Maturation of Cutaneous Sensory Neurons From Normal and NGF-Overexpressing Mice

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Ritter, Amy M., C. Jeffery Woodbury, Kathryn Albers, Brian M. Davis, and H. Richard Koerber. Maturation of cutaneous sensory neurons from normal and NGF-overexpressing mice. J. Neurophysiol. 83: 1722–1732, 2000. In the rodent, cutaneous sensory neurons mature over the first two postnatal weeks, both in terms of their electrical properties and their responses to mechanical stimulation of the skin. To examine the coincidence of these events, intracellular recordings were made from neurons in the dorsal root ganglion (DRG) in an in vitro spinal cord, DRG, and skin preparation from mice between the ages of postnatal day 0 and 5 (P0–P5). We also examined mice in which nerve growth factor (NGF) is overexpressed in the skin. NGF has been shown to be involved in a number of aspects of sensory neuron development and function. Therefore we ask here whether excess target-derived NGF will alter the normal course of development, either of somal membrane properties, physiological response properties, or neuropeptide content. In wild-type mice, somal action potentials (APs) were heterogeneous, with some having simple, uninflected falling phases and some displaying an inflection or break on the falling limb. The proportion of neurons lacking an inflection increased with increasing age, as did mean conduction velocity. A variety of rapidly and slowly adapting responses could be obtained by gently probing the skin; however, due to relatively low thresholds and firing frequencies, as well as lack of mature peripheral receptors such as hairs, it was not possible to place afferents into the same categories as in the adult. No correlation was seen between the presence or absence of an inflection on the somal AP (a marker for high-threshold mechanoreceptors in adult animals) and either peripheral threshold or calcitonin-gene related peptide (CGRP) content. Small differences in the duration and amplitude of the somal AP were seen in the NGF-overexpressing mice that disappeared by P3–P5. Excess target-derived NGF did not alter physiological response properties or the types of neurons containing CGRP. The changes that did occur, including a loss of the normal relationship between AP duration and conduction velocity, and a decrease in mean conduction velocity in the inflected population, might best be explained by an increase in the relative proportions of myelinated nociceptors. Of greatest interest was the finding that in both NGF overexpressers and wild-type mice, the correlation between mechanical threshold and presence or absence of an inflection on the somal spike is not apparent by P5.

INTRODUCTION

The maturation of sensory neurons of the dorsal root ganglion (DRG) takes place over a protracted period that, in the rodent, extends into the first two postnatal weeks (Fitzgerald and Fulton 1992). During this time, the axons of sensory neurons become myelinated, and receptor structures in the skin mature. Responses can be elicited from the skin as early as embryonic day 17 (E17) (Fitzgerald 1987a); however, the firing rates and adaptation properties of the receptors do not fully resemble those of the adult until after postnatal day 14 (P14) (Koltzenburg et al. 1997). The biophysical properties of the neurons also mature over this time. Somal action potential duration becomes shorter over the first two postnatal weeks as conduction velocity increases. As animals mature, more neurons are encountered lacking an inflection or “hump” on the falling limb of the somal spike (Fulton 1987), although uninflected neurons can be recognized as early as E19 (Mirnics and Koerber 1997).

In adult animals, the presence or absence of such a hump is of some significance, because it is a reliable indicator of physiological function for myelinated afferents. Afferents responding to high-intensity, potentially noxious stimulation of the skin [high-threshold mechanoreceptors (HTMRs)] are always found to have an inflected spike, whereas low-threshold mechanoreceptors that respond to hair movement, gentle pressure, or vibration do not (Koerber et al. 1988; Ritter and Mendell 1992; Rose et al. 1986). Among unmyelinated afferents (C-fibers), there is some species variability. In the cat (Traub and Mendell 1988) both high-threshold and low-threshold mechanoreceptive C-fibers have inflected spikes, whereas in the mouse, a class of physiologically unidentified C-fiber has been reported that lacks an obvious inflection, but that possesses a very long afterhyperpolarization (Yoshida and Matsuda 1979; Yoshida et al. 1978).

The development of electrical properties and the maturation of peripheral physiology occur in tandem and may be interrelated. How this process is regulated is not known. However, neurotrophins have been shown to be critically involved in a number of other aspects of sensory neuron development and function, including the regulation of peripheral response properties. This family of related molecules includes nerve growth factor (NGF), neurotrophin-3 (NT-3), brain-derived neurotrophic factor (BDNF), and neurotrophin 4/5 (NT-4/5). They are produced by the targets of sensory neurons and act embryonically to regulate differentiation of sensory neuron precursors and mediate target-dependent survival (reviewed in Davies 1994). NGF, in particular, has been shown to regulate the survival, development, and physiological response properties of nociceptors (Mendell et al. 1999), whereas other neurotrophins appear to regulate the survival and mechanical sensitivity of restricted subclasses of low-threshold mechanoreceptors (Airaksinen et al. 1996; Carroll et al. 1998).
In the adult animal, high affinity receptors for NGF, NT3 (McMahon et al. 1994), and possibly BDNF (Wright and Snider 1995) are confined to largely nonoverlapping subpopulations of sensory neurons, indicating that there may be some specificity with regards to the subclasses of sensory neurons that individual neurotrophins affect. However, in the neonate, the distribution of high-affinity receptors is much more widespread, and individual sensory neurons may respond to more than one neurotrophic factor. For instance, TrkA, the high affinity receptor for NGF, is found on 70–80% of neurons at birth in the rat and declines to its adult distribution on ~40% of neurons over the first three weeks after birth (Bennett et al. 1996; Molliver and Snider 1997).

Given that TrkA is widely distributed in DRG neurons in the neonate, and declines over the period of time that sensory neurons and their targets are maturing, it is possible that NGF is involved in the maturation of sensory neurons. To test this, we examined sensory neurons from mice in which NGF is overexpressed in the skin (Albers et al. 1994). Normally, NGF begins to be expressed around E11 (in whisker pads) (Davies et al. 1987), which is around the time that sensory axons begin innervating the skin. NGF levels fall precipitously around E15, which corresponds to the onset of cell death in lumbar regions (Coggeshall et al. 1994). In this line of NGF overexpressers, the NGF transgene is linked to the K-14 keratin promoter. Transgene expression begins around E11, as endogenous NGF expression is falling, and NGF levels remain high in the skin and other keratinized structures throughout adult life. These mice have hypertrophied sympathetic and sensory ganglia and increased innervation of the skin (Albers et al. 1994; Mendelson et al. 1996) and, behaviorally, are hyperalgesic (Davis et al. 1993). In this study, we have examined early postnatal changes that occur in the physiology of sensory neurons. We have addressed whether the maturation of somal membrane properties is correlated with the maturation of peripheral physiology, and whether these changes occur normally in the presence of excess target-derived NGF.

METHODS

NGF-overexpressing male mice and wild-type female mice were obtained from the colony maintained at the University of Kentucky (Albers et al. 1994) and bred in-house at the University of Pittsburgh animal facilities.

Mouse pups aged P0–P5 were cooled on ice, then perfused through the heart with ice-cold Ringer solution (composition in mM: 127 NaCl, 1.9 KCl, 1.2 KH2PO4, 1.3 MgSO4, 2.4 CaCl2, 26 NaHCO3, and 10 D-glucose). The animal was decapitated, eviscerated, and submerged in a recirculating, oxygenated (95% O2-5% CO2) bath of cold Ringer (17–19°C). A dorsal laminectomy was performed to expose the spinal cord, which was dissected out along with several (3–8) thoracic DRGs, attached dorsal cutaneous nerves (DCNs), and a large block of trunk skin innervated by those nerves. Spinal cord, DRGs, and skin were pinned out in a silicone elastomer (Sylgard)-coated block of trunk skin innervated by those nerves. A dorsal laminectomy was performed to expose the spinal cord, which was dissected out along with several (3–8) thoracic DRGs, attached dorsal cutaneous nerves (DCNs), and a large block of trunk skin innervated by those nerves. Spinal cord, DRGs, and skin were pinned out in a silicone elastomer (Sylgard)-coated block of trunk skin innervated by those nerves. The animal was decapitated, eviscerated, and submerged in a recirculating, oxygenated (95% O2-5% CO2) bath of cold Ringer (17–19°C). A dorsal laminectomy was performed to expose the spinal cord, which was dissected out along with several (3–8) thoracic DRGs, attached dorsal cutaneous nerves (DCNs), and a large block of trunk skin innervated by those nerves. Spinal cord, DRGs, and skin were pinned out in a silicone elastomer (Sylgard)-coated block of trunk skin innervated by those nerves. The animal was decapitated, eviscerated, and submerged in a recirculating, oxygenated (95% O2-5% CO2) bath of cold Ringer (17–19°C).

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Intracellular recordings were made from DRG neurons using thin-walled glass electrodes filled with 1 M potassium acetate and 5% neurobiotin with impedances of 80–100 MΩ. When a neuron was impaled that could be driven by stimulation of the DCN, and that had an action potential amplitude of at least 50 mV, the skin was searched with a blunt glass probe for the cell’s receptive field. Receptive field properties were characterized using glass probes and mechanical thresholds determined using calibrated Von Frey hairs. Data were digitized (Neuro Data Neuro-Corder model DR-484) and recorded on VHS tape for off-line analysis. For each neuron, latencies to DCN stimulation, resting membrane potential, and input resistance were measured. In addition, single sweeps were used to measure the other action potential parameters illustrated in Fig. 1A.

A single cell per ganglion was labeled with neurobiotin (75% duty cycle, 2–4 nA for 2–10 min). At the end of the experiment, DRGs were removed and fixed in 4% paraformaldehyde for 20 min, then transferred to 30% sucrose in 0.1 M phosphate buffer. Single, serial 20- to 25-μm sections were collected in small wells. Neurobiotin (Vector) was visualized using FITC-conjugated avidin (Vector, incubation of 1–2 h), and calcitonin-gene related peptide (CGRP) staining in the same sections was performed using rabbit anti-CGRP antiserum (Chemicon, overnight at 4°C). CGRP was then visualized using Cy3-conjugated goat anti-rabbit secondary. After final washes, sections were mounted and fluorescence was viewed using a Leica confocal microscope under 100 oil. Eight optical sections were obtained through a depth of 5 μm for each fluorophore. Tissue was included in the analysis only if the following two criteria were met: only a single, brightly labeled neurobiotin-positive cell was found in the ganglion, and the quality of the CGRP staining was optimal.

FIG. 1. A: schematic illustrating the different parameters that were measured for each spike: 1, latency; 2, resting membrane potential; 3, amplitude; 4, rise time; 5, baseline duration; 6, afterhyperpolarization (AHP) duration at half-amplitude; 7, AHP amplitude; 8, overshoot. B: uninflected spike from a postnatal day 1 (P1) wild-type animal. C: spike from a P1 nerve growth factor (NGF)-overexpresser with a small inflection. D: spike from a P2 wild-type animal with a much more obvious inflection. Vm, voltage; dV/dt, derivative of membrane voltage. Calibration for B–D is 10 mV, 5 ms.
RESULTS

Intracellular recordings were made from 72 neurons in 26 wild-type mice, and 108 neurons from 10 NGF-overexpressing mouse pups. Of the 26 wild-type mice, 21 were control littermates of the transgenics, but 5 were control mice of a different strain (Swiss Webster, Hilltop). No differences were found between the cellular properties of DRG neurons in the two wild-type groups, so the results from the two strains were pooled. Within the control and NGF-overexpressing groups, data were subdivided according to age: neurons from mice aged P0–P2, and neurons from mice aged P3–P5. Within the two age groups, data were further subdivided into neurons lacking an inflection on the falling limb of the action potential (Fig. 1B), and those with an inflection (Fig. 1D). This was determined by examining the derivative of the spike (dV/dt, Fig. 1A). A truly “broad” inflected spike, such as the one in Fig. 1D, would typically have a bend of nearly 90° in the negative-going portion of the derivative, which corresponds to the change in velocity giving rise to the “hump” in the falling limb of the spike. An uninflected spike with fast kinetics such as that in Fig. 1B would have no angle at all (no change in velocity) in the negative-going part of the derivative. In some spikes, the inflection was not obvious to the naked eye, but could be detected by examining the derivative, in which a change in velocity was apparent (Fig. 1C).

A graph of conduction velocity versus baseline duration for the entire sample from wild-type and NGF-overexpressing animals is presented in Fig. 2. For the wild-type animals (Fig. 2A), the distribution of data points is much the same as has been reported for DRG neurons of adult cat and rat (Harper and Lawson 1985; Koerber et al. 1988; Ritter and Mendell 1992; Villière and McLachlan 1996). An inverse relationship between conduction velocity and baseline duration exists, such that neurons with slower conduction velocities have, on average, longer duration action potentials than those with faster conduction velocities. As might be expected given the incomplete state of myelination at these ages, the range of conduction velocities sampled was much compressed compared with adults. Only a few neurons were sampled conducting over 1 m/s, and most of these were found in the P3–P5 age group. The graph illustrates another feature apparent in adults: the baseline duration of uninflected neurons is generally shorter than inflected neurons for any given conduction velocity. Figure 2A also illustrates several differences between the two age groups. As has been reported, baseline duration decreased as age increased, and conduction velocity increased (Fulton 1987). Overall, spikes became narrower and faster with increasing age.

In contrast, the plot of baseline duration versus conduction velocity for somal spikes from NGF overexpressers (Fig. 2B) did not display the clear relationship between these two parameters as in controls. All of the values for baseline duration...
clustered within a narrow range of conduction velocities. In Fig. 2, C and D, only the data from animals aged P3–P5 is plotted against conduction velocity, and regression lines were calculated separately for inflected and uninflected neurons. In the wild-type animals, a significant correlation was seen between conduction velocity and baseline duration for both types of cell (P < 0.01, for both). In the NGF overexpressers, a significant correlation existed for the inflected neurons (P < 0.01), but not for uninflected neurons (P > 0.5). Uninflected neurons in the NGF overexpressers displayed a wide range of baseline durations that did not correlate well with conduction velocity.

The compression in conduction velocity range is illustrated again in Fig. 3, where the means ± SE of nine different parameters are broken down by age and presence or absence of inflection for the two groups. A comparison of mean conduction velocity for the different groups (Fig. 3A) indicates that the inflected neurons sampled in the NGF overexpressers had, overall, slower conduction velocities than those in wild-type animals, which contributes to the clustered appearance of the graph in Fig. 2B. This trend was also apparent in the uninflected population but did not reach statistical significance. Also of interest is the skewing in the sampling at P3–P5 between the wild-type animals and NGF overexpressers (Fig. 3B). At P0–P2, in both groups, the overwhelming majority of neurons sampled were inflected, with uninflected neurons making up ~10% of the sample. By P3–P5, uninflected neurons had increased to ~40% of the sample in wild-type animals. However, uninflected neurons were still scarce in NGF overexpressers, remaining at 12%.

Surprisingly, very few consistent differences were noted between the electrical properties of neurons from wild-type and NGF overexpressing mice. The spikes of inflected neurons from P0–P2 NGF-overexpressing animals were significantly

![Graphs of various parameters](http://jn.physiology.org/)

**FIG. 3.** Summary of spike properties of dorsal root ganglion (DRG) neurons broken down by age (P0–P2 or P3–P5) and by presence or absence of an inflection in wild-type and NGF-overexpressing mice. +, spikes with inflections; −, those without. Asterisks indicate where values in controls and NGF-overexpressers were significantly different, P < 0.01, χ² test for B, 2-tailed Student’s t-test for all others. Because there was only a single data point for wild-type uninflected spikes at P0–P2, that bar has been omitted from the graphs.
shorter in duration than their wild-type counterparts (Fig. 3C), and this difference arose from a faster rising portion of the spike (Fig. 3D). The longer duration in the wild-type group might be attributed to more neurons exhibiting an inflection in the rising limb of the spike often seen at this age (Fulton 1987) (see also Fig. 6); however, this was not the case. Similar numbers of neurons in control and NGF overexpressers displayed such an inflection (50% of the wild-type P0–P2 neurons, as compared with 44% in the NGF overexpressers at P0–P2). Concomitant with the faster rise time, spikes from P0–P2 NGF-overexpressing animals also had slightly larger amplitude (Fig. 3E), and more of an overshoot (Fig. 3F). Resting membrane potential was elevated in inflected neurons sampled at P3–P5 in the NGF overexpressers (Fig. 3I), as was spike overshoot (Fig. 3F). One might have expected spike amplitude to be larger in this group as well, but this did not reach statistical significance (P = 0.06). No differences were seen in the length of the afterhyperpolarization (AHP; Fig. 3G) or in input resistance (Fig. 3H) between wild-types and NGF-overexpressers. The large standard error in the value for AHP duration in the P3–P5, NGF-overexpressing uninflected population was due to a single neuron with an extraordinarily long AHP, that may have been a type A neuron described by Yoshida et al. (1978).

Immunostaining

In NGF-overexpressing mice, it has been reported that, whereas the total number of trigeminal ganglion neurons increases twofold, there is a disproportionate (5-fold) increase in the number of neurons expressing TrkA (Goodness et al. 1997). Normally, TrkA is highly (>90%) co-localized with CGRP (Averill et al. 1995). As might be expected, the numbers of CGRP-immunoreactive DRG neurons are increased in NGF-overexpressers (Davis, unpublished observations), and the density of CGRP-immunoreactive fibers increases dramatically in the skin (Albers et al. 1994) and spinal cord (Mendelson et al. 1996). The increase in the numbers of TrkA and CGRP positive neurons and the increase in the density of CGRP immunoreactive fibers may result from the rescue during development of neurons that normally express CGRP/Trk A. Alternatively, NGF overexpression may induce de novo expression of CGRP in populations of neurons that normally do not express it. To distinguish between the two possibilities, neurons were labeled with neurobiotin, and their CGRP content was correlated with their somal spike shape. A total of 13 neurobiotin-stained neurons were recovered from wild-type animals, and 17 from NGF overexpressers. Sections from DRGs double labeled for both neurobiotin and CGRP are shown in Fig. 4. As might be expected (Lawson et al. 1996), many neurons with inflections in both types of animals were immunopositive: 71% (5/8) in wild-type animals and 58% (8/13) in NGF overexpressers (Fig. 4, M–P). In addition, in both types of animals, a proportion of uninflected neurons were CGRP-positive. In wild-type animals, 60% (3/5) of uninflected neurons were positive (Fig. 4, A–D), a surprisingly large percentage given that uninflected neurons are associated with low-threshold mechanoreceptors in adults. Only 1/5 (20%) of uninflected neurons in NGF-overexpressers was CGRP positive. The finding that NGF overexpression does not significantly alter the proportions of inflected or uninflected neurons that express CGRP does not support the hypothesis that large numbers of neurons are changing their peptidergic phenotype.

Peripheral physiology

We were able to determine adequate stimulus and thresholds for a total of 42 neurons in NGF overexpressers (22 at P0–P2 and 20 at P3–P5), and 21 neurons in wild-type mice (6 at P0–P2 and 15 at P3–P5). Few units were characterized at P0–P2 in the wild-type mice because these neurons were extremely fragile, and we were unable to hold the penetrations long enough to adequately type most of them. Unlike adult neurons, which generally depolarize as the penetration deteriorates, these neurons would hyperpolarize. As membrane potential became more negative, the somal spike would fail to invade, leaving only the initial segment (IS) spike. As it became more negative still, the IS spike would gradually disappear, and no spike could be evoked even by intracellular injection of current.

By stimulating the skin, both rapidly and slowly adapting units could be identified. Rapidly adapting units had small, discrete receptive fields, but many slowly adapting units would respond with a few spikes when probing gently over a large area of skin (approximately half the size of the nerve territory). These units had one or two small spots where slowly adapting responses could be elicited at lower thresholds, and we presume that responses generated at other areas of the skin were in response to stretch. A few “slowly-rapidly adapting” units were identified, that sustained their discharge for a few seconds, but eventually adapted. Such units have been described in immature animals (Koltzenburg et al. 1997).

Although we were able to separate units into the broad categories of slowly and rapidly adapting, it was difficult to further separate neurons into the classes normally seen in adult animals. Although a few tylotrich hairs have erupted by birth, the majority of the pelage does not fill in by P5 (Payne et al. 1991), and we were never able to associate any of our rapidly adapting units with hair movement. Receptive field typing was especially problematic for slowly adapting units. A few units were encountered with short-duration, uninflected action potentials, that gave a brisk, slowly adapting discharge from the skin and that may have been slowly adapting type I or II units. However, the majority of slowly adapting neurons fired at relatively low frequencies, had inflected spikes, and discharged at higher frequencies to increasing intensity of stimulation. Their mechanical thresholds were too low to call them nociceptors (see Peripheral thresholds) and their frequency of discharge too low to call them SAs. Thus the rather stereotyped responses seen in adults were not evident at these ages.

Peripheral thresholds

The range of mechanical thresholds found in both wild-type and NGF-overexpressers at these ages was much lower than values published for adult animals. The highest threshold value at P0–P2, 2 mN, was within the range seen for low-threshold mechanoreceptors from an in vitro preparation of adult mouse skin (Koltzenburg et al. 1997), and even at P3–P5 thresholds were well below the range at which HTMRs from adult rats in
vivo respond (5–25 mN: Lynn and Carpenter 1982; 14–100 mN: Leem et al. 1993). The cumulative sum distribution of mechanical thresholds is illustrated in Fig. 5. Two points are illustrated by this graph. The first is that at P3–P5, there was no difference in thresholds between wild-type and NGF-overexpressing mice, indicated by the fact that the cumulative sum distributions for the two groups overlap completely. The second is that thresholds in the NGF-overexpressing mice increased with age. We would expect the same to be true in the wild-type mice; however, too few units were characterized in wild-type animals, 2 days old to make the same comparison across age groups.

At these ages, spike shape was not a reliable indicator of mechanical threshold. Figure 6 shows somal spikes and physiological responses from two slowly adapting units recorded from wild-type animals, one from a P3 mouse, and one from a P4 mouse. Mechanical thresholds for both units are low: 0.3 mN for the P3 unit (Fig. 6A), and 1 mN for the P4 unit (Fig. 6B), but the cell with the lower mechanical threshold (Fig. 6A) is the one with the inflected spike. Graphs in which mechanical threshold is plotted against baseline duration at different ages in the two types of mice are shown in Fig. 7. These graphs illustrate that the presence or absence of an inflection per se was not a good predictor of threshold. For any given threshold value, our sample contained neurons with inflected spikes and those without. The mean mechanical threshold for inflected

**FIG. 4.** Examples of identified neurons double-labeled for calcitonin-gene related peptide (CGRP). Each row illustrates the somal spike, neurobiotin staining, CGRP staining, and an overlay of neurobiotin and CGRP for 1 neuron. In both wild-type and NGF overexpressing animals, neurons with inflected spikes could be CGRP positive or negative, as could neurons with uninflected spikes. Calibration for traces of somal spikes are 20 mV, 5 ms, and for photomicrographs 10 μm.
spikes in wild-type animals was 1.28 ± 0.69 (SD) mN, and in NGF overexpressers, 1.93 ± 1.94 mN. For uninflected neurons, mean threshold in wild-type animals was 2.76 ± 3.26 mN, and in NGF overexpressers 1.66 ± 0.61 mN. There were no significant differences between mean thresholds of inflected and uninflected neurons in either set of animals (\(P > 0.05\), Mann-Whitney \(U\) test), nor were there significant differences between thresholds from wild-type animals and NGF-overexpressers.

**DISCUSSION**

**Normal development**

Results obtained here confirm and extend earlier findings on the postnatal maturation of somal membrane properties of DRG neurons (Fulton 1987). As animals increased in age, the average duration of the somal spikes of DRG neurons became shorter, conduction velocities increased, and there was an increase in the number of neurons sampled that lacked an inflection on the falling limb of the spike.

Peripheral response properties also matured over the first few postnatal days. Thresholds for activation from the skin started off low and increased between \(P0–P2\) and \(P3–P5\). However, even at \(P3–P5\), most thresholds were clearly within a range that would be considered innocuous, and, moreover, the mechanical thresholds for neurons with inflected spikes and those with uninflected spikes were indistinguishable. This is in contrast to the case in the adult, where there are clear differences in peripheral threshold between myelinated afferents with inflected action potentials, and those with uninflected ones (Koerber et al. 1988; Ritter and Mendell 1992; Rose et al. 1986). Thus the functional classifications of “HTMR” and “LTMR” are not distinguishable at very early ages, because nearly all sensory neurons are low threshold, irrespective of somal spike shape.

Neonatal animals are hyperalgesic and hyperreflexic compared with adults (Fitzgerald et al. 1988; Holmburg and Schouenborg 1996). It has been speculated that the hyperalgesia results from transient inappropriate synapses made by low-threshold mechanoreceptors within areas of the spinal cord normally innervated by C-fiber nociceptors (Fitzgerald et al. 1994). However, given that putative HTMRs are not particularly high threshold at these ages, no more so than putative LTMRs, it seems that the hypersensitivity of the flexion reflex may be due in part to a low threshold for activation at the periphery. A caveat of this hypothesis is that we have no way of knowing how many of the inflected neurons in our sample will remain unmyelinated and be classified in the adult as C-fibers. However, it is known that myelinated nociceptors as well as C-fibers contribute to the flexion reflex, and under

**FIG. 5.** Cumulative sum distributions for threshold values for neurons at different age groups in wild-type and NGF-overexpressing mice. Values for wild-type mice aged \(P0–P2\) were not plotted, because there were only 6 data points (see Fig. 7).

**FIG. 6.** Physiological response properties from 2 neurons, from a \(P3\) wild-type animal (A) and one from a \(P4\) wild-type animal (B). For each cell, the somal spike along with its derivative is shown, and a slowly adapting response elicited by a supra-threshold stimulus. Arrow beneath the trace in A denotes the break, or inflection, in the falling phase visible in the differentiated record. Vertical calibration for all traces is 10 mV.

A P3 Wild Type, Threshold 0.3 mN

B P4 Wild Type, Threshold 1 mN
conditions of spinalization it is even possible to unmask weak inputs from LTMRs (Weng and Schouenborg 1998).

The stereotyped physiological response properties of cutaneous mechanoreceptors in adult animals were not evident in the neonate. In part, this may be due to the immature state of the somal spike: because their spikes are, as a population, longer in duration than they will be when fully mature, they may not be able to sustain the high firing frequencies found in the adult. Another contributing factor is likely to be the immature state of the peripheral end-organs within the skin. For instance, although hair follicles are present in the skin at this time, the hairs themselves do not erupt until after P3 (Payne et al. 1991). Although it is likely that some of our rapidly adapting afferents were hair follicle afferents being stimulated by pressure, without the adequate stimulus of hair movement, it is impossible to definitively type them as such. Likewise, we sampled many slowly adapting units: because most fired at relatively low frequencies, we could not definitively type them as SAI or SAII, and because they all had relatively low thresholds, we could not definitively type them as mechanonociceptors. Despite our inability to find many units that were convincingly “nociceptive” in the neonates, we are able to find such units in an identical preparation taken from animals aged P14 and older, indicating that the low thresholds are probably not an artifact of being maintained in vitro (Woodbury, unpublished observations). Instead, they are probably related to the immature state of the integument at these ages.

Our findings are in contrast to a report by Fitzgerald (1987b) in which it was stated that mechanical thresholds for HTMRs and LTMRs were “normal” in the neonate. The two studies differ in methodology: the Fitzgerald study was done in vivo and examined hindlimb afferents, whereas the current study was done in vitro, and we recorded from thoracic afferents. However, the author describes a class of pressure receptors that are common in very young neonates (comparable to the ages studied here), but scarce by P14. Some of the pressure receptors described by Fitzgerald probably correspond to the slowly adapting units described in this report. It was suggested that the thresholds of pressure receptors will fall as they mature into LTMRs, but based on our findings that thresholds increase with age, we suggest that thresholds of some afferents will rise as they mature into HTMRs. The two hypotheses are not mutually exclusive: examination of the central projections of some of our slowly adapting afferents that had been stained with neurobiotin indicates that they are a mixed population, some having collaterals confined to laminae I and II, with others projecting into III and IV (Woodbury et al., in preparation; Ritter et al. 1998). Thus at very early postnatal ages there appears to be a population of immature afferents that are homogeneous in terms of spike shape, threshold, and periph-
eral response properties, but heterogeneous in terms of eventual phenotype.

NGF overexpressers

Somal membrane properties of sensory neurons in neonatal mice overexpressing NGF in the skin differed consistently from those of wild type animals only at the earliest time points (P0–P2). At these ages, neurons in NGF overexpressers had spikes with faster rise times, shorter durations, and larger amplitudes. These differences may be a result of slightly larger neurons as a result of the excess NGF, and so less damage incurred as a result of the penetrations. There may also be a real difference in the ionic basis for the rising portion of the spike, primarily carried by sodium current, between wild-type mice and NGF-overexpressers that disappears as animals mature. Multiple isoforms of the sodium channel α-subunit have been cloned and identified (reviewed in Black and Waxman 1996), and the pattern of expression of these isoforms changes during development (Felts et al. 1997). The type III isoform is expressed in DRG neurons at E17 but not in the adult and reappears after axotomy (Waxman et al. 1994). Its expression in cultured, adult DRG neurons can be suppressed by addition of NGF (Black et al. 1997). Perhaps the excess NGF causes a change in the time course over which this channel subunit is replaced by other forms, and speeds the maturation of the sodium current in these neurons. After a few days, currents in neurons from wild-type animals would “catch up,” and so the differences in somal spike shape would disappear.

NGF overexpressers were found to have an increased proportion of neurons with inflected spikes at P3–P5 relative to controls. One possibility is that excess NGF caused most neurons to retain their inflection, and indeed there is evidence that NGF regulates currents in the inflected population of DRG neurons. The inflected action potentials in DRG neurons are resistant to tetrodotoxin (TTX) (Morita and Kateyama 1989; Ritter and Mendell 1992; Villière and McLachlan 1996; Wadell and Lawson 1990; Yoshida et al. 1978), which is due largely to a TTX-resistant sodium current with slow kinetics (Kostyuk et al. 1981; Roy and Narahashi 1992) that has been identified in this subpopulation of DRG neurons (Oyelose et al. 1997; Rizzo et al. 1994). Neurons with inflected spikes also express the SNS sodium channel α-subunit (Dib-Hajj et al. 1996), thought to underly a TTX-resistant current (Akopian et al. 1996; Sangameswaran et al. 1996). Addition of exogenous NGF to a cut nerve stump partially rescues α-SNS expression, TTX-resistant currents, and inflections in large cutaneous and small, presumably unmyelinated sensory neurons after axotomy (Dib-Hajj et al. 1998; Oyelose et al. 1997). If NGF is capable of up-regulating this current in nonaxotomized DRG neurons, this may explain the increased incidence of neurons with inflected somal spikes.

Another, more parsimonious, explanation is that the increase in the proportion of inflected neurons can be accounted for by differential effects on the proliferation and/or survival of different subpopulations of DRG neurons promoted by the elevated levels of NGF. In this line of mice, the total number of trigeminal ganglion neurons is increased twofold, but those that express TrkA are increased fivefold (Goodness et al. 1997). Also, as adults, these mice have been shown to have an increase in the number of myelinated nociceptors (Stucky et al. 1997). Therefore it is likely that the increase in the percentage of inflected neurons observed in the overexpressers reflects an increase in the percentage of myelinated nociceptors rather than the maintenance of a “hump” by neurons that ordinarily lack one.

Proliferation of myelinated nociceptors would also explain the lack of correlation between baseline duration and conduction velocity that is apparent in the sample as a whole. The inverse correlation between these two parameters is extremely robust and has been reported in every species examined: cat (Cameron et al. 1986; Koerber et al. 1988; Rose et al. 1986), rat (Harper and Lawson 1985; Mirnics and Koerber 1997; Ritter and Mendell 1992; Villière and McLachlan 1996; Wadell and Lawson 1990), and pigeon (Gorke and Pierau 1980). Its absence here is striking. Yet, if we were sampling many neurons from a very homogeneous population (myelinated nociceptors) in which all the units had very similar conduction velocities, this would create such a graph. In addition, overrepresentation of this group in our sample at the expense of the faster-conducting units might explain the decrease in mean conduction velocity in the P3–P5 inflected group.

No clear-cut differences were found between wild-type and NGF-overexpressing mice in the types of neurons that contained CGRP immunoreactivity. In adult rats, CGRP expression is not confined to a discreet physiological or morphological class of DRG cell. It is found in a small percentage of fast, uninflected neurons, in a subset of myelinated, inflected neurons with comparatively short afterhyperpolarizations (Lawson et al. 1996), as well as in a subset of C-fibers (Lawson 1995). In both wild-type and NGF overexpressers, there were some inflected and some uninflected neurons that were CGRP positive. In the overexpressers, many more inflected than uninflected neurons were double labeled (58% inflected, 20% uninflected), which is in good agreement with percentages found in myelinated afferents from adult rat (Lawson et al. 1996). In wild-type animals, we found an unusually high percentage (60%) of uninflected neurons that were immunonegative, but this represents a very small sample (3/5). If excess NGF caused a conversion of neurons that do not normally express CGRP, we might have expected proportionally more uninflected neurons in the NGF overexpressers to be CGRP immunoreactive, but this was not the case. Of methodological concern is the possibility that the presence of neurobiotin in the cell body may have obscured the presence of CGRP immunoreactivity in some cases. This would result in false negatives and an underestimation of the percentage of double labeled neurons. We have no reason to believe that there were systematic differences in the amount of neurobiotin injected into neurons in experiments done on wild-type and those done on overexpressers that would lead to more false negatives in one group than the other.

Conclusions

Over the first few postnatal days, as somal membrane properties of DRG neurons mature, the maturation of peripheral response properties lags behind. In adults, HTMRs and LTMRs are clearly distinguishable on the basis of spike shape and threshold. In contrast, in neonatal animals up to at least 5 days of age, inflected and uninflected neurons do not separate out into physiologically distinguishable groups. Overexpression of
NGF does not substantially interfere with or accelerate the maturation of electrical properties, peptide expression, or peripheral response properties (i.e., elevation of peripheral thresholds); however, changes in the relative proportions of different types of DRG neurons caused by the excess of NGF obscures the relationship between peripheral fiber conduction velocity and the baseline duration of the somal spike normally exhibited by DRG cells.

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