Physiological Properties of Mouse Skin Sensory Neurons Recorded Intracellularly In Vivo: Temperature Effects on Somal Membrane Properties

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INTRODUCTION

Skin sensory neurons of dorsal root ganglia (DRGs) represent an exceedingly diverse assemblage marked by notable heterogeneity across multiple physiological, anatomical, and molecular properties (reviewed in Brown 1981; Burgess and Perl 1973; Scott 1992; Willis and Coggeshall 2004). Although most information surrounding relationships between these disparate properties has been obtained piecemeal from studies conducted in larger mammalian models (Brown et al. 1977; Fang et al. 2006; Hoheisel et al. 1994; Lawson et al. 2002; Leah et al. 1985; Light and Perl 1979; Sugiiura et al. 1986; Xu and Zhao 2001), the recent advent of ex vivo somatosensory system preparations from mice has greatly facilitated such investigations across multiple developmental time points in both normal and transgenic animals (Albers et al. 2006; Woodbury and Koerber 2003; Woodbury et al. 2001, 2004).

The power of these diminutive murine preparations for studies of sensory neuron biology is evidenced not only by the fact that all classes of sensory neurons can be intracellularly recorded, physiologically characterized, and stained for immunocytochemical studies from their somata as in larger species (e.g., Fang et al. 2006; Lawson et al. 2002; Leah et al. 1985; Xu and Zhao 2001) but precisely because of their small size, staining of the central arbors of these same afferents is also within reach from the DRG for routine analyses of laminar termination patterns in the spinal dorsal horn; in neonates, peripheral terminals in the skin are within reach as well (Woodbury and Koerber 2001; Ye and Woodbury 2006). In this species, therefore correlations between somal biophysical properties, axonal conduction velocity (CV), peripheral response properties, molecular markers, and terminal morphology can now be investigated in the same afferent.

The unparalleled resolution afforded by this detailed single-neuron approach has provided a number of novel insights into sensory neuron biology, particularly with respect to the anatomical, functional, and molecular diversity of nociceptors (Albers et al. 2006; Woodbury and Koerber 2003; Woodbury et al. 2004). In light of the growing prominence of mouse models in studies of nociceptive mechanisms and the critical importance of an accurate understanding of nociceptor biology to pain management, it is imperative that these findings obtained under manifestly artificial conditions are evaluated under more natural conditions to validate this in vitro work. Therefore as a first step toward this end, we have developed a new in vivo spinal cord/DRG preparation for combined structure/function analyses of individual, physiologically identified skin sensory neurons in adult mice.

The present report represents the first on the properties of mouse primary afferents recorded with intracellular techniques at normal temperatures. Studies were conducted at thoracic levels to facilitate comparison with similar in vitro studies in our lab. These studies revealed that a number of physiological properties of sensory neurons, from response properties in the periphery to somal membrane properties in the DRG, were critically dependent on temperature. In some populations, the effects of alteration in temperature radically altered “diagnostic” physiological properties and thus perceived functional identity of cutaneous afferents. Some of these findings were previously presented in abstract form (Boada and Woodbury 2006; Woodbury and Boada 2006).

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METHODS

The present in vivo electrophysiological experiments were conducted on young adult Swiss–Webster mice of either sex ranging in age from postnatal day (P) 28 to P72 and weighing between 25 and 40 g. All procedures used in the present experiments were approved by the Institutional Animal Care and Use Committee of the University of Wyoming.

Surgical procedures

Mice were deeply anesthetized with ketamine and xylazine [90 and 10 mg/kg, respectively, administered intraperitoneally (ip)] and hair over the lateral flank was clipped and shaved between dorsal and ventral midlines. Areflexive animals were then intubated and ventilated (Minivent, Harvard Apparatus, Holliston, MA) with humidified oxygen. The scalp was incised to perform a craniotomy and the cerebral cortex aspirated bilaterally full thickness. Exposed tissues were covered with gelatin foam and sealed with petroleum jelly. The EKG was monitored throughout to assess level of analgesia. In later experiments, end-tidal CO2 (microCapStar, CWE, Ardmore, PA) and carotid pressure (Mikro-Tip, AD Instruments, Colorado Springs, CO) were also monitored and maintained within normal limits (the latter by supplemental lactated Ringer, ip). Decorticate animals were immobilized with β-tubocurarine (Sigma, St. Louis, MO).

As illustrated in Fig. 1A, a dorsal midline incision was made in trunk skin and DRGs at thoracic levels T11/T12 and adjacent spinal cord were exposed by laminectomy. Desiccation was prevented by continuous superfusion of oxygenated artificial cerebrospinal fluid [aCFS (in mM): 127.0 NaCl, 1.9 KCl, 1.2 KH2PO4, 1.3 MgSO4, 2.4 CaCl2, 26.0 NaHCO3, and 10.0 D-glucose]. The spinal column was secured using custom clamps and the preparation then transferred to a preheated (30–34°C) recording chamber where the superfusate was slowly raised to 37°C (MPRE8, Cell MicroControls, Norfolk, VA). Pool temperature adjacent to the DRG was monitored with a small thermocouple (IT-23, Physitemp, Clifton, NJ). Rectal temperature adjacent to the DRG was monitored with a small thermocouple (IT-23, Physitemp, Clifton, NJ). Rectal temperature (RET-3, Physitemp) was maintained at 34 ± 1°C with radiant heat. All thermal monitoring and output devices were calibrated (CL-740A, Omega, Stamford, CT).

Electrophysiology

DRG somata were impaled with quartz microelectrodes (80–150 MΩ) containing 20% Neurobiotin (Vector Laboratories, Burlingame, CA) in 1 M potassium acetate. Intracellular penetrations with a resting membrane potential of ≲ −45 mV were characterized further. DC output from an Axoclamp 2B (Axon Instruments/Molecular Devices, Sunnyvale, CA) was digitized and analyzed off-line using Spike2 (CED, Cambridge, UK). Sampling rate for intracellular recordings was 21 kHz throughout (Power1401, CED). In three experiments, cells sampled initially at 21 kHz were also subsequently sampled at 100 kHz to compare resulting spike waveforms (n = 13 cells, including five low-threshold mechanoreceptors, four myelinated nociceptors, and four unmyelinated nociceptors). In all cases, these different sampling rates produced spikes that were essentially indistinguishable both qualitatively and quantitatively, as exemplified in Fig. 1B. All quantitative analyses of somal membrane properties (below) were performed on cells sampled at the lower rate.

Mechanically sensitive neurons

On stable impalement, the skin was searched with a fine sable-hair brush to locate the peripheral receptive field (RF). For afferents requiring higher intensities, subsequent searches used increasingly stiffer probes and finally sharp-tipped watchmaker forceps. Neurons with RFs located near the incision site were excluded because of possible injury effects. Afferents with cutaneous RFs were distin-
guished from those with deep RFs by displacing skin to ensure that RFs tracked rather than remaining stationary. Mechanical thresholds were characterized with calibrated von Frey filaments (Stoelting, Wood Dale, IL). Adaptation rate was frequently evaluated using micromanipulator-based probes; responses to skin stretch and vibratory stimuli were also tested in many cells. In all cases, RFs were characterized with the aid of a zoom stereomicroscope.

Thermal sensitivity was tested using a $3 \times 5$-mm pellet stimulator (Yale Instrument Repair and Design Shop, New Haven, CT). To improve thermal conductivity and standardize heat transfer, a small drop of water was used to displace insulating air trapped at the peltier/skin interface; a second IT-23 thermocouple, sandwiched between peltier and epidermis, was used to measure water temperature and thus the actual temperature experienced by the epidermal surface. Stimuli below freezing (indicated by a sudden exothermic reaction) and $>5$ s above $53^\circ$C were normally avoided to prevent tissue damage and areas of preexposed skin were monitored.

Mechanically insensitive cells

Mechanically sensitive RFs could not be located in about 20% of impaled cells. Outside mechanical stimuli (above), additional testing of such “silent” cells was limited to innocuous radiant warming or cooling stimuli; noxious thermal (or electrical) search stimuli were not used to avoid sensitizing other afferents throughout the dermatome. Further, because whole nerve electrical search stimuli were not used in these experiments (resulting from the complexity of peripheral innervation patterns [below] and short dorsal roots at these segmental levels) and the presence of Neurobiotin in our electrodes prevented indiscriminate current injection (below) we were unable to obtain information on the somal spike properties of “silent” neurons or whether they innervated cutaneous or deep tissues; thus such afferents will not be discussed further.

Somaal membrane properties

Active membrane properties of all identified sensory neurons analyzed included the amplitude and duration of the positive-going spike and afterhyperpolarization of the action potential (AP), along with the maximum rates of spike depolarization and repolarization; durations were measured at half-amplitude rather than baseline to minimize hyperpolarization-related artifacts. Passive properties analyzed included input resistance, time constant, inward rectification, and, where possible, rheobase; all but the latter were determined by injecting incremental hyperpolarizing current pulses ($\leq 0.1$ nA, 500 ms) through balanced electrodes.

Evoked spike latency

Because intact thoracic DRGs serve multiple nerves, spike latency was obtained by stimulating the RF at the skin surface using a bipolar electrode (0.5 Hz), performed subsequent to natural stimulation to prevent potential alterations in RF properties. Because we were interested in latency from terminals, all measurements were obtained using the absolute minimum intensity required to excite neurons consistently without jitter, as exemplified in Fig. 1C; significantly shorter latencies, seen at traditional (i.e., two- to threefold threshold) intensities and presumably reflecting spread to more proximal sites along axons, were rejected. Stimuli ranged in duration from 50 to 100 $\mu$s; utilization time was not taken into account. Conduction distances were measured for each afferent on termination of the experiment by inserting a pin through the RF (marked with ink at the time of recording) and carefully measuring the distance to the DRG along the closest nerve.

In two experiments, compound action potentials (CAPs) were recorded from dorsal cutaneous nerves (DCNs) at these segments. CAPs were evoked with bipolar electrodes inserted into the skin at a single point and thus sampled a small fraction of axons in the nerve; to increase signal-to-noise ratios, the latter were transected near the DRG, recorded with suction electrodes, DC amplified, and averaged ($>10 \times$) across multiple stimulus intensities.

Neuronal labeling

After physiological characterization, selected neurons were iontophoretically injected with Neurobiotin (+0.5–1.5 nA, 10–68 nA·min total). The number of cells stained per DRG was no more than two and depended on adequate dorsoventral separation within the dermatome. Decorticate animals were heparinized 5–9 h later and transcendally perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Spinal cord and DRGs were dissected free and processed histologically to visualize Neurobiotin as detailed elsewhere (Woodbury et al. 2001).

Statistical analyses

Skin sensory neurons were categorized according to peripheral response properties to natural stimuli. For each identified group, all neurons meeting acceptance criteria (see RESULTS) were included in analyses of physiological parameters. The latter measurements were compared across groups using standard parametric (Student’s $t$-test, one-way ANOVA, linear regression) or nonparametric (Mann–Whitney $U$ test, Kruskall–Wallis) tests depending on normality; evaluations of significant comparisons between at least three groups were performed using various post hoc tests (e.g., Tukey’s, Dunn’s). For some neurons, spike first derivatives were examined using runs tests. Statistical tests were carried out across multiple packages (OriginPro 7.5, Northampton, MA; InStat/Prism, San Diego, CA; JMP, Cary, NC). Descriptive data are presented as means ± SE.

RESULTS

The present report includes observations from a total of 362 dorsal root ganglia (DRGs) sensory neurons that were recorded intrasomally from thoracic levels T11–T12 in a new in vivo adult mouse spinal preparation. Of this total, 92 neurons were recorded in both preliminary and subsequent experiments at lower temperatures (DRG $\approx 30^\circ$C) to allow comparisons with previous recordings from these same ganglia in ex vivo preparations (Albers et al. 2006; Woodbury et al. 2001, 2004). However, in view of the absence of baseline information in this species in vivo, most efforts concentrated on temperatures approximating normal physiological conditions.

In all, 270 sensory neurons were recorded from a total of 90 mice of either sex in which DRG temperatures were tightly controlled around normal core temperatures ($37 \pm 0.5^\circ$C). Of these, 137 physiologically identified and well-characterized cutaneous afferents, including 69 low-threshold mechanoreceptors (LTMRs) and 68 nociceptors, were included in detailed analyses. Intracellular recordings ranged from 35 to 150 min with little to no drift in resting membrane potential throughout, and all analyzed neurons fulfilled requirements for complete characterization across multiple physiological parameters, including information on adequate stimuli across multiple modalities, active and passive membrane properties, evoked spike latency, and peripheral conduction velocity (CV); further, all included cells satisfied the following requirements: resting membrane potential more negative than $-45$ mV, action potential (AP) amplitude $>30$ mV (all but three were $>40$ mV), and the presence of spike afterhyperpolarization (AHP); passive membrane properties indicative of poor impalement were
also grounds for exclusion. Representative somal APs and peripheral response properties of analyzed neurons are shown in Fig. 2; physiological properties of this sample are presented in Tables 1 and 2.

**Cutaneous afferents**

Cutaneous receptive fields (RFs) were found throughout the dermatomes spanning the lateral flank from the dorsal to ventral midlines (Fig. 1A). Thus RFs were contained within the innervation territories of dorsal cutaneous nerves (DCNs) used in previous in vitro studies, as well as lateral and ventral cutaneous branches of the spinal (i.e., intercostal) nerve. Although encountered in preliminary experiments at lower temperatures, no spontaneously active cutaneous afferents were observed in experiments where skin temperature was maintained at normal levels (32–33°C). Because of the search stimuli used (see METHODS), all cutaneous afferents studied were mechanosensitive; purely thermosensitive neurons were not observed. Further, because a priori knowledge of spike shape or CV that could bias functional characterization was not available, all afferents were tested with a standardized protocol of natural stimuli before latency determination and classification by CV.

**LATENCY AND CV.** Consistent differences in evoked spike latencies were seen among functional subtypes of cutaneous afferents as shown in Fig. 3A. Latencies of many groups fell within narrow bounds regardless of RF location on the trunk. Greater variance was observed in calculated CV values than in spike latency, and less variance in CV was seen in unmyelinated than in most myelinated groups (e.g., Fig. 3B), possibly reflecting distance measurement errors and/or immaturity of myelination state within our age window. However, CVs were well above those described for mice in previous in vitro studies; because rectal temperatures were approximately 34°C, these values still are likely underestimates for this species.

For the purpose of subclassification by CV, compound action potentials (CAPs) were recorded in DCNs after electrical skin stimulation. Curiously, low-intensity stimulation recruited three peaks corresponding to myelinated fibers. The fastest peak had a latency shorter than that of the fastest cutaneous afferents in our sample (Table 1), suggesting the presence of Aα fibers in this “cutaneous” nerve (Fig. 1D; see Noncutaneous afferents, below). For cutaneous afferents, the “valley” between the second and third CAP peaks, corresponding to a CV of 11 m/s, was used as the Aβ/δ cutoff in all subsequent analyses; this value fit well with CV distributions of most functional subpopulations of LTMRs. Because a clear...
Skin temperatures (Bury et al. 2001) was absent in preliminary recordings at lower sensitivity normally diagnostic of these afferents (e.g., Wood-A, Table 1). Further, the exquisite dynamic cutoff (Fig. 4) preparation had unexpectedly slow CVs very near the A/C Pacinian corpuscles.

Cutoff between A and C fibers was not evident in these CAP recordings, 1.5 m/s was selected arbitrarily based on findings from polymodal nociceptors that fell at or below this value. In light of the broad age range in these experiments, however, such cutoff values are regarded as approximations only.

Receptive field properties

RA LTMRS. Three rapidly adapting (RA) LTMR subtypes could be discerned based on differences in RF properties and CVs (Table 1); all were silent to thermal stimuli. These included guard and down (D) hair follicle afferents (HFAs), and units classified simply as “RA.” The latter exhibited highly phasic response properties and CVs that were essentially indistinguishable from Aβ HFAs, although mechanical thresholds were higher and direct skin contact appeared to be required at threshold intensities; all tested followed vibratory stimuli ≤440 Hz in a 1:1 fashion, suggesting innervation of Pacinian corpuscles.

Interestingly, some D-HFAs (verified by central anatomy; in preparation) had unexpectedly slow CVs very near the A/C cutoff (Fig. 4A, Table 1). Further, the exquisite dynamic sensitivity normally diagnostic of these afferents (e.g., Woodbury et al. 2001) was absent in preliminary recordings at lower skin temperatures (~24–26°C).

SA LTMRS. Slowly adapting (SA) LTMRs fell into two discrete groups based on mechanical thresholds and response properties; none responded to thermal stimuli. One group (n = 4) exhibited mechanical thresholds <0.07 mN and responded with an irregular pattern of discharge to maintained stimuli similar to type I receptors. The second group exhibited higher thresholds (0.74 mN) and responded with a highly regular pattern of discharge. Importantly, however, two additional “Type II” afferents, originally included in this group based on identical thresholds and response properties, were discovered subsequently to be muscle afferents and hence reclassified (see Noncutaneous afferents, below). Because others from this second group were not stained centrally, their identity is unknown and they are tentatively included in Table 1.

MECHANO-COLD SENSORY NEURONS. A small number of myelinated afferents were encountered with low mechanical thresholds and large (>3 mm²), ill-defined RFs that responded vigorously to cooling (Fig. 2); none responded to heating. Peripheral CVs spanned the Aβ/Aδ cutoff (Table 1; Figs. 3B and 4). Interestingly, similar afferents exhibited spontaneous activity in early experiments at lower temperatures (not shown). The physiological and anatomical properties of this enigmatic group form the basis for a separate report (in preparation).

A-NOCICEPTORS. Myelinated nociceptors spanned a broad range of CVs that included some of the fastest seen among...
cutaneous afferents. However, the majority in our sample (18/21) had CVs in the A\(\beta\) range (Table 1). Because A\(\beta\) nociceptors satisfying all criteria for inclusion in analyses were few (n = 3), and these were largely indistinguishable from A\(\delta\) nociceptors in peripheral response properties, all afferents were combined in subsequent analyses.

These afferents exhibited a broad range of mechanical thresholds (Table 1). A few were relatively sensitive and responded with a distinctly regular SA discharge as described previously (Woodbury and Koerber 2003), whereas others were particularly insensitive and required probing the skin with sharp-tipped forceps and/or noxious skin pinch. The vast majority showed no response to noxious thermal stimuli despite repeated applications (e.g., <0 and >55°C) and were thus classified as high-threshold mechanoreceptors (A-HTMRs); those showing thermal sensitivity (n = 2) failed to respond to the first test and their weak response to repeated testing may have reflected sensitization.

Unmyelinated sensory neurons

C-LTMRs. Putative unmyelinated LTMRs were rarely encountered (n = 2) in these experiments; recordings to date have not met criteria required for inclusion in analyses.

C-nociceptors. Two distinct groups of unmyelinated nociceptors were encountered. A small number responded to mechanical but not thermal stimuli and were classified as C-HTMRs (Table 1). One cell, included in this category on the basis of CV, exhibited somal properties identical to those of A-HTMRs, whereas another with a slightly higher CV exhibited somal properties identical to those of C-polymodals (e.g., Fig. 4); misclassification thus remains a possibility in the absence of central anatomy or markers of myelination state (see also Alvarez et al. 1993).

Most unmyelinated afferents encountered were C-polymodal nociceptors that responded well both to mechanical and to thermal stimuli; the few tested also responded to dilute acid...
or histamine applied onto or under the skin, respectively. Mechanical thresholds were relatively low (Table 1) and all were localized using a fine sable-hair brush; afterdischarge to moderately suprathreshold mechanical stimuli was common (e.g., Fig. 2). Although these cells fired well to heating (threshold = 44.4 ± 1.0°C), cooling also produced an initial burst of APs (threshold = 12 ± 1.6°C), followed by an intermittent, low-frequency discharge throughout the cold stimulus. Curiously, a rebound response was also evident when temperatures rose quickly back toward baseline (Fig. 2).

Somal membrane properties

PASSIVE PROPERTIES. Passive and active somal membrane properties of cutaneous afferents recorded at normal core values (36.5–37.5°C) are shown in Table 2. Across subtypes, few consistent differences were observed in passive membrane properties, with the exception of C-polymodal afferents that showed on average greater input resistance and time constant than other afferent groups (P < 0.05; Kruskal–Wallis). Analyses of inward rectification (not shown) also failed to reveal consistent differences with the small hyperpolarizing currents used in the present experiments (i.e., ≤0.1 nA). When observed, however, it was seen only in LTMRs and C-polymodal nociceptors, in partial agreement therefore with a previous report in cat using larger currents (e.g., 2–5 nA; Koerber et al. 1988).

ACTIVE PROPERTIES. Consistent differences were observed between the APs of LTMRs and nociceptors (e.g., Fig. 2) across most parameters analyzed.

Spike amplitude. Overall, nociceptor spike amplitudes were significantly greater (69.9 ± 1.7 mV) than those of LTMRs (47.7 ± 1.4 mV; P < 0.001, Mann–Whitney) as reported previously (Fang et al. 2005; Koerber et al. 1988; Woodbury and Koerber 2003). As a group, C-nociceptor spikes were larger than those of A-HTMRs (P < 0.01, Kruskal–Wallis) and more than double those of many LTMRs (similar findings were observed in AHP amplitudes; Table 2). A-HTMR spikes were also larger than those of LTMRs (P < 0.01, Kruskal–Wallis), independent of peripheral CV. Unexpectedly, however, A-HTMR spikes exhibited little to no overshoot (Fig. 1, B and C; see also Figs. 2, 5, and 6); the latter was typically substantial in C-nociceptors (e.g., Figs. 1B and 2) and a common occurrence in A-HTMRs in previous reports.

Spike duration. As expected from previous recordings, consistent differences were also seen in spike durations (measured at half-amplitude, or D50) of functional subtypes as illustrated in Fig. 2. At one extreme were the short-duration spikes of LTMRs (Table 2), which lacked a hump, or inflection, on their falling phase; at the other extreme were the longer-duration and clearly inflected spikes of C-nociceptors; these two groups overall were essentially nonoverlapping (P < 0.001, Kruskal–Wallis).

Surprisingly, however, A-HTMR spikes were much narrower than anticipated from previous work and lacked any sign of an inflection on the falling phase; the absence of an inflection, a qualitative feature normally diagnostic of these afferents, was a consistent finding across all A-HTMRs regardless of D50. CV, presence of neurobiotin, or sampling rate used (e.g., Fig. 1B; see Temperature effects on somal membrane properties, below). As seen in the plot of CV versus D50 (Fig. 4A), cutaneous afferents clustered in two distinct groups largely reflective of myelination state. Although D50 values of A-HTMRs were statistically different from those of both

FIG. 5. Somal AP properties of identified skin sensory neurons recorded at different DRG temperatures. A–C: examples of somal APs from A-HTMRs with similar peripheral CVs recorded in different experiments illustrating the range of variation observed at different temperatures; black and gray traces represent APs recorded at 37 and 30°C, respectively. Superimposed traces of the shortest- (A) and longest-duration APs (B) encountered at each temperature. C: superimposed AP traces from Aβ HTMRs recorded in these same experiments; hyperpolarization is evident in the Aβ HTMR recorded at 30°C. Note the inflections on the falling limbs of APs recorded at 30°C, which are evident as a break in the first derivatives (shown below); by contrast, all APs recorded at 37°C lacked such an inflection. CVs for these afferents (at 37 and 30°C, respectively) were: 4.3 and 6.0 (A), 5.8 and 4.6 (B), 15 and 13 m/s (C). B: relationship between somal spike duration (D50) and Log10 peripheral CV among identified skin sensory afferents recorded from DRGs held at 30°C; note the longer durations overall and the separation between A-HTMRs and LTMRs at this reduced temperature (cf. Fig. 4A). Calibration bars: 20 mV, 2 ms (for all).
LTMRs and C-nociceptors on average \( (P < 0.05, \text{Kruskal–Wallis}; \text{Table 2}) \), there was considerable overlap between individual A-HTMRs and LTMRs (Fig. 4A). Among Aδ afferents, for example, 50% of HTMRs (nine of 18) exhibited \( D_{50} \) values \( \leq 0.6 \) ms, the maximum observed among D-HFAs; conversely, 80% of D-HFAs (12/15) exhibited \( D_{50} \) values \( \geq 0.4 \) ms, the minimum among A-HTMRs (Fig. 4A, inset). This overall similarity in LTMR and A-HTMR spike widths was also evidenced by analyses of maximum rates of depolarization and repolarization (Table 2). Whereas C-nociceptor spike kinetics differed substantially from other afferent subtypes, LTMRs and A-HTMRs showed broadly overlapping values with an essentially identical slope corresponding to the repolarizing limb of the spike (Fig. 4B).

**AHP duration.** The above-cited findings reveal that spike width is of limited usefulness for diagnosing individual A-HTMRs from LTMRs at normal core temperatures. However, a strong correlation was seen between AHP duration (at half-amplitude) and different functional subgroups of cutaneous afferents. Regardless of CV, APs of nociceptive afferents overall exhibited significantly longer AHPs than those of LTMRs (Table 2). This was especially true for A-HTMRs, which typically exhibited far more prolonged AHPs than even C-nociceptors \( (P < 0.05, \text{Kruskal–Wallis}) \). As evident in Fig. 4C, C-nociceptors clustered in a relatively tight group in plots of CV versus AHP. By contrast, A-HTMRs, although exhibiting some overlap in AHP duration with LTMRs, spanned a remarkably broad range that was largely independent of CV. Such differences in AHP duration between afferents were evident despite similar mean resting membrane potentials (Table 2).

The strongest evidence that A-HTMRs formed an independent group based on somal membrane properties was seen by plotting spike duration against AHP duration in log–log plots as shown in Fig. 4D, which revealed a clear separation of basic functional subgroups of cutaneous afferents. In combination, these two parameters showed a strong positive correlation \( (P < 0.001, R^2 = 0.338) \), with longer-duration spikes tendency to be associated with longer AHP durations. Thus despite the considerable overlap in spike duration, the slightly longer average spike widths and long-duration AHPs of A-HTMRs clearly distinguished the latter from LTMRs on the basis of these two parameters combined.

**Temperature effects on somal membrane properties**

Despite the ability of off-line analyses (above) to reveal significant differences in A-HTMR and LTMR APs, an important question nevertheless remains. Why have previous studies found such robust differences in spike width between these two populations? One possibility stems from the fact that our earlier recordings in vitro (Albers et al. 2006; Woodbury and Koerber 2003) as well as many recent in vivo recordings (see Fang et al. 2005 and references therein) were performed at lower temperatures.

As a first test, a subsequent set of in vivo experiments was conducted in four mice in which DRGs were maintained at 30 ± 0.5°C; littersmates of similar age and weight were used. Because of reduced heat input from this colder superfusate, core temperatures averaged 33°C compared with 34°C in previous experiments, possibly resulting in a slight discrepancy in peripheral CV comparisons across data sets. Because hyperpolarization tended to be more common at these reduced
temperatures, analyses focused solely on spike width and shape. A total of 37 cutaneous afferents (19 LTMRs, six A-HTMRs, and 12 C-nociceptors) at these lower controlled temperatures satisfied basic acceptance criteria for spikes (above); these findings were concordant with those from preliminary experiments ($n = 55$ cutaneous afferents) with similarly low yet less precisely controlled temperatures.

These experiments revealed profound differences in AP properties across identified populations of sensory neurons recorded at 30 versus 37°C, as exemplified by Fig. 5, A–C and seen in comparisons between Fig. 4A and Fig. 5D. Differences were evident in all populations, although effects were greatest among nociceptors across these different temperatures; indeed, differences in some LTMR populations (e.g., Pacinian corpuscle afferents) were largely negligible, whereas all nociceptor subtype exhibited much longer duration spikes compared with recordings at 37°C. For example, the mean $D_{50}$ of C-nociceptors was 2.5 ms at 30°C versus 1.5 ms at 37°C (Table 2). The mean $D_{50}$ of A-HTMRs was 1.2 ms at 30°C versus 0.6 ms at 37°C; further, their spikes now showed a clear inflection on the falling limb, which was absent at 37°C. The overall effect on A-LTMRs was less, with a mean $D_{50}$ of 0.6 ms at 30°C versus 0.4 ms at 37°C; however, all comparisons within these groups across different temperatures were significant ($P < 0.01$ for all, Mann–Whitney). Interestingly, some LTMR subpopulations (e.g., HFA) appeared to be more susceptible to temperature than others (e.g., Pacinian afferents). Nonetheless, comparing Figs. 4A and 5D illustrates that at 30°C, A-HTMRs no longer overlap LTMRs and begin to approximate C-nociceptor spike shape. A similar inverse correlation was also evident in AP amplitude (not shown); as exemplified in Fig. 5, most A-HTMRs exhibited overshooting spikes at 30°C.

SERIAL RECORDINGS ACROSS DIFFERENT TEMPERATURES. The above-reported findings were obtained by sampling from heterogeneous populations in different animals. Therefore to obtain a better understanding of how temperature affects AP properties outright, later experiments took advantage of the remarkable stability in our preparation to compare active and passive properties of cutaneous afferent somata at different temperatures in continuous (i.e., serial) recordings. Thus recordings initially obtained at 37°C were compared with those from the same afferent at different DRG temperatures. Most recordings were obtained as temperatures were progressively reduced (minimum $\approx 28°C$), although some were also obtained as temperatures were increased (maximum $\approx 41°C$). Temperatures were stabilized for 5 min before recording APs and only those recordings showing complete recovery were accepted for further analyses. Representative examples obtained at 37, 30, and again at 37°C are shown in Fig. 6.

These serial recordings revealed striking effects of temperature on the kinetics of active membrane properties of individual sensory neuron somata. Essentially all analyzed AP parameters changed as temperature was reduced from 37 to 30°C. Importantly, the amplitude and duration of spikes and AHP increased, in some cases dramatically, as temperature was reduced (Fig. 6, A–D); by contrast, reciprocal changes occurred as temperatures were increased above normal core values, resulting in smaller-amplitude and shorter-duration spikes (not shown). These effects of temperature were extremely rapid and highly reproducible; as such, temperature control enabled selective “tuning” of AP properties. As seen in Fig. 6, $E$ and $F$, these effects extended to a greater or lesser degree across all afferent subtypes examined. Interestingly, the AP parameters of nociceptors, including A-HTMRs, tended to exhibit greater temperature dependency than did LTMRs; in some, $>200\%$ change in $D_{50}$ was observed between 30 and 37°C. The greatest impact was seen on the repolarization phase of A-HTMR spikes. Indeed, although less pronounced than in population studies at lower temperatures (Fig. 5), an inflection appeared de novo on the falling limb of A-HTMR spikes that previously lacked any sign of one at 37°C, clearly evident in comparisons of spike first derivatives in Fig. 6C. Although these findings are confined to a small subset of our total population and are therefore viewed as preliminary, this greater temperature dependency of A-HTMR spike properties likely accounts for the lack of robust separation in spike width between A-HTMRs and LTMRs in studies at 37°C. Additional changes in overall AP shape were also seen; for example, notable changes in AHP shape (e.g., Fig. 6B) were observed in many afferents including A-HTMRs; however, such changes were inconsistent within otherwise homogeneous functional groups and thus highly unpredictable. Importantly, APs were not simply “taller/fatter” versions at lower temperatures, but often assumed appreciably different appearances.

One potential explanation for these effects of temperature on active properties is a more fundamental effect on passive membrane properties. As exemplified in Fig. 7, variations in temperature had a profound effect on the somal input resistance (Ri; and membrane time constant, not shown) of individual cutaneous afferents. Temperature-dependent changes in electrode resistance (recorded extracellularly) constituted only a small fraction (i.e., $<5\%$ with 150-MΩ electrodes) of observed changes in passive membrane properties. As with active properties (above), temperature effects on passive properties were greater overall on nociceptors than on LTMRs (Fig. 7C). Combined with dynamic effects on AP shape (above), these findings suggest that temperature likely exerts differential effects on the constellations of ion channels expressed across these different sensory neuron populations.

Noncutaneous afferents

Although excluded from analyses, multiple noncutaneous sensory neurons were encountered at these segmental levels; because of the lack of in vivo recordings from thoracic DRGs in mice, the paucity of similar recordings in other species, and the critical relevance of some to identification of cutaneous afferents (above), the most commonly encountered are described here.

One relatively common group ($n = 15$) exhibited a distinctive bursting discharge that would continue for hours at fairly regular intervals. As seen in Fig. 8A, somal APs were indistinguishable from unmyelinated afferents (cf. Fig. 2) and bursts contained six to 12 APs. Their peripheral targets are presumably visceral, although all stimuli to date, including vigorous manipulations of abdominal viscera through the body wall, have failed to impact activity levels.
Another relatively common population innervated skeletal muscle stretch receptors. As seen in Fig. 8B, somal APs were similar to cutaneous LTMRs (cf. Fig. 2). Within this group, two distinct types were observed. Most were readily identifiable as spindle afferents, with ongoing discharge that was tightly correlated with ventilation, or stimulus-evoked discharge that could be reproducibly modulated by alternating pressure to the flank (not shown); in either case, RFs failed to track with skin and remained fixed with deeper tissues.

In other cases, however, these neurons were essentially impossible to distinguish a priori from cutaneous afferents. For example, ongoing activity was lacking and RFs tracked with displaced skin and also responded to skin stretch. Moreover, their small (≈1 mm²), spotlike RFs exhibited relatively low mechanical thresholds (0.74 mN) and minor suprathreshold force gave rise to a highly regular, SA Type II–like response (Fig. 8B). In addition, their terminals could be excited with low-intensity electrical stimuli from the skin surface unlike other deep afferents. In fact, that they were not cutaneous afferents per se was revealed only after the recovery of labeled central terminals (n = 3). As seen in Fig. 8B, they exhibited the classic central morphology of Ia afferents (Brown 1981; Nakayama et al. 1998). Their peripheral targets are unknown, although the tracking of RFs with skin suggests cutaneous musculature (see latency and CV above; cf. Holstege and Blok 1989).

**DISCUSSION**

The present report describes findings from intracellular studies of cutaneous afferents in adult mice using a novel in vivo spinal cord/DRG preparation. The primary rationale behind the development of this preparation was to begin to assess the extent to which findings from ex vivo mouse somatosensory system preparations (Albers et al. 2006; Woodbury et al. 2001, 2004) translate to more natural conditions. Our results largely validate such in vitro recordings for most afferent classes; however, we also observed significant consequences to sensory neuron physiology resulting from the reduced temperatures in previous recordings.

**Peripheral response properties of cutaneous afferents**

Using this in vivo preparation we were able to sample a broad spectrum of cutaneous afferents that have been characterized in vitro from the same region of skin, and it is therefore possible to compare findings from similar populations under radically different recording conditions. This is of particular
interest for nociceptors because our previous in vitro studies have reported mechanical thresholds typically well below those in other species (e.g., Beitel and Dubner 1976; Bessou and Perl 1969; Burgess and Perl 1967; LaMotte and Campbell 1978; Leem et al. 1993: Lynn and Carpenter 1982; Lynn et al. 1995; Meyer et al. 1991; Simone and Kajander 1997; Treede et al. 1998).

For most afferent categories, there was substantial agreement between peripheral response properties recorded in vitro and in vivo. For example, mechanical thresholds and response properties of most LTMR groups appear to have been unaffected by prolonged in vitro conditions. The mechanical and heat thresholds of C-polymodal nociceptors also appear to have been unaffected (Albers et al. 2006; Woodbury et al. 2004). The same may also extend to myelinated nociceptors because the range of mechanical thresholds and overall lack of heat sensitivity observed in vitro (Woodbury and Koerber 2003; Woodbury, personal observations) was also observed in vivo (see RESULTS). Therefore these in vivo findings provide general validation for previous in vitro studies of mouse trunk skin afferents and confirm that mechanical thresholds of nociceptors innervating this region are substantially below those innervating thicker glabrous skin in the same species (Cain et al. 2001).

However, the present studies also found differences in both the types of afferents encountered and the response properties of certain populations. Some populations, such as Pacinian corpuscle afferents, were relatively common in the present experiments, likely reflecting the fact that such corpuscles reside predominantly in subcutaneous connective tissues (Kumamoto et al. 1993) that are dissected away in ex vivo preparations (Woodbury et al. 2001); In addition, myelinated MC HTMRs have only recently been confirmed in vitro (Ye and Woodbury, unpublished observations; see also Hunt and McIntyre 1960). Differences in response properties were also evident in some populations. In vivo, C-polymodal nociceptors responded not only to heat, but also routinely to cold stimuli as well as the rewarming phase as temperatures returned to baseline levels. The latter rebound response appears to be novel (Albers et al. 2006; Bessou and Perl 1969; Burgess and Perl 1973; Cain et al. 2001; Koltzenburg et al. 1997; Lynn et al. 1995; Shea and Perl 1985; Simone and Kajander 1996; Woodbury et al. 2004) and warrants additional studies of the underlying mechanisms.

Somal properties of skin sensory neurons at core temperatures

These studies found clear differences in somal membrane properties between functionally identified categories of skin sensory neurons, in accord with previous studies (Djouhri and Lawson 2001; Fang et al. 2005; Koerber et al. 1988; Woodbury and Koerber 2003). Nevertheless, striking differences were observed between these and earlier studies in that all afferent populations exhibited spike durations that were substantially less than reported previously. For example, spike width of C-nociceptors in the present study had a mean D50 of 1.5 ms compared with 2.7 ms for similar afferents recorded from trunk skin in vitro (Albers et al. 2006). Although baseline spike duration was not routinely measured for reasons noted earlier (see METHODS), such measurements in C-nociceptors, to allow comparisons with in vivo studies in other rodent species, also showed substantial reduction; indeed, median baseline durations for such afferents in rats and guinea pigs (~6 ms; Djouhri and Lawson 1999; Fang et al. 2005) were roughly double that (2.8 ms) found in the present study. This general finding of shorter-duration spikes was especially notable in myelinated nociceptors. Unlike other studies to date, the latter lacked any sign of an inflection on the falling phase and tended to cluster with LTMRs, rather than occupying intermediate values between LTMRs and C-nociceptors (Djouhri et al. 1998; Fang et al. 2005; Koerber et al. 1988; Ritter and Mendell 1992; Woodbury and Koerber 2003).

Although recording at normal core temperatures likely accounts for the unexpectedly narrow spikes of myelinated nociceptors (below), sampling bias could also play a role. These afferents exhibit a broad spectrum of peripheral CVs, spike widths, and mechanical thresholds (Burgess and Perl 1967; Djouhri and Lawson 2004; Woodbury and Koerber 2003) and both spike width and mechanical threshold tend to be inversely correlated with CV (Burgess and Perl 1967; Koerber et al. 1988; Light and Perl 1979; Woodbury and Koerber 2003). Given the use of mechanical search stimuli, our sample was likely skewed toward afferents with lower thresholds (Meyer et al. 1991). Nevertheless, most exhibited CVs in the Aδ range and many required highly noxious forces for activation. Importantly, spike widths were narrow throughout, with even the most insensitive and/or slowest conducting A-HTMRs tending to cluster with LTMRs. That is, we found little correlation between spike width and CV or mechanical threshold among myelinated nociceptors in these studies. Future studies of HTMRs with CVs lying close to the A/C-fiber cutoff are warranted; near this boundary, information from nonphysiological sources may be required (Lawson and Waddell 1991; Light and Perl 1979; Sugiura et al. 1986).

Temperature dependency of somal membrane properties

The present studies revealed that recording temperature had profound effects on both active and passive somal membrane properties of sensory neurons. With respect to AP properties, spike amplitude and duration displayed a consistent, inverse correlation with recording temperature across all neurons; at temperatures below core values, spike amplitude and duration were greater, whereas reciprocal effects were observed above core values. Such temperature-dependent “tuning” of somal spike properties was seen to varying degrees across all afferents and may largely reflect changes in input resistance.

As noted earlier, spike widths of myelinated nociceptors were exceptionally narrow at 37°C and tended to approach those of LTMRs. Reducing temperature to match that used in previous in vitro experiments (Woodbury and Koerber 2003; see also Djouhri and Lawson 1999; Fang et al. 2005) resulted in a pronounced shift in duration to longer values. This effect was greatest among nociceptors, leading to complete separation of A-HTMR and LTMR spike widths at lower temperatures. Moreover, an inflection appeared de novo on the falling limb of myelinated nociceptor spikes at reduced temperatures. Such temperature-dependent effects were more pronounced in population studies than serial recordings from single afferents, likely reflecting the fact that temperatures were allowed to stabilize longer in the former compared with the latter (i.e., ≥2 h vs. 5 min, respectively). Regardless, these findings have
important bearing on studies involving analyses of spike shape and kinetics, whether in whole animals or isolated cells, and reveal that the broad, inflected spikes widely used to diagnose A-nociceptors are a product of recording at lower temperatures; such spikes are not representative of this nociceptor class under normal homeothermic conditions in this species.

The only potential somal “fingerprint” of myelinated nociceptors detected at normal core temperatures was a long-duration AHP (reviewed in Hille 2001) and that calcium entry coincides with the “hump” on the repolarizing limb of spikes (Blair and Bean 2002). The presence of broad AHPs in spikes lacking this hump is therefore striking. Moreover, that these AHPs are longer (approximately twofold) than in spikes with humps reveals an elemental difference between A- and C-nociceptors that warrants further study. Techniques enabling cell-specific dissection of TTX-R currents and calcium regulatory mechanisms (e.g., Blair and Bean 2002; see also Coste et al. 2007; Helton et al. 2005; Lu et al. 2006) at normal core temperatures in these distinct neuronal classes may be required to resolve this issue. The differential responses A- and C-nociceptor spikes to changing temperatures (above) may offer a new tool to help identify A-nociceptors in future studies of isolated cells.

The effects of temperature on neuronal kinetics are well established (Hodgkin et al. 1952; reviewed in Hille 2001). High Q10 values of channel gating lead to exponential changes in voltage-dependent conductances and thus tight thermal control in analyses involving membrane properties is essential. Such nonlinear effects, coupled with differential temperature-dependent conductances in many ion channels (e.g., Alloui et al. 2006; Askwith et al. 2001; Kang et al. 2005; Maingret et al. 2006; Fang X, Djouhri L, McMullan S, Berry C, Waxman SG, Okuse K, Lawson SN), suggest that minor variations in temperature may play a substantial role in neuronal function. Indeed, changes in input resistance from thermal modulation of ionic conductances would have greater impact on sensory neuron terminals than somata and thus subtle modulation of these conductances in the skin could lead to profound alterations in neuronal sensitivity at different temperatures (Viana et al. 2002). Ultimately, intact terminals may thus be required in attempts to tease apart mechanisms underlying the contributions of these temperature-dependent effects to the overall functioning of the somatosensory system. Although ex vivo preparations provide a valuable tool for such studies, the present findings highlight a clear need to verify results from reduced preparations using whole animal approaches, and at temperatures that are of physiological relevance to the particular species.

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