The regularity of sustained firing reveals two populations of slowly adapting touch receptors in mouse hairy skin

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Running title: Slowly adapting touch receptors in mice

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Abstract

Touch is initiated by diverse somatosensory afferents that innervate the skin. The ability to manipulate and classify receptor subtypes is prerequisite for elucidating sensory mechanisms. Merkel cell-neurite complexes, which distinguish shapes and textures, are experimentally tractable mammalian touch receptors that mediate slowly adapting type I (SAI) responses. The assessment of SAI function in mutant mice has been hindered because previous studies did not distinguish SAI responses from slowly adapting type II (SAII) responses, which are thought to arise from different end organs, such as Ruffini endings. Thus, we sought methods to discriminate these afferent types. We developed an epidermis-up ex vivo skin-nerve chamber to record action potentials from afferents while imaging Merkel cells in intact receptive fields. Using model-based cluster analysis, we found that two types of slowly adapting receptors were readily distinguished based on the regularity of touch-evoked firing patterns. We identified these clusters as SAI (coefficient of variation=0.78±0.9) and SAII responses (0.21±0.9). The identity of SAI afferents was confirmed by recording from transgenic mice with GFP-expressing Merkel cells. SAI receptive fields always contained fluorescent Merkel cells (N=10), whereas SAII receptive fields lacked these cells (N=5). Consistent with reports from other vertebrates, mouse SAI and SAII responses arise from afferents exhibiting similar conduction velocities, receptive field sizes, mechanical thresholds and firing rates. These results demonstrate that mice, like other vertebrates, have two classes of slowly adapting light-touch receptors, identify a simple method to distinguish these populations, and extend the utility of skin-nerve recordings for genetic dissection of touch receptor mechanisms.

Keywords: Touch, Merkel cell, mechanotransduction, somatosensory, SAI
Introduction

In mammals, the sense of touch is initiated by more than a dozen morphologically and physiologically distinct sensory afferents in the skin. These somatosensory afferents encode a wide range of stimuli, including hair movement, light touch, vibration, texture and pain (Halata 1993; Lumpkin and Caterina 2007; Perl 1992). Whether these disparate receptor subtypes share common mechanotransduction molecules remains unknown. Moreover, the developmental pathways underlying the physiological diversity of mammalian touch receptors are only now being uncovered (Bourane et al. 2009; Luo et al. 2009; Seal et al. 2009). The answer to these questions relies on the ability to selectively label, accurately classify and isolate different receptors for molecular and physiological studies.

With only a few exceptions, physiologically identified responses have been linked to morphologically distinct cutaneous receptors largely through post hoc anatomical correlations (Iggo and Muir 1969; Chambers et al. 1968, 1972). The best characterized light touch response is the slowly adapting type I (SAI), which was identified as arising from Merkel cell-neurite complexes through a painstaking combination of ex vivo recording, neuronal tracing and post hoc histological analysis (Woodbury and Koerber 2007). Atoh1, which is specifically expressed in Merkel cells in the skin (Lumpkin et al. 2003), serves as a molecular marker of these complexes. Very recent molecular and histological studies have also established markers for low-threshold C-mechanoreceptors and rapidly adapting myelinated mechanoreceptors (Bourane et al. 2009; Luo et al. 2009; Seal et al. 2009); however, discrimination of individual subclasses within these groups remains elusive.

Cutaneous mechanosensitive afferents in vertebrate models have traditionally been divided primarily by conduction velocity in physiological assays (Gasser 1941). Fast, myelinated afferents, or A-afferents, are subdivided into Aβ- and Aδ-afferents, whereas unmyelinated afferents are dubbed C-fibers. In addition to conduction velocity, cutaneous afferents are often divided into touch receptors and nociceptors based on sensory threshold. Noxious levels of force primarily activate A-
mechanonociceptor (AM) afferents and nociceptive classes of C-fibers, though some groups report that up to 20% of Aβ afferents respond to these force levels (Djouhri and Lawson 2004). AM afferents are thinly myelinated, falling primarily into the Aδ conduction velocity range, and are responsible for quick, prickling pain sensations, whereas unmyelinated nociceptors convey slow, sustained responses (Lumpkin and Caterina 2007; Stucky et al. 2001). Low-threshold C-fibers, which represent a rare population of unmyelinated afferents, appear to be important for the onset of mechanical hypersensitivity during inflammation or injury (Loken et al. 2009). Low-threshold Aδ afferents include down hair receptors (D-hairs or DH), whose response properties and extreme sensitivity place them firmly in the category of light touch receptors.

Most cutaneous Aβ afferents are low-threshold mechanoreceptors that can be divided into slowly adapting and rapidly adapting (RA) categories based on whether they maintain action potential discharge throughout a sustained mechanical stimulus. Based on their low mechanical thresholds and cutaneous location, it is likely these somatosensory afferents subserve the sensation of touch. RA afferents fire only in response to a changing stimulus, providing the brain with a neural image of moving or vibrating stimuli (Johnson 2001). Sensory structures associated with RA responses include hair-follicle afferents, Meissner’s corpuscles and Pacinian corpuscles, which are innervated by early Ret+ neurons (Bourane et al. 2009; Horch et al. 1977; Johnson 2001; Luo et al. 2009; Zimmermann et al. 2009).

Slowly adapting afferents maintain firing during sustained indentation and have been divided into two types in all vertebrate models except mice. SAI responses convey high-resolution spatial information to the brain and are thought to be responsible for our ability to discriminate texture, curvature, patterns such as Braille, and some component of proprioception (Edin 2001; Goodwin et al. 1997; Johansson and Flanagan 2009; Johnson and Lamb 1981; LaMotte and Srinivasan 1993; Phillips and Johnson 1981). The Merkel cell-neurite complexes that generate SAI responses are located in highly touch-sensitive skin structures including finger tips, whisker follicles and touch domes of hairy skin (Iggo and Muir 1969; Merkel 1875; Woodbury and Koerber 2007). SAIII
responses have been postulated to arise from Ruffini endings (Chambers et al. 1972); however, direct evidence supporting this correlation is still lacking.

Mice share similar classes of cutaneous mechanosensitive afferents with other mammals and have myriad genetic tools available to manipulate identified cell populations. Past characterization of touch receptors in mice has, however, differed somewhat from that of other mammals, as prior studies have reported an absence of SAII responses or have not distinguished SAI from SAII afferents (Boada and Woodbury 2007; Cain et al. 2001; Kinkel et al. 1999; Koerber and Woodbury 2002; McIlwrath et al. 2007; Wetzel et al. 2007; Woodbury et al. 2001; Woodbury and Koerber 2007). This limitation complicates the detailed study of either afferent type.

Most investigations of cutaneous mechanosensitive afferents in mice have been performed in ex vivo skin-nerve preparations, the majority of which mounted the epidermal surface facing down to allow superfusion of the dermis with oxygen-rich saline solution. In this configuration, mechanical stimuli are applied to the dermal surface, which does not mimic stimuli encountered by the living animal. This inverted configuration has been proposed to account for the lack of resolution of SAII from SAI responses (Lewin and Moshourab 2004). If so, multiple classes of slowly adapting afferents might be discerned in mice by stimulating touch receptors from the epidermal surface.

To test this possibility, we developed a novel chamber for ex vivo skin-nerve recording that allows the skin to be perfused from below, exposing a dry epidermal surface for stimulation and direct visualization of fluorescently labeled touch receptors. We used this preparation to assess conduction velocity, mechanical threshold, receptive field size and response patterns to maintained stimuli from both the dermal and epidermal surfaces for direct comparison and for comparison to previous studies. We then employed model-based multivariate cluster analysis to query the number of distinct slowly adapting afferent populations in mouse hairy skin, validating the results using genetic tools. The ability to resolve these cutaneous mechanosensitive afferents in the mouse is essential for future studies using genetic manipulations to investigate mechanotransduction mechanisms in these two receptors.
Materials and Methods

Animals and dissection

All animal use was conducted according to the Guide for the Care and Use of Laboratory Animals (NIH), and was approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine and the Department of Defense. When indicated, recordings were made from Atoh1/nGFP transgenic mice expressing enhanced GFP (GFP) in Atoh1-expressing cells, including Merkel cells (Lumpkin et al. 2003). Mice were generated in a BDF1 or mixed genetic background. In a few cases, wild-type mice were injected with SynaptoGreen/FM1-43 (2–3 mg/kg; Biotium) from 16–72 hours prior to recording to label Merkel cells (Maricich et al. 2009; Meyers et al. 2003).

Adult mice (≥6 weeks of age) were sacrificed by inhalation of isoflurane followed by cervical dislocation. The posterior half of the animal was shaved and the remaining stubble removed by applying a depilatory agent (SurgiCream; Ardell) for 10–15 min. The saphenous nerve and innervated skin of the hindlimb was dissected largely as previously described (Koltzenburg et al. 1997; Reeh 1986; Zimmermann et al. 2009). The plantar surface of the foot was attached to a wedge with double-sided tape to ease manipulation and stabilize the leg. To increase the length of accessible nerve, we removed the viscera along with skin and fascia proximal to the knee joint. The saphenous nerve and the trunk of the femoral nerve were dissected away from the femoral artery and underlying muscle, tied with nylon string at the level of the lumbar plexus and severed. To facilitate fiber teasing, at least two centimeters of nerve was obtained beyond the point of attachment to the skin. An incision was made on the lateral side of the leg from knee to heel, and the skin of the leg and the dorsal aspect of the hindpaw were removed. Care was taken to keep the connection between nerve and skin intact while dissecting between the underlying musculature and the fascia that contains the branching nerve fibers. The tissue was periodically washed with synthetic interstitial fluid (SIF, in mM: 108 NaCl, 3.5 KCl, 0.7 MgSO₄, 26 NaHCO₃, 1.7 NaH₂PO₄, 9.5 sodium gluconate, 5.5 glucose, 7.5 sucrose and 1.5 CaCl₂, saturated with 95%O₂/5%CO₂; pH 7.4) throughout the dissection to keep it moist and oxygenated and to desanguinate the dissection field if
necessary. Additional sucrose (up to 20 mM) was used to adjust the osmolality to a level comparable with mammalian interstitial fluid (290–305 mmol/kg). SIF ion concentrations were within 3% of those reported for subcutaneous interstitial fluid (Fogh-Andersen et al. 1995; Gilanyi et al. 1988).

The total time required by a trained experimentalist from the moment of animal sacrifice to the complete removal of both skin-nerve preparations was ≤2 h. The resulting skin-nerve preparations were maintained at 4°C in SIF until recording (≤6 h). No difference has been noted in either the viability of the two preparations or the populations of afferents detected. Fewer fibers overall have been recorded from the left-leg preparation, but this is largely due to the increased likelihood of damage to the preparation during dissection by right-handed experimenters, irrespective of dissection or recording order.

Recording chamber

A custom recording chamber (Fig. 1A) was mounted on the base of a brain slice chamber containing a heating element (Harvard Apparatus BSC-BU). The perfusion chamber is rhomboid with axes of 1.5” (38 mm) and 2.625” (67 mm) and a depth of 0.375” (9.5 mm). The smaller, ovoid recording chamber adjoins the perfusion chamber at one of the obtuse vertices, and is 0.625” by 1.25” (16 by 32 mm). The bottom of the perfusion chamber was coated with silicone-elastomer (Dow Corning Sylgard), on top of which was pinned a nylon wick (L’eggs winter-weight pantyhose). The elasticity of the Sylgard and nylon wick substrates did not contribute significantly to the force adaptation of the system, as repeated stimuli at 30-s intervals to the substrate alone produced non-adapting force traces with highly repeatable amplitudes. During recording, the chamber contained ~3.5 mL of SIF, and was perfused at a rate of ~4 mL/min with a micro-annular gear pump (Mikrosysteme mzr-2921). Bath temperature was maintained at 32°C with a temperature controller and reported by a bath thermistor (Harvard Apparatus TC-202A).
Ex vivo skin-nerve preparations were mounted with the epidermis facing up in the perfusion chamber and the nerve threaded into the adjacent recording chamber (Fig. 1A, C). Mineral oil (Sigma-Aldrich cat# M3516) was layered over the SIF in the recording chamber, with the SIF-oil interface just below the surface of a raised platform (0.75 cm²). The severed end of the nerve was placed on the platform, stripped of its perineurium and teased into isolated nerve bundles of up to a few tens of axons. Axon bundles were draped over a gold recording electrode, and extracellular potentials were recorded via a differential amplifier (A-M Systems model 1800) while stimulating the skin. Receptive fields were located with a rounded glass probe, and their size was estimated by touching the skin with a fine glass probe (~0.5 mm in diameter). Mechanical threshold was defined as the lowest calibrated monofilament (von Frey hair) that elicited a response from a receptive field in ≥50% of trials. Von Frey hair forces ranged from 0.08–300 mN, though filaments above 10 mN were not necessary for classifying low-threshold mechanosensitive afferents.

To estimate conduction velocity, biphasic electrical stimuli 0–35 V in amplitude and 100 µs in duration were delivered from a pulse stimulator (A-M Systems model 2100) to receptive fields via a tungsten electrode (World Precision Instruments). The latency of the action potential peak from the stimulus artifact was measured, and the distance from the stimulating electrode to the recording electrode was measured with a calibrated eyepiece reticle. Conduction velocity was calculated as the quotient of distance to latency.

The preparation was visualized with a fluorescence stereomicroscope (Olympus SZX16) equipped with 0.5X/0.075 NA and 2.0X/0.3 NA objective lenses, epifluorescence filters (Chroma) and a 300-W Xenon lamp (Sutter). Fluorescent images of Merkel cell-neurite complexes from Atoh1/nGFP mice (Lumpkin et al. 2003) were acquired with a CCD camera (Olympus DP-71). In some cases, high-resolution images were captured post-recording by marking a grid around the touch dome with a waterproof marker for localization, and then visualizing skin whole-mounts with a
confocal microscope equipped with a 40X/1.25 NA objective lens (Leica DM IRBE). Images were processed in ImageJ (Abramoff 2004) with the Bio-Formats plugin (Linkert 2009).

In some cases, high-resolution mapping of receptive fields and measurement of von Frey thresholds was carried out on both the epidermal and dermal surface for the same afferent. Receptive fields were mapped in these cases at 20–50X magnification using a calibrated eyepiece reticle and fine forceps (Dumont #5). (The working distance of this objective, ~2 cm, was insufficient for von Frey hairs or our mechanical indenter, but more than enough for mapping with hand-held forceps.) After receptive field mapping and threshold determination, the skin was carefully flipped over without disturbing the teased nerve fibers, and the same procedure carried out on the other surface for comparison. The order of mapping (dermis or epidermis first) was alternated to avoid a systematic change in sensitivity due to the passage of time or skin relaxation with subsequent manipulations. Action potential shape and the location of the receptive field center did not change when flipping the skin. When possible, the skin was flipped more than once to allow repeated measurements. Note that the working distance of the objective used for fluorescence (2X) was insufficient to accommodate our mechanical stimulator or von Frey hairs, meaning only the receptive field mapping is possible with fluorescent imaging.

**Mechanical stimulation**

Families of mechanical displacements were delivered using a custom-built indenter (Fig. 1B), with stimulus order randomized using atmospheric noise (Random.org). A 3.4-mm diameter (9.2 mm$^3$) MACOR (Corning) filleted cylinder was mounted to a motorized Z-stage driven by a linear actuator (Ultra Motion model D-A.25AB-HT17-2-BR/4) that was wired in parallel to a stepper motor controller (Applied Motion Products 3540i) configured for $2 \times 10^4$ steps per revolution. The indenter had a maximum travel of 50 mm and moved in 0.32-μm increments. Typical stimuli were ≤2 mm and were performed with accelerations ≤1.27 μm/ms$^2$ and average velocities ≤40 μm/ms. Generated pressures under the probe tip ranged from 1–250 kPa, roughly matching the pressure
generated by von Frey filaments of $\leq 10$ mN (Fig. S1). A digital signal from the motor controller was sampled to mark the onset and termination of probe movement.

During mechanical stimulation, the applied force was constantly monitored in real time by a load cell (Honeywell model 31) and amplified via an inline amplifier (Honeywell model 060-6827-02). The indenter was controlled via handheld remote or custom software. Displacement steps were 5-s in duration and were delivered at 30-s intervals. Displacement families were performed in 0.1- to 0.2-mm increments between the minimum displacement required to elicit a response and the maximum displacement with forces in the linear range of the force transducer (~1.7 N). Up to three displacement families were delivered to each afferent's receptive field.

**Data acquisition and analysis**

Data were digitized via a PC data acquisition card (Data Translation model DT304) at the following sampling frequencies: extracellular potentials (12 kHz), applied force (250 Hz), stimulator movement (250 Hz) and bath temperature (30 Hz). Electrophysiological data were processed in Sciworks Experimenter software (Datawave). Action potentials were detected and sorted in real time to isolate single units. Captured action potential waveforms were compared to confirm spike identity between experiments. Offline analysis was also carried out in Experimenter, with the resulting data exported into Microsoft Excel, Matlab (MathWorks), R (www.r-project.org) or Igor Pro (Wavemetrics) for further analysis, plotting and statistics. Autocorrelation on extracted spike times was performed in MatLab.

For analysis of dynamic and static phases of touch-evoked responses, the point at which the indenter began its movement was treated as $t=0$. The indenter typically reached its final position 40–50 ms into each stimulus. To ensure that any firing during stimulus onset, as well as the initial rapidly adapting phase of stimulus maintenance, was captured, the dynamic phase was defined as $t=0$–200 ms, encompassing at least one time constant of rapid adaptation. Dynamic firing rates were calculated from the mean inter-spike interval (ISI) during the dynamic phase. The static phase was
defined as the response between 2 and 4.5 s. Coefficient of variation (CoV) for each stimulus was calculated as the standard deviation of static phase ISIs divided by mean static ISI.

Model-based cluster analysis was carried out in R using the MClust package (Fraley and Raftery 2002; 2006) to determine the number of classes of slowly adapting Aβ afferents responses from mouse recordings. Univariate model possibilities include equal variance (E) or variable variance (V) Gaussians. Multivariate models tested by the software included: spherical clusters of equal or variable diameters; ellipsoid models with equal or variable size and shape, with orientations either aligned with the coordinate axes, aligned in the same orientation or of variable orientation. Shorthand notations for each model indicate equal (E), variable (V) or inapplicable (I) variance for volume, shape and orientation, in that order. Models tested were: EII (equal volume spheres), VII (variable volume spheres), EEI (equal volume and shape ellipsoids aligned with the coordinate axes), VEI (variable volume, equal shape ellipsoids along the coordinates), EVI (equal volume variable shape along the coordinates), VVI (variable volume and shape along the coordinates), EEE (equal volume, shape and orientation), EEV (equal volume, shape with variable orientation, VEV (variable volume, equal shape and variable orientation) and VVV (variable volume, shape and orientation).

The independent variables used in these analyses were mean static ISI, minimum dynamic ISI, minimum static ISI and the CoV of static ISIs. All combinations of these variables, as well as each individual variable, were tested. A hierarchical clustering method was used to determine the best description of each data set for 1–9 clusters. For each combination of model, input data and number of clusters, Bayesian information criterion (BIC) was calculated as a measure of how well each model described the data with a penalty for the number of free parameters. By comparing minimum BIC across all combinations, we determined the best model and number of clusters to describe the data. BIC was a useful metric for model comparison because it is an absolute value comparable across fits and models, and it severely penalizes model complexity, which is important to protect against overfitting a small number of observations.
Results

To distinguish subtypes of cutaneous mechanosensitive afferents in mice, we used a novel epidermis-up *ex vivo* skin-nerve recording chamber (Fig. 1A). This system uses a nylon wick to perfuse the skin from below, allowing mechanical stimuli to be applied directly to the dry surface of the epidermis, as occurs *in vivo*. We also designed a custom mechanical stimulator (Fig. 1B) to deliver rapid, well-defined displacement stimuli with on- and offset times of <200 ms. These stimuli provided compressive stresses, or pressures, of \( \leq 200 \text{ kPa} \), which were monitored in real-time via a force transducer mounted in series with the displacement probe. Rapid-onset stimuli inevitably produce some ringing, especially with a force transducer in the system. This ringing decays to within the noise well before the static phase of the response. Perfusion rates were adequate to collect data from healthy preparations for 4–6 hours, but low enough that the skin rested securely on the perfusion wick (Fig. 1C). Under these conditions, slowly adapting low-threshold afferents were observed up to four h after transferring skin-nerve tissue from 4°C to the recording chamber.

To determine whether the sensitivity of low-threshold touch receptors differed in this epidermis-up recording configuration compared with epidermis-down recordings, we directly compared receptive field size and von Frey thresholds in both configurations for the same afferents (Fig. 2). Consistent with our hypothesis, we observed significantly smaller receptive field areas and lower von Frey thresholds \( (p=0.01, N=7, \text{ Wilcoxon signed-rank}) \) when displacement stimuli were applied to the epidermal surface. Median receptive field areas were 0.17 mm\(^2\) when measured from the epidermal surface and 0.26 mm\(^2\) from the dermal side (Fig. 2A; \( p=0.036, N=7, \text{ paired Student’s } t \) test). Mechanical thresholds (Fig. 2C–D) measured from the epidermal surface were similar to those published by Woodbury *et al.* in the only other reports of an epidermis-up *ex vivo* preparation (Woodbury et al. 2001; Woodbury and Koerber 2007). These values are lower than those reported from epidermis-down reports (Table 1). Importantly, fluorescently labeled touch receptors could not be imaged from the dermal surface, making the epidermis-up configuration necessary for confirming afferent identity with genetically encoded fluorescent labels.
The epidermis-up *ex vivo* preparation enables precise classification of all previously identified classes of touch-sensitive afferents using conduction velocity, receptive field extent, von Frey threshold and the response to a sustained stimulus (Fig. 3). Single-unit isolation was informed by action potential shape and receptive field. In agreement with published reports (McIlwrath et al. 2007; Wetzel et al. 2007; Woodbury and Koerber 2007), conduction velocity values were used to separate Aβ- (>9 m/s), Aδ- (1.0–11 m/s) and C-fibers (<1.0 m/s). Some units with response properties usually associated with Aβ-afferents (SAI, SAII and RA) in our preparation had conduction velocities as low as 9.3 m/s and D-hairs and AM fibers conducted as fast as 10.4 m/s. Due to overlapping conduction velocity ranges for these populations, fibers were assigned “Aβ” or “Aδ” designations based on their physiological response properties instead of using a hard cutoff at 10 m/s. Mean conduction velocities (± SD) for Aβ-, Aδ- and C-fibers were 12.5±2.0 m/s (N=27), 6.0±2.3 m/s (N=46) and 0.41±0.08 m/s (N=24), respectively. Receptive field extents matched previous reports (Table 1), and were an especially useful parameter in separating D-hair afferents, which had oblong receptive fields 3–4 mm in length and ~1 mm wide, with the long axis of the receptive field aligned with that of the leg. Other afferent types’ receptive fields were more symmetrical and, for most types, varied from punctate (<0.5 mm) to several millimeters in diameter. Mechanical thresholds were useful in distinguishing AM afferents, whose thresholds exceeded 1.5 mN (Median=6mN, N=19), consistent with reports from *in vivo* studies (Table 1). D-hair, RA and SA afferents had highly overlapping ranges of mechanical thresholds, with median thresholds for all three groups of 0.08 mN (N=45, 14 and 32, respectively).

Because of the similar receptive field characteristics of most Aβ afferents and overlapping conduction velocity distributions of Aβ and Aδ afferents, controlled mechanical stimuli were often necessary to discriminate among mechanosensitive afferents with conduction velocities greater than 9 m/s. D-hair afferents displayed robust rapidly adapting responses to very light touch stimuli (<0.08 mN), but maintained a low static firing rate throughout suprathreshold sustained stimuli, a behavior not seen in Aβ-RA afferents at any intensity level and consistent with other reports of D-
hair properties in mice (Koltzenburg et al. 1997). AM afferents responded with low firing rates that adapted very slowly and lacked the robust on/off responses of D-hair receptors (Boada and Woodbury 2007; Koltzenburg et al. 1997). RA afferents fired exclusively during probe movement across force levels, making their classification relatively simple.

Slowly adapting afferents had little or no discharge at rest, fired rapidly during the onset of a mechanical stimulus, maintained firing throughout a sustained displacement and sometimes produced a burst of action potentials at stimulus offset. We noted that some of these afferents responded to mechanical stimuli with a highly irregular sustained discharge of action potentials, whereas others displayed a more regular ISI during the adapted response (Fig. 3). This difference in firing regularity is readily detectable during recording by playing responses through a speaker (Fig. S2). Because irregular firing is a hallmark of vertebrate SAI responses, these results suggest that SAI responses represent only a subset of murine Aβ afferents with slowly adapting responses.

We next sought to determine whether mouse SAI responses could be reliably distinguished from other slowly adapting responses using the epidermis-up ex vivo preparation. To do so, we analyzed responses from 17 slowly adapting afferents, nine of which were recorded from Atoh1/nGFP mice (or wild-type mice injected with FM1-43) to determine whether their receptive fields co-localized with Merkel-cell-containing touch domes. As detailed in Methods, we performed model-based cluster analysis and calculated BIC to assess how well each model described the data with a penalty for increasing free parameters.

We found that several multivariate models yielded BIC<-100, the lowest of which was a five-cluster fit to static CoV of ISIs, minimum dynamic ISI and mean static ISI. In this model, afferents that innervated touch domes failed to cluster together, indicating that the analysis was unsuitable for distinguishing SAI afferents.

By contrast, analysis of two variables (CoV and min. dynamic ISI) identified two distinct clusters, one of which encompassed all fluorescently labeled touch-dome afferents. A model of two equal volume ellipsoid clusters oriented along the coordinate axes (EEI) yielded a minimized BIC of
-142 for a two-cluster model (Fig. 4A). Other models, including equal-sized ellipsoids with freely
oriented axes with the same orientation (EEE) or variable orientation (EEV) also predicted two
clusters but had minimum BIC >-142.

We noted that the two clusters identified by this analysis separated conspicuously along the CoV
axis (Fig. 4B); therefore, we reasoned that this coordinate alone might be sufficient to separate the
clusters. Indeed, univariate analysis of CoV produced a minimum BIC for two equal-variance
clusters. Despite a higher BIC (-1), the results from this classification were identical to those from
the best multivariate model, yielding clusters with mean CoVs of 0.21 and 0.78 (SD=0.9). The
Gaussian distributions that describe these two clusters best separated at CoV=0.49. As described
below, we designated these two clusters as SAI (CoV>0.49, \( N=9 \)) and SAII responses (CoV<0.49,
\( N=8 \)) based on their firing properties and receptor morphologies.

SAI afferents responded with irregular firing rates to sustained displacements of the skin (Fig. 5),
as reported previously (Iggo and Muir 1969). Peak firing rates \( \leq 668 \) Hz were observed for SAI
responses (mean=393±177 Hz). Their punctate receptive fields encompassed only the extent of a
touch dome and generally had von Frey thresholds <0.5 mN (median=0.08 mN, \( N=21 \)). In two
cases, we observed SAI afferents that innervated two touch domes. In \( \text{Atoh1/nGFP} \) or FM1-43-
injected mice (Haeberle et al. 2004; Lumpkin et al. 2003), SAI receptive fields completely co-
localized with groups of Merkel cells (Fig. 5A, B; \( N=10 \)).

The slowly adapting A\( \beta \) afferents excluded from the SAI cluster displayed physiological
properties consistent with those SAII afferents in other species (Table 1). Most notably, they
exhibited highly regular firing patterns during displacements that elicited a mean firing frequency
\( \geq 15 \) Hz (Fig. 6C), with peak firing frequencies of \( \leq 633 \) Hz (mean=380±162 Hz). Additionally, their
receptive fields ranged from punctate to 2 mm (median=1 mm, \( N=8 \)) in diameter. Finally, they
displayed mechanical thresholds of up to 4 mN (median=0.3 mN, \( N=14 \)), suggesting that these
afferents are slightly less sensitive to compressive stimuli than are SAI afferents. In recordings from
mice with fluorescently labeled Merkel cells, SAII afferent receptive fields showed no overlap with
fluorescent Merkel cells (Fig. 6A; \( N=5 \)), indicating that mouse SAI afferents do not innervate touch domes.

Stimulus-response plots revealed that the mean firing rates for SAI and SAI afferents were comparable during both dynamic and static response phases (Fig. 7A–B). A histogram of normalized ISIs (Fig. 7C) illustrates the differences in ISI variability during static-phase responses. SAI intervals (\( N=3348 \) normalized intervals) encompass a much broader distribution than the tightly clustered peak for SAI intervals (\( N=1533 \) normalized intervals). This difference in ISI variability allows the use of CoV as a simple discriminant, and is audibly detectible during recording (Supplemental Fig. S2).

Discussion

Ex vivo skin-nerve preparations provide an expedient alternative to in vivo recordings and have been used for over two decades to investigate mechanisms of peripheral somatosensory transduction (Reeh 1986). Here we used a modified ex vivo skin-nerve preparation to discriminate classes of slowly adapting responses in mice for the first time. In this recording setup, the epidermis is dry and exposed to air, allowing direct stimulation of epidermal touch receptors on their physiologically relevant surface. The orientation of our preparation also allows direct imaging of fluorescently labeled end organs while recording. These capabilities represent a significant step toward experiments to rapidly identify, manipulate and assess the function of sensory structures that transduce touch, mimicking in vivo mechanical thresholds and firing rates in an ex vivo preparation.

Most prior approaches to ex vivo recording used a “flipped” system where the epidermis lies against a substrate and the dermis is superfused with saline. This is an excellent method to ensure that the preparation stays well-perfused, but may not be necessary given a previous finding that epidermal exposure to air is sufficient to maintain SAI responsiveness in the absence of blood flow in vivo (Findlater et al. 1987). Additionally, stimulating the dermal surface of the skin has drawbacks for precise testing of light-touch responses. It is a non-native stimulus for cutaneous
receptors and provides slightly elevated values for receptive field area and mechanical threshold (Fig. 2), presumably due to force buffering and spreading in the dermis, and the slipperiness of the wet corium surface (Lewin and Moshourab 2004). Consistent with published reports (Woodbury and Koerber 2007), we find that liquid on the receptive field can make accessing the receptive field difficult, as the finest standard von Frey filaments ($\leq 0.08$ mN) are deflected away by surface tension or obscured by diffraction, making precise filament positioning and accurate threshold determination much more difficult.

Mechanical thresholds for identified sensory afferent types are consistent across species in vivo. Recordings of myelinated, touch-sensitive afferents in human hairy skin report the presence of SAI and SAII afferents with median mechanical thresholds of 0.45 and 1.3 mN, respectively (Vallbo et al. 1995). In the cat, minimum forces to elicit a response in SAI afferents were $\sim 0.01$ mN (Iggo and Muir 1969). Similarly, at least one study of rat in vivo recordings reported mechanical threshold values of $<0.05$ mN (Lynn and Carpenter 1982), although others reported thresholds of up to 14 mN (Leem et al. 1993a). All of the aforementioned models, including humans (Vallbo et al. 1995), cats and rabbits (Brown and Iggo 1967) and even reptiles (Kenton et al. 1971), have two distinct classes of slowly adapting touch receptors, making the two in vivo reports from mice a notable exception (Table 1). Neither study separated SAI from SAII responses, a feature they share with ex vivo characterizations of mouse cutaneous afferents.

We previously published an unbiased survey of mechanosensitive afferents in wild-type mice using our epidermis-up approach (Maricich et al. 2009). We found afferents with mechanical thresholds ranging from 0.08 mN to 254 mN ($N=97$); however, the vast majority ($N=94/97$) had thresholds $\leq 10$ mN. We found 24 C-fibers (25.8%), 13 AM A$\delta$-afferents (14.0%), 33 D-hair A$\delta$-afferents (35.5%), 10 RA A$\beta$-afferents (10.8%), 6 SAI A$\beta$-afferents (6.5%) and 7 SAII A$\beta$-afferents (7.5%). These results agree with in vivo and ex vivo reports in mice (Table 1), with the exception that we separate slowly adapting afferent types I and II (Boada and Woodbury 2007; Cain et al. 2001; Koerber and Woodbury 2002; McIlwraith et al. 2007; Wetzel et al. 2007; Zimmermann et al. 2007).
2009). We did not observe fibers with unambiguously Aβ conduction velocities (>11 m/s) that had von Frey thresholds ≥6 mN, the cutoff for nociceptors used by Zimmerman et al (2009).

It is worth noting that the bulk of the literature reports mechanical threshold as the approximate measured force of the smallest von Frey filament to consistently (≥50%) elicit a response, despite the finding that compressive stress/pressure is a more reliable predictor of firing rates in slowly adapting mechanoreceptors (Ge and Khalsa 2002). We follow this convention for mechanical threshold reporting for easier comparison to previous literature and because there is not a straightforward conversion of these forces into pressures. This is an inherent limitation of von Frey filaments, as the bending of these filaments provides an irregular contact surface through which the force is applied. With that limitation in mind and assuming a simple cylinder perpendicular to the skin surface, the pressures applied by von Frey filaments in the light touch range (<10 mN or ~200 kPa) occupy the same pressure range as our mechanical indenter equipped with a filleted cylindrical probe (Supplemental Fig. S1).

We found that mouse SAI and SAII afferents can be reliably classified based on the regularity of firing, as measured by the CoV of static-phase ISIs. This parameter has previously been used to quantitatively discriminate slowly adapting units in hairy skin of the cat, with SAI response CoV generally <0.3 and SAI response CoV >0.5 (Chambers et al. 1972). CoV has also been used to discriminate slowly adapting responses in sinus hair follicles from rat and cat (Baumann et al. 1996; Cahusac and Mavulati 2009; Gottschaldt et al. 1973), though the value generally used in those studies to segregate whisker units (0.1 in rat, 0.2–0.5 in cat) was lower than the discriminant produced by our analysis (0.49). A few studies have reported regular responses arising from identified touch domes (Horch et al. 1974; Yasargil et al. 1988), but in both cases this required either deliberate damage to a portion of the touch dome or careful placement of very fine probes to stimulate specific regions of the touch dome. The relatively large size and perpendicular approach of our mechanical stimulus probe is designed to eliminate exactly these intra-touch dome edge effects, minimizing the possibility of spuriously regular SAI responses.
Classifying slowly adapting afferents by their static phase irregularity is not without complications, as SAII responses lose much of their characteristic regularity at firing frequencies <15–20 Hz (Fig. 6C; [Horch et al. 1974; 1977]). We observe static firing rates of 20–40 Hz at ~100 kPa stimulus intensities (comparable to von Frey filaments <10 mN), above the minimum firing rate reported by Horch et al. to be necessary to distinguish SAI from SAII responses. At these stimulus intensities and response firing rates, the difference in regularity is audibly detectable when listening to neural responses during recording (Fig. S1). Previously published ex vivo preparations typically reported SA firing rates below 10-20 Hz during the static phase, including the only previously published epidermis-up recording method, which is a likely explanation for the lack of afferent type discrimination (Kinkelin et al. 1999; Koerber and Woodbury 2002; McIlwrath et al. 2007).

It is possible that the responses we identify as SAII afferents do not share identity with the classically defined SAII response in the hairy skin of cat (Chambers et al. 1972), rat (Leem et al. 1993a; Leem et al. 1993b; Lynn and Carpenter 1982; Reeh 1986) and macaque (Harrington and Merzenich 1970). Although their receptive field sizes, mechanical thresholds and highly regular sustained firing rates closely match those of SAII afferents in other species, these afferents did not differ in stretch sensitivity or spontaneous firing rates compared with SAI afferents. This may explain some of the previous difficulty in separating these two light touch responses in mice, as SAII afferents have been consistently reported to be directionally stretch sensitive and to have higher spontaneous firing rates than SAI afferents in non-murine species (Chambers et al. 1972; Zimmermann et al. 2009). A few publications have hypothesized the existence of a third SA light touch receptor in humans (Edin 2001), but conclusive molecular, morphological or statistical differentiation is lacking. Boada and Woodbury (2007) noted the presence of some regularly firing slowly adapting receptors in vivo, which they postulated to innervate musculature. In our ex vivo preparation, the major muscle mass is removed, leaving only small cutaneous muscles present. It is possible that our SAII afferents innervate this remnant cutaneous musculature, but because these sensors are exquisitely sensitive to epidermal touch, they functional as cutaneous mechanoreceptors.
independent of the structure they innervate. Conclusive identification of SAII receptors awaits the discovery of a selective marker; however, our results clearly demonstrate that a significant proportion (~50%) of slowly adapting Aβ afferents in the hairy skin of mice arise from receptors other than Merkel-cell neurite complexes.

Widely used epidermis-down *ex vivo* systems have valuable advantages. The dermis is much more permeable to chemical agents than the epidermis, making epidermis-down recording the preferred method for pharmacological studies in the intact skin (Kirchhoff et al. 1992; Steen and Reeh 1993). Nonetheless, the epidermis-up recording system may be preferable to epidermis-down recording for physiological studies of light touch receptors, as firing rates and mechanical thresholds more closely match those reported *in vivo* in mice and other mammals. Fine distinction of slowly adapting afferents in a genetically tractable model will allow exploitation of the full potential of mouse genetics to tease apart the mechanisms underlying touch sensation. Simply labeling Merkel cells by expressing eGFP in *Atoh1*-expressing cells, for example, allowed us to image the live end organs of SAI afferents during recording, confirming in living skin the observations of Iggo and Muir (1969) and Woodbury and Koerber (2007). This strategy can be implemented to easily identify other cutaneous receptors, and to pave the way for genetic dissection of the cellular and molecular pathways that transduce touch.

**Acknowledgements**

We thank A. Nelson for dissection assistance, K. Firozi for care and maintenance of the mouse colony, and J. Howard and E. Martinson for assistance with chamber design and fabrication. We also thank C. Cowen, D. Yatsenko and members of the Lumpkin lab for insightful comments on the manuscript.

**Grants**

This work was supported by NIAMS grant AR051219 to E. A. Lumpkin, by DARPA grant HR0011-08-1-0072 to G. J. Gerling, National Library of Medicine grant T15LM009462 to
Figure Legends

Fig. 1. Schematic of the epidermis-up ex vivo skin-nerve preparation (A). The hindlimb skin of a mouse is mounted in a perfusion chamber, with the attached saphenous nerve resting on a mirror in the adjoining recording chamber. The skin is perfused with SIF, which is saturated with 95% O2/5% CO2. Bath temperature is monitored by a thermistor (Therm) and maintained at 32°C. The preparation is visualized with a fluorescence-equipped stereomicroscope (Micro) connected to a camera and computer for image capture. The computer also controls electrical stimuli from a stimulus generator and mechanical stimuli via a motor controller. The motor controller drives a stepper motor (M) mounted to a rigid arm and an X/Y stage. A ceramic probe is mounted to the end of a cantilever arm with an in-line force transducer (FT). Force data are sent to the computer via an amplifier and captured along with differential recordings of extracellular potentials in a nerve bundle. Extracellular potentials are sent in parallel to an oscilloscope and a speaker for aural detection of action potentials. B: Image of the mechanical stimulator. C: A skin-nerve preparation mounted in the recording chamber. The edge of the skin is denoted by a dotted black line. Pigmented areas of skin are in the active growth phase (anagen) of the hair cycle.

Fig. 2. Receptive field areas and von Frey thresholds measurements are higher on the dermal surface than the epidermal surface. Measurements of RF area and von Frey threshold were made from both the epidermal and dermal surfaces on the same set of afferents by flipping the skin over during recording (N=7). Receptive field size was calculated as the area of an ellipsoid with measured diameters, and had median values of 0.17 mm² (epidermal) and 0.26 mm² (dermal). A: Fold change in RF area from epidermal to dermal surface. Each connected pair of points indicates a single afferent, and shows the change in measured receptive field size due to skin orientation, with the absolute size of the dermal receptive field indicated at right (mm²). B: Superimposed action potentials from one of the afferents used in the epidermis vs. dermis comparisons. Ten action
potentials are from an epidermal stimulus (black) and 10 are from a dermal stimulus (grey). C: Von Frey force thresholds also increased when measured from the dermal surface (p=0.01, Wilcoxon one-tailed signed-rank). Only three pairs are visible due to overlap of multiple afferents (N=7). D: The same data as shown in (C), but with von Frey forces converted to minimum potential pressures, based on the assumption of a simple cylindrical probe. Because von Frey filaments do not increase in diameter monotonically, this conversion to pressure causes a spurious reversal of some trends in the data.

Fig. 3. Flow chart for classifying touch receptors in the epidermis-up mouse skin-nerve preparation. After locating a mechanosensitive afferent with a mechanical search, A-afferents and C-fibers can easily be distinguished based on their conduction velocities. Aδ-fibers can usually be distinguished from Aβ-fibers by conduction velocity, but there is some overlap between the two populations. SA, RA, and any remaining AM or D-hair fibers can then be distinguished by mechanical thresholds, receptive field sizes and adaptation properties. SAI and SAII responses are differentiated by the regularity of their static-phase firing rates, which is quantified as the coefficient of variation of inter-spike intervals (see Figs. 4–6). Plots in each afferent-type box shows a typical response, as instantaneous firing frequency versus time, to a 5-s touch stimulus. Twenty mechanically-evoked action potentials from the example fiber are superimposed to the right of each plot to show matching waveforms.

Fig. 4. Two classes of slowly adapting mechanoreceptors exist in mice. Model-based cluster analysis on up to four variables including minimum dynamic ISI, mean static ISI, CoV of static ISIs and minimum static ISI was performed on 17 slowly adapting fibers using the Mclust package for R. A: The model and cluster number combination with the lowest BIC that grouped together fluorescence microscopy-confirmed SAI fibers was equal size and shape ellipsoids with their axes aligned with the coordinate axes (EEI, hollow circles). Minimum BIC with this model was -142 at two clusters. Other models shown are equal size and shape ellipsoids with either the same orientation (EEE, black squares) or freely oriented (EEV, grey triangles). Other models tested had
minimum BIC above -100. B: All fibers used for cluster analysis plotted along the two discriminant variables. Fiber classification based on the best model chosen by the analysis from (A) is indicated by an open circle or gray triangle. Clusters for the best fit model were ellipsoids of the same volume with long axes perpendicular to the CoV coordinate. Clusters are indicated as dashed ellipses with radii of 3 X SD centered around the mean of each cluster. C: Univariate cluster analysis of CoV for all fibers yielded a higher BIC (-1), but categorized the fibers into the same clusters and provided the lowest BIC of any other single variable. The best fit was to an equal variance model (open circles) with two clusters. Results of variable variance model fitting are shown as grey triangles. D: Mean coefficient of variation (CoV) of inter-spike interval values plotted as a histogram of all fibers. For each afferent, values were calculated as the mean of the CoV of all stimuli. There is a bimodal distribution with maximal separation at 0.49. Afferents confirmed as SAI fibers by fluorescent microscopy lie within the cluster with CoV > 0.49, leading to the designation of these two populations as SAI and SAII.

Fig. 5. SAI receptive field images and responses to touch stimuli. Micrographs (A and B) are shown of the Merkel-cell cluster innervated by this SAI afferent. Arrowheads indicate the same three Merkel cells in both images. A: An epifluorescence micrograph of the touch dome demonstrating eGFP fluorescence (green) in Merkel cells in the living skin-nerve preparation. Asterisk denotes the position of the guard hair. B: Confocal z-series projection of the same touch dome shows 22 Merkel cells in the cluster. C: Recorded force values during a family of 5-s displacements. D: Voltage traces showing action potential trains for each stimulus. The SAI afferent responds to increased force with an increase in firing rate while maintaining its irregular firing pattern. Responses are shown from static-phase pressures of ~30–150 kPa. This particular afferent was chosen as an example of the hallmark irregularity of SAI responses, although its firing rate falls below the mean for SAI afferents. There is an RA unit present in this recording as well, visible at the end of the top two traces as a ~200-μV peak that was easily discriminated and did not interfere with data analysis.
Fig. 6. SAII receptive field image and responses to touch stimuli. A: Micrograph of the skin of an FM1-43-injected mouse from which the recordings in (B and C) were taken. The receptive field of this afferent (dashed ellipse) does not overlap with two labeled touch domes (asterisks) visible in this field. B: Force family for the voltage traces shown below. Each stimulus lasted for 5 seconds. Some mechanical ringing is visible at the onset of each stimulus, which decayed to below baseline well before the static phase. C: The SAII afferent responded with low, stochastic firing to very light stimuli (top trace), but more intense stimulation elicited robust and highly regular spike trains. When CoV values across stimulus intensity are averaged prior to afferent comparisons, the impact of low-firing rate responses is reduced. To avoid classification error, it is important to use stimuli sufficient to exceed this spurious irregularity range (<15–20 Hz). Responses are shown from ~25–150 kPa static pressures. Two additional units are visible as low-amplitude action potentials, one at all stimulus intensities and one at only the highest two stimulus levels.

Fig. 7. Firing rate data for SAI responses (open circles) and SAII responses (gray squares) are shown in A and B (error bars denote ± standard errors of the mean). Data are individual stimuli across afferents in 200-kPa bins, demonstrating that SAI and SAII afferents produce similar firing rates in response to displacement stimuli. A: Mean firing rate during the dynamic phase (t=0–200 ms, calculated as the inverse of the mean ISI) plotted against peak dynamic force. B: Mean firing rate for the static phase (2–4.5 s) plotted against the mean static-phase force. (N=113 stimuli from 11 SAI afferents and 27 stimuli from 7 SAII afferents). C: Histogram of normalized static-phase inter-spike intervals for SAI (black outline) and SAII (solid grey) responses. Note the wide dispersion of intervals in SAI responses relative to SAII. To allow comparison of intervals across stimulus intensities and afferents, each interval is normalized to the mean interval for their stimulus of origin (SAI, N=3348 intervals; SAII, N=1533 intervals).

Fig. S1. Constant displacement stimuli applied in our experiments have comparable compressive stresses to the minimum stress applied by von Frey filaments. Grey diamonds are calculated pressures (stress, in kPa) for each von Frey filament with a linear regression line. Calculated von
Frey pressures assume perpendicular contact with the end of a cylinder, making these values underestimates of the pressures actually applied by the filaments. The black line shows the pressure/force relationship for our cylindrical probe over the force range typically used in our experiments. The dashed box highlights the pressure range of the von Frey filaments most commonly used to probe the function of light touch receptors.

Fig. S2. Slowly adapting afferents separated by coefficient of variation produce audibly discriminable firing patterns. SAI response firing patterns are quite irregular, characterized by the random “popcorn popping” pattern of action potentials during a sustained stimulus (Fig. S2: SAI.wav). SAI responses, by contrast, present extremely regular trains of action potentials, one example of which is (Fig. S2: SAII.wav).
References


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Table 1: Reported properties of touch-sensitive afferents in various preparations

<table>
<thead>
<tr>
<th>species</th>
<th>preparation</th>
<th>Conduction velocity (m/s)</th>
<th>Mechanical threshold (mN)</th>
<th>Fraction of afferents (%)</th>
<th>Firing rates (Hz)</th>
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<tr>
<td></td>
<td></td>
<td>(means)</td>
<td>(medians)</td>
<td>(of all afferents)</td>
<td>Peak</td>
<td>Dynamic</td>
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(ge and Khalsa 2002)