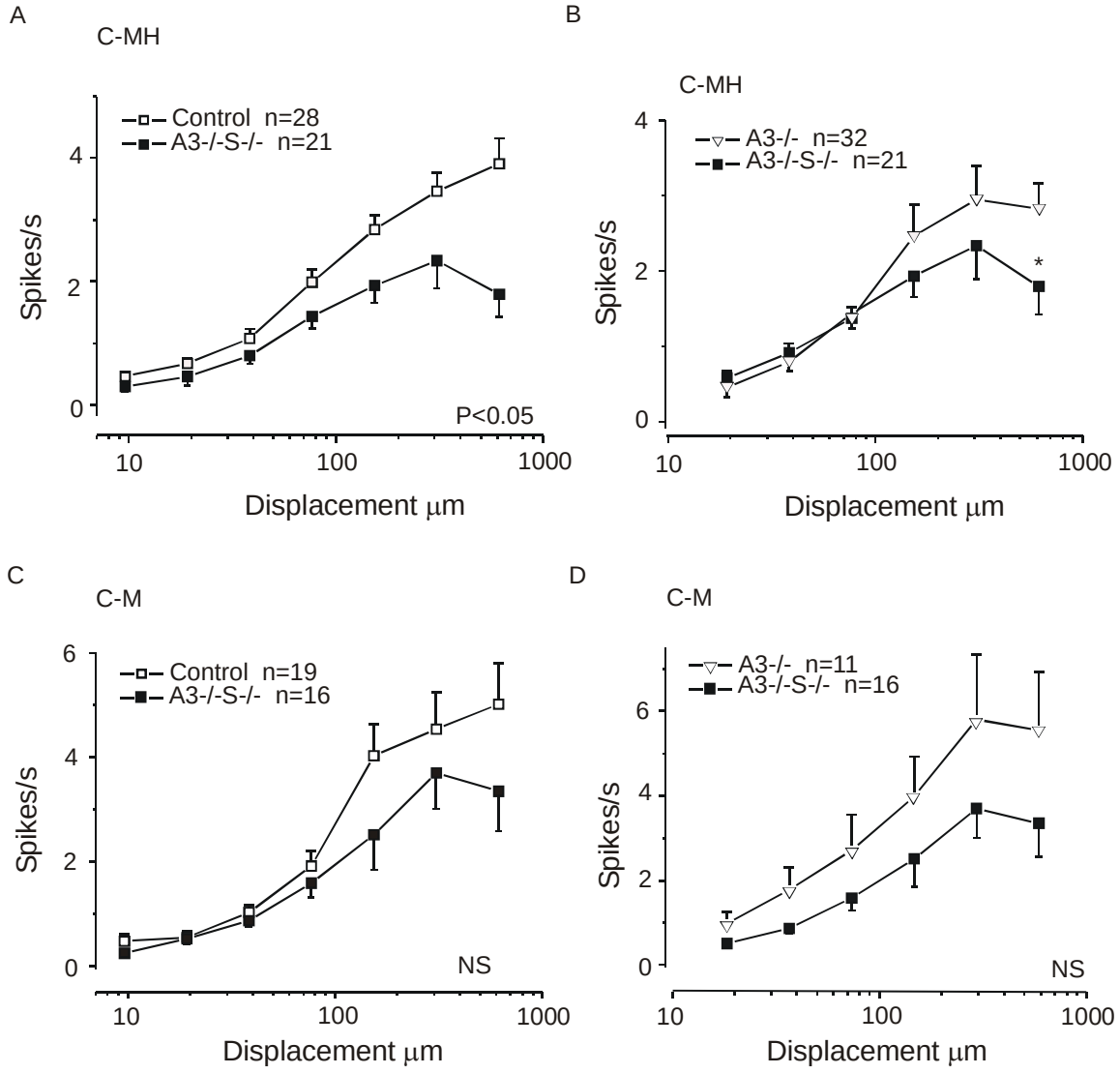

Figure 23: Stimulus-response function of C-MH and C-M

Intensity coding plots of C-MH for A3-/-S-/- compared to controls (A) and compared to A3-/- (B). In (B) significant difference (p< 0.05, ANOVA) between A3-/- and A3-/-S-/- C-MH (*) is evident in firing rates at the highest stimulus intensity, but not at lower intensities. Average responses of C-M for A3-/-S-/- and controls in (C) and A3-/-S-/- and A3-/- in (D). Bars indicate SEM. NS: non-significant.