Receptive Properties of Embryonic Chick Sensory Neurons Innervating Skin

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Koltzenburg, Martin and Gary R. Lewin. Receptive properties of embryonic chick sensory neurons innervating skin. *J. Neurophysiol.* 78: 2560–2568, 1997. We describe a new in vitro skin-nerve preparation from chick embryos that allows detailed study of the functional properties of developing sensory neurons innervating skin. Functionally single sensory afferents were isolated by recording from their axons in microdissected filaments of the cutaneous femoralis medialis nerve, which innervates skin of the thigh. A total of 157 single neurons were characterized from embryos [embryonic *days 17–21* (*E17–E21*), $n = 115$] and hatchlings up to 3 wk old ($n = 42$). Neurons were initially classified on the basis of their conduction velocity; those conducting below 1.0 m/s were being classified as C fibers and faster conducting fibers as A fibers. The proportions of A and C fibers encountered in embryonic and hatchling preparations were not very different, indicating that myelination and axon growth proceeds quite slowly over the period studied. Afferent fibers that could subserve nociceptive and nonnociceptive functions were identified in the time period studied. Subpopulations of low-threshold myelinated afferent units exhibited rapidly or slowly adapting discharges to constant force stimuli and could have tactile functions. Many afferent fibers responded to noxious heat and were excited and sensitized by exposure to inflammatory mediators, suggesting that they are nociceptors. The behavior of these units changed in several respects over the period studied. The discharge of C fibers to noxious heat increased with age as did their mechanical thresholds. A substantial population of heat-responsive neurons (34% of the A fibers) present in embryos were not encountered in hatchling chicks. This indicates that substantial changes in the physiological response properties of sensory afferents occur after hatching. We conclude that this new preparation can be used for quantitative assessment of the receptive properties of developing sensory neurons and has considerable potential for the investigation of factors, such as neurotrophins, that specify and influence the functional phenotype of sensory neurons during embryonic development in vivo.

INTRODUCTION

Over the past 30 years the physiological properties of sensory neurons innervating skin in mammals has been extensively investigated, showing that primary afferents can be classified on the basis of their responses to adequate mechanical, thermal, or chemical stimuli (Meyer et al. 1994; Perl 1992; Perl and Burgess 1973). These studies have demonstrated that the receptive-field properties of afferent neurons are highly correlated with their neurochemical, biophysical, and neuroanatomic characteristics (Brown 1981; Koerber and Mendell 1992; Lawson 1992; Smith and Frank 1988). The remarkable specificity of their central connections is probably best exemplified by the fact that selective electrical microstimulation of single cutaneous afferents in humans results in elementary sensations that are specific for each subgroup of afferents (Torebjörk et al. 1987). Thus the way that these receptive-field properties develop in relation to other aspects of the sensory neuron phenotype during embryogenesis is therefore of general neurobiological interest.

Only few studies have investigated the physiological properties of developing cutaneous afferents in rats and cats. Myelinated sensory neurons innervating the footpad of neonatal kittens exhibit a profound increase in sensitivity in the first weeks of life (Ferrington et al. 1984; Ferrington and Rowe 1980). Fitzgerald (1987a,b) recorded primary afferent activity at even earlier stages of development in fetal and neonatal rat pups, both before and during myelination of sensory fibers. The main conclusion of these studies was that the majority of primary afferent types in adult animals could already be distinguished at early developmental stages. Because these early experiments were performed in vivo in very small animals, the stimuli used were necessarily qualitative.

In the present investigation we have adopted an in vitro skin-nerve preparation technique previously developed in the rat (Reeh 1986) to record from both myelinated and nonmyelinated fibers of a purely cutaneous nerve in the chick embryos as young as embryonic *day 17* (*E17*) (Hamburger and Hamilton stage 43, HH 43; hatching takes place on *day 21*) (Hamburger and Hamilton 1951). Besides the clear practical advantages of stable recording conditions and the ability to apply a wide range of controlled quantitative stimuli, this chick preparation is attractive for several other reasons. Because the chick embryo is easy to manipulate early in development, it has been extensively used by embryologists (Hamburger 1980; Le Dourain 1982). For example, much evidence regarding the neurotrophic factor requirements of sensory neurons was first discovered using the chick model (Davies 1994; Levi-Montalcini and Hamburger 1951; Lewin and Barde 1996), and there is an extensive literature describing the biophysical properties of cultured embryonic chick dorsal root ganglion neurons (Gottmann et al. 1988; Nowicki 1992). The few isolated reports describing the physiological properties of primary afferents in the adult chicken indicate that they largely resemble those in mammals (Gentle 1989). The present paper is the first description of the functional response properties of sensory neurons in the developing chick. These results will allow us to eventually relate the differentiation of primary

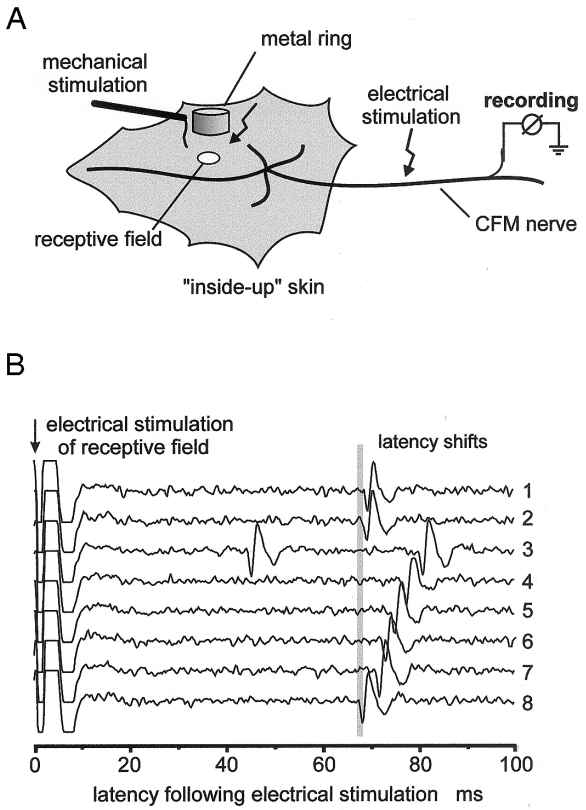

FIG. 1. *A*: schematic drawing of the preparation. CFM nerve, cutaneous femoralis medialis nerve. *B*: series of electrical stimulation (interstimulus interval 3 s) to determine the conduction velocity of single units (sweep 1–2) and ''marking'' procedure to ascertain identity of electrically and naturally evoked unitary potentials. This was accomplished by demonstrating a characteristic latency shift of the electrically evoked action potentials produced by an interspersed mechanical activation (sweep 3) of the unit that gradually recovered (sweep 4–8).

afferent receptive properties to their biophysical, neuroanatomic, and neurochemical phenotype. Thus one can investigate in more detail those factors that govern normal development of primary afferent phenotypes.

METHODS

Skin-nerve preparation

The chick skin-nerve in vitro preparation (Fig. 1) was modified from rat, which has been described in detail previously (Kress et al. 1992; Reeh 1986). In the case of embryonic chicks, a window was first made in the egg above the air sac and the animal decapitated in ovo. Hatchling chicks were first killed by CO_2 inhalation followed by decapitation. After clipping and gentle removal of the feathers, the skin of the hindlimb was carefully dissected together with the attached cutaneous femoralis medialis (CFM) nerve. The nerve was dissected free to the crural plexus to ensure a sufficient length of nerve for recording. The preparation was then placed ''inside-up'' in an organ bath to facilitate oxygenation and drug accessibility through the corium side of the skin (Fig. 1) and superfused (15 ml/min) with an oxygen-saturated modified synthetic interstitial fluid (SIF) solution containing (in mM) 123 NaCl, 3.5 KCl, 0.7 $MgSO_4$, 1.7 NaH_2PO_4, 2.0 $CaCl_2$, 9.5 sodium gluconate, 5.5 glucose, 7.5 sucrose, and 10 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.4 ± 0.05 and temperature of 32 ± 0.5°C.

Recordings

Using sharpened watchmaker forceps, filaments were teased from the desheathed nerve, and functionally single sensory neurons were recorded extracellularly using a low-noise differential amplifier. Receptive fields of primary afferents were identified with a mechanical search stimulus (manual probing of the skin with a glass rod), which is known to activate >90% of the cutaneous afferents in mammals in this preparation (Kress et al. 1992). Only units with a signal-to-noise ratio of more than 3 were used for further analysis. The conduction velocity of each axon was determined by electrically stimulating the receptive field with supramaximal square-wave pulses (duration 0.1–1.0 ms, interstimulus interval 1–5 s) after insertion of a Teflon-coated steel needle electrode with an uninsulated tip diameter of 10 μm. The median conduction distance of units recorded from embryonic preparations was 25 mm (range 12–50 mm) and in hatchling preparations 32 mm (range 17–55 mm). To ascertain the identity of the naturally and electrically evoked activity, a ''marking'' procedure (Fig. 1) was routinely carried out (Kress et al. 1992). The latency of action potentials of unmyelinated and thin myelinated afferents is stable when regularly stimulated with supramaximal electrical stimuli. However, when the same unit is activated by a short natural stimulus, e.g., mechanical probing, in the interval between two electrical stimuli, there is a characteristic latency shift, which recovers gradually over several seconds (Iggo 1958; Schmelz et al. 1995).

Mechanical stimulation

The mechanical threshold of each unit was determined with calibrated von Frey monofilaments with uniform tip diameter of 0.8 mm. The weakest von Frey filament used in this study exerted a bending force of 1 mN because smaller monofilaments were incapable of reliably penetrating the surface tension of the bathing solution. Where possible, each unit was subjected to a standard protocol of stimuli consisting of mechanical, thermal, and chemical stimuli. Constant force stimuli were applied with a feedback-controlled probe with a tip diameter of 0.8 mm placed perpendicularly onto the most sensitive spot of the receptive field. Each stimulus began with an adaptation period of 5 s using a force of 1 mN after the probe had made contact with the skin. In very sensitive units this minimal force could evoke a discharge that in most cases settled within 5 s. Then the force rose within 200 ms to a preset 10-s-long force plateau that varied between 5 and 300 mN after which it returned to the adaptation force of 1 mN for 5 s before the probe was lifted off the tissue. In pilot experiments we determined that this stimulus configuration was suitable for clearly differentiating the adaptation properties of the afferents. Stimuli were delivered in ascending order every minute. Longer interstimulus intervals might have been advantageous to completely avoid interstimulus interaction. However, in initial detailed recordings we determined that desensitization was most noticeable when a high force stimulus was followed by a very low force stimulus and that most of this interference could be observed within 30 s. Thus the protocol used in the present study was a practical compromise between relatively little interstimulus interaction and a desirable speed of data acquisition.

Thermal and chemical stimulation

At the end of the series of mechanical stimuli, the neurons' receptive field and its surroundings were isolated with a self-sealing metal ring (6–12 mm diam), the fluid within the ring was manually removed with a syringe, and a thermocouple was gently applied to measure intracutaneous temperature. Cold stimuli were delivered by giving a 10-ml bolus injection of ice-cold SIF solution that resulted in a temperature nadir of 4–6°C, after which the tempera-

ture returned passively to baseline within 1–2 min. Care was taken not to apply the force of the injection stream directly to the receptive field to avoid a nonspecific mechanical activation of very sensitive units. A true cold discharge was only scored when the unit discharged during the drop of temperature until the nadir was reached and control injections of fluid at 32°C did not evoke this discharge. When temperature had returned to baseline, a standard heat stimulus was delivered from the epidermal side of the preparation through the translucent bottom of the organ bath. A halogen bulb was focused onto the receptive field, and temperature rose linearly within 15 s from 32 to 47°C, thus giving a rate of 1°C/s. Where possible, a chemical stimulation of the receptive field was performed. Thus, 75 s after the end of the heat ramp, the receptive field was superfused with a mixture of inflammatory mediators containing 10 μM bradykinin, serotonin, histamine, and prostaglandin E_2 at 32°C for 180 s using a roller pump. The flow of the pump was adjusted to 3 ml/min to ensure rapid fluid exchange and replenishment of the mediators. A second heat stimulus was delivered 30 s after the end of the chemical stimulation. Thus the time interval between heat stimuli was 5 min, which in rats results in no significant change of an average heat response in a population of heat-sensitive nociceptors (Koltzenburg et al. 1992; Lang et al. 1990).

Data analysis and statistical tests

All data were collected with custom-made data acquisition software running on a PC, and action potentials were subsequently analyzed with a template matching program (Forster and Handwerker 1990). Further quantitative analysis of the recorded neural responses was carried out with custom-designed software. For construction of the stimulus response functions for mechanical stimulation, all action potentials were counted that were recorded in a time period of 11 s after the onset of a force stimulus. This time window was chosen because it contained all spikes during the rise time, the plateau, and the discharge during the release of the stimulus. Stimulus response functions for heat responses were calculated by averaging the discharges of all fibers tested and calculating a linear regression of the running average of this histogram for the time of the temperature increase (Koltzenburg et al. 1992). A unit was scored as chemically sensitive when the average discharge exceeded 0.1 impulses/s for the duration of the superfusion.

All values are given as means ± SE and interquartile range between the 25th and 75th percentile. Appropriate statistical tests were applied after fulfillment of necessary prerequisites (Sachs 1978) using the Statistica software package by Statsoft. Where *P* values are stated, they are always based on two-tailed distributions.

RESULTS

Conduction velocities

A total of 13 chicken embryos (between *E17* and *E21*) and 7 hatchling chicks between 4 and 21 days posthatching were used in this study. Measurement of compound action potentials from the whole nerve was used to estimate the conduction velocity range of axons within the CFM nerve, and a representative example of such record from an embryonic preparation is shown in Fig. 2. The slowest compound potential began between 0.6 and 0.8 m/s and generally had the largest amplitude. The conduction velocity of the fastest potential started at 5–6 m/s and was usually small in amplitude. A third compound was often present with intermediate values for both conduction velocity and amplitude. Since <5% of the axons in cutaneous nerves of the chick are myelinated before hatching (Saxod and Bouvet 1982), the large late component probably represents unmyelinated fibers.

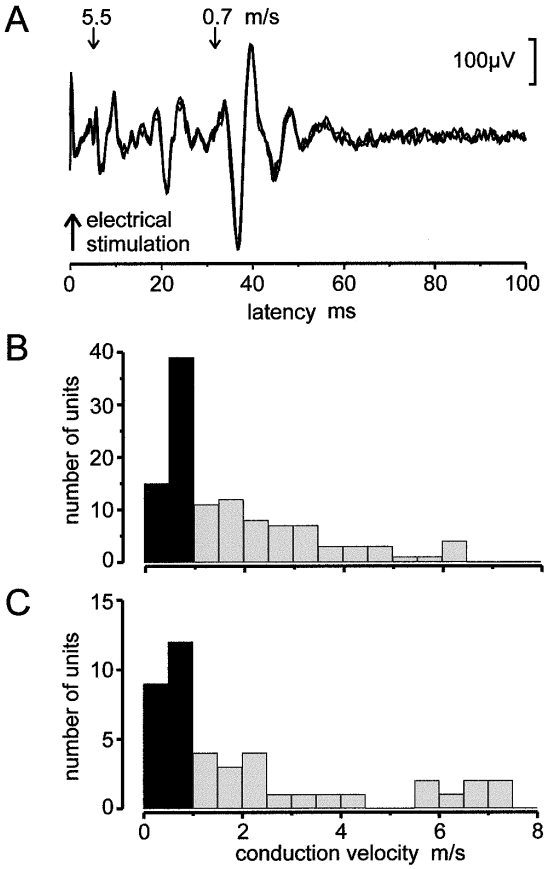

FIG. 2. *A*: several times superimposed sweep of the compound action potential after supramaximal electrical stimulation of the CFM nerve. The largest and slowest conducting peak represents afferents conducting slower than 0.7 m/s (indicated with an arrow). The fastest but smallest compound action potential representing A-fiber afferents conducted at a maximal velocity of 5.5 m/s. Distributions of the conduction velocities of single units recorded from embryonic (*E17–E21; B*) and posthatching (4–21 days; *C*) chick. Fibers conducting at <1.0 m/s were considered to be unmyelinated and shown in black. Note that in the older animals the fastest afferents increase their conduction velocities slightly, but the overall distribution is very similar.

The conduction velocities of the single units recorded fell well into the cutoff values as defined by the compound action potentials (Fig. 2). As demonstrated in the developing rat, cutoff values for the conduction velocity of afferents in postnatal pups cannot be used as in the adult to separate unmyelinated from myelinated fibers (Fitzgerald 1987b). However, afferents that will acquire a myelination sheath in later life already conduct faster at earlier stages of development than those units that remain unmyelinated (Fitzgerald 1987b). Accordingly, we chose a cutoff value of 1.0 m/s to differentiate between presumptive A and C fibers in the present investigation.

A total of 157 single afferents were recorded in all preparations, 115 of which were studied in embryonic preparations (between *E17* and hatching). Of those, 54 (47%) were C fibers and had a mean conduction velocity of 0.65 ± 0.18 (SE) m/s. The remaining 61 (53%) A fibers had a mean conduction velocity of 2.7 ± 1.4 m/s with the fastest unit conducting at 6.2 m/s.

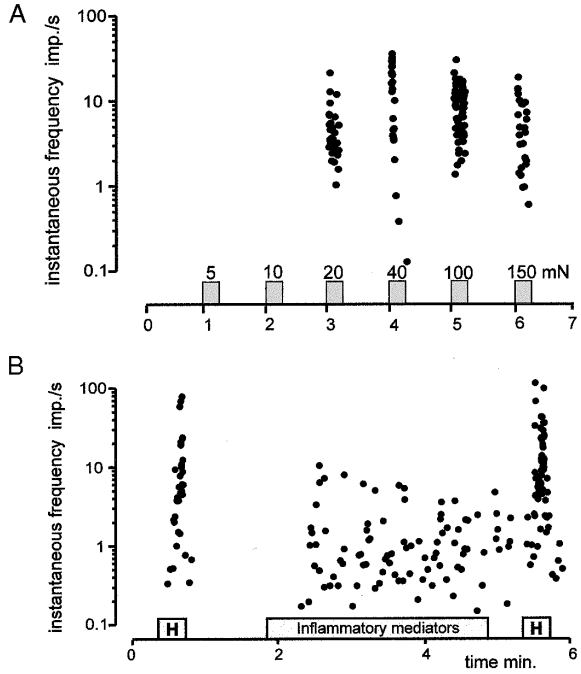

FIG. 3. Examples of C-fiber units recorded from embryonic chick. *A*: response of a unit to increasing constant force stimuli. This unit was subsequently tested for a response to noxious cold, heat, and inflammatory mediators but did not respond and was consequently classified as mechanosensitive C fiber (CM fiber). *B*: response of a mechano-heat-sensitive C fiber (CMH-unit, mechanical response not shown) during 2 noxious heat stimuli before and after exposure to inflammatory mediators (10 μM of each bradykinin, serotonin, histamine, and prostaglandin E_2). Note that the unit displays a vigorous response to chemicals and an increased response to the 2nd heat stimulus indicating sensitization.

In the hatchling preparations (4–21 days posthatching) 42 afferents were recorded, and of these 21 (50%) were C fibers with a mean conduction velocity of 0.54 ± 0.16 m/s. The mean conduction velocity of the remaining 21 (50%) A fibers was 3.7 ± 2.4 and thus, significantly faster than in the embryonic preparations ($P < 0.05$, *t*-test). Therefore the separation between the conduction velocities of A- and C-fiber afferents increases with age (Fig. 2). Ultrastructural examination of cutaneous nerves shows that myelination in chicken proceeds, in contrast to postnatal mammals, very slowly and is not complete until well into adulthood (Saxod and Bouvet 1982). Consistent with the small alterations of conduction velocity shown in the present study, the diameter of unmyelinated axons does not change, and axon size and myelin thickness of A fibers increases only slightly during the time period studied here (Saxod and Bouvet 1982). This means that functional changes of the receptive properties observed in the present study cannot be explained by major changes in myelination or conduction velocity of axons, but rather reflects changes in the receptive apparatus of sensory neurons.

Functional classification of units recorded

After identification of the receptive field with a mechanical search stimulus, neurons from both embryonic and hatchling preparations were further characterized by their responses to quantitative mechanical, thermal, and chemical stimuli (Figs. 3 and 4). C fibers generally exhibited a slowly adapting discharge to sustained mechanical stimuli (Figs. 3*A* and 4). Many of these fibers also responded to thermal and chemical stimulation (Fig. 3*B*). Whereas most C fibers had slowly adapting mechanosensitive properties, 41% of the A fibers ($n = 49$ tested) in embryonic or hatchling chick were rapidly adapting and discharged only at the onset or offset of the stimulus (Fig. 4). Quantitatively, these rapidly adapting responses were relatively uniform across a series of increasing force stimuli, presumably because the rise time of the force ramp (200 ms) was kept constant (Fig. 4). The remaining slowly adapting A fibers exhibited a proportional increase in discharge with increasing stimulus strength.

As determined with calibrated von Frey hairs, A fibers (1.0 mN, quartile range 1.0 mN, $n = 51$) had significantly ($P < 0.001$, *U* test), lower mechanical thresholds than C fibers (2.8 mN quartile range 1.0 mN, $n = 45$) in the embryonic chick (Fig. 5*A*). In preparations from hatchling chicks, there was a significant increase in the von Frey thresholds of both A fibers (2.4 mN, quartile range 1.0 mN; $n = 21$, $P < 0.05$, *U* test) and C fibers (median 5.7 mN, quartile range 4 mN, $n = 21$; $P < 0.01$, *U* test).

The mechanical sensitivity of a subpopulation of the embryonic A and C fibers was tested in more detail by constructing stimulus response functions using a range of suprathreshold stimuli (from 5 to 300 mN). The average stimulus response functions of both slowly adapting A and C fibers increased monotonically between 5 and 100 mN and then reached a plateau (Fig. 5*B*). However, the discharge evoked in A fibers was consistently higher than that in C fibers ($F_{1,210} = 19.3$, $P < 0.001$, analysis of variance).

Responses to nonmechanical stimuli

Thermal and chemical stimuli were used to further classify the neurons. It is clear that on the basis of mechanical responsiveness alone it would be difficult to unequivocally differentiate between nociceptive and nonnociceptive primary afferents. In mammals virtually all mechanosensitive C fibers recorded in vitro are nociceptors (Kress et al. 1992), but a large fraction of the myelinated fibers fulfill nonnociceptive

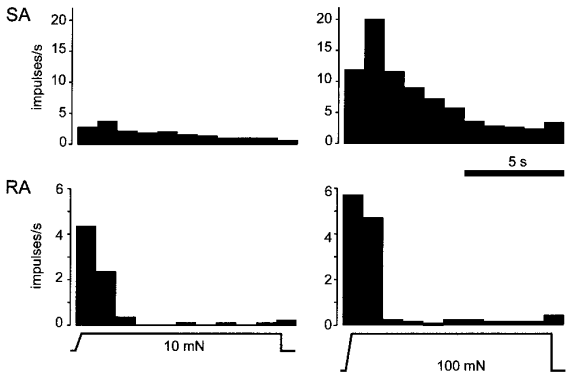

FIG. 4. Mean responses of all the tested slowly (*top*) and rapidly (*bottom*) adapting A fibers at 2 stimulus strengths of 10 and 100 mN. The stimulus was of 10 s duration and each bin (1 s) represents the mean activity of all the recorded fibers during this stimulus. A total of 29 slowly adapting A fibers and 19 rapidly adapting A fibers were used to construct the histograms. It is obvious that the slowly adapting A fibers code the increase in stimulus strength quite well, and their stimulus response properties for the entire range of stimuli used are shown in Fig. 5*B*.

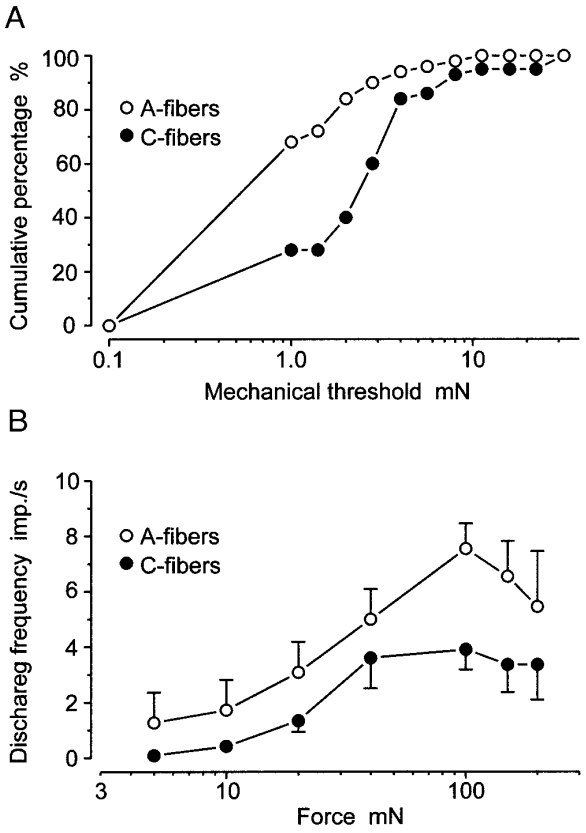

FIG. 5. *A*: plot of the cumulative mechanical threshold distribution determined with calibrated von Frey hairs for A and C fibers recorded in embryonic preparations. Distributions were obtained from 45 C fibers and 51 A fibers. *B*: stimulus response functions of slowly adapting A or C fibers recorded in embryonic preparations. Data were obtained from between 6 and 29 fibers per stimulus for A fibers; some fibers were not tested at all stimulus strengths, especially the strongest stimuli for fear of damaging the fibers. For C fibers each point represents the mean of between 10 and 29 fibers; error bars are standard error of the mean.

functions (Airaksinen et al. 1996; Koltzenburg et al. 1997). Because responses to noxious heat or algesic chemicals are known to be a unique characteristic of nociceptors (Handwerker and Reeh 1991; Meyer et al. 1994), we used these stimulus modalities as a tool to ascertain the nociceptive function of units (Fig. 3).

C fibers

Many of the 38 C fibers tested in preparations from embryonic chick responded to a nonmechanical stimulus (summarized in Table 1). Thus 32% of the neurons were excited by noxious heat and were classified as mechano-heat–sensitive C fibers (CMH fiber), and another 8% responded to both noxious heat and cold (''mechano-heat-cold sensitive,'' CMHC fiber). Superfusion of the receptive field with a mixture of inflammatory mediators (10 μM bradykinin, serotonin, histamine, and prostaglandin E_2) excited 60% of the tested CMH fibers as defined by a discharge exceeding 1 impulse/10 s, and 80% showed an increased heat response thereafter (Fig. 3). Moreover, 21% of the C fibers that did not initially respond to heat did so after exposure to inflammatory mediators. Thus a large proportion of the embryonic C fibers respond to nonmechanical modalities, which is consistent with the view that these neurons subserve a nociceptive function.

TABLE 1. *Proportion of C and A fibers from embryonic chick responding to thermal and chemical stimuli*

	Thermal Response	Response to Mediators	Sensitized to Heat
CMH	32% (12/38)	60% (6/10)	80% (8/10)
CMHC	8% (3/38)	66% (2/3)	66% (2/3)
CM	60% (23/38)	21% (4/19)	21% (4/19)
AMH	36% (12/33)	50% (6/12)	42% (5/12)
AMC	9% (3/33)	0% (0/2)	0% (0/2)
AM	55% (18/33)	0% (0/18)	28% (5/18)

The numbers of neurons tested for thermal and chemical responses are shown; 38 A and 33 C fibers were tested for their response to a standard noxious heat or noxious cold stimulus. On the basis of their responses to these stimuli, they could be classified into CMH or AMH (mechano-heat sensitive), into CMHC (mechano-heat and cold sensitive), into AMC (mechano-cold sensitive) or into CM or AM (only mechanoreceptive). The numbers and proportions of these receptor types are listed in Thermal Response. In addition, subpopulations of these receptor types were further tested for a response to algogenic chemicals (Response to Mediators) and for the ability of these chemicals to sensitize the neuron to a 2nd heat stimulus or to induce a de novo response to heat (Sensitized to Heat).

In the hatchling chicks, the proportion of 16 C fibers responding to noxious thermal and chemical stimuli was very similar to that found in embryonic preparations (Tables 1 and 2). However, the heat response of CMH fibers in hatchling chicks was more robust as the standard stimulus elicited more action potentials than in fibers recorded from embryonic chick (Fig. 6). A striking feature of the response to a heat ramp was the prolonged afterdischarge that persisted during the passive cooling of the receptive field after the end of the heat stimulus. This feature was observed in afferents from either embryonic or hatchling chick and contrasts with responses of heat-sensitive cutaneous nociceptors in rats that typically stop firing after the intracutaneous temperature has passed its zenith (Kress et al. 1992). In hatchling chicks 12% of the mechanosensitive C fibers also responded to cold, but these units ($n = 2$) responded neither to noxious heat nor to chemicals (Table 2).

A fibers

Among 33 embryonic A fibers tested, 15 (45%) also responded to noxious heat, cold, or chemical mediators (36% AMH and 9% AMC summarized in Table 1). There was no significant difference between the proportions of embryonic

TABLE 2. *Proportion of C and A fibers from hatchling chick responding to thermal and chemical stimuli*

	Thermal Response	Response to Mediators	Sensitized to Heat
CMH	44% (7/16)	71% (5/7)	66% (4/6)
CMC	12% (2/16)	0% (0/2)	0% (0/2)
CM	44% (7/16)	29% (2/7)	29% (2/7)
AMC	12% (2/16)	0% (0/2)	50% (1/2)
AM	88% (14/16)	0% (0/14)	7% (1/14)

Same specifications as Table 1; refers only to units tested from preparations taken from hatchling chicks.

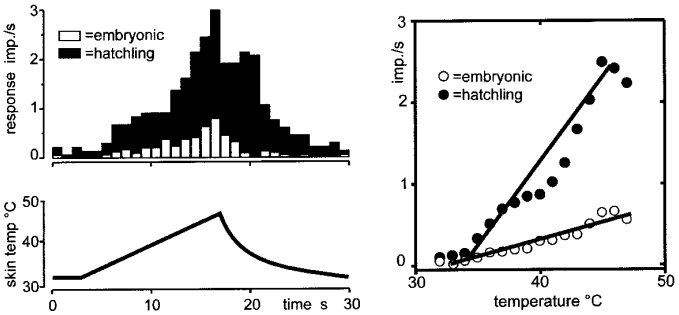

FIG. 6. Average heat response and stimulus response functions of C fibers tested in preparations from embryonic or hatchling chick. *Left*: mean response of all the tested CMH fibers before, during, and after the heat ramp in embryonic preparations ($n = 38$) and hatchling chicks ($n = 16$). *Right*: stimulus response functions are plotted for both populations by plotting the mean response against the skin temperature.

A and C fibers responding to heat ($P > 0.5$, χ^2 test) or inflammatory mediators ($P > 0.1$, χ^2 test; Table 1). Since an additional five units became heat sensitive after exposure to inflammatory mediators, a total of 61% of the embryonic mechanosensitive A fibers responded also to nonmechanical stimulus modalities and could therefore have nociceptive functions. Although the median mechanical thresholds of polymodal units was the same as for the remaining unimodal A fibers (1 mN), the conduction velocities of the putative nociceptors was significantly slower (1.9 ± 1.2 vs. 2.6 ± 0.6 m/s, $P < 0.05$, *t*-test) at this stage of development.

In the hatchling chick, none of the 16 neurons tested were heat sensitive compared with 36% of embryonic A fibers ($P < 0.05$, Fisher's exact test). Moreover, only 7% of the mechanosensitive A fibers (1/14 AM fibers) showed a small response to heat following exposure to inflammatory mediators compared with 28% (5/18 AM fibers) in the embryo (Tables 1 and 2). The hatchling A fibers tested for heat sensitivity were derived from all seven preparations, and the median age of these preparations was 14 days posthatching. There was no trend for more A fibers to be encountered in hatchling chicks, thus a subpopulation of A fibers probably lost their heat sensitivity after hatching.

Sensitization of the heat response after exposure to inflammatory mediators

Mature mammalian heat-sensitive polymodal nociceptors usually have stable average heat responses when tested at 5- to 10-min intervals, but many fibers show an increased heat response after exposure to inflammatory mediators (Koltzenburg 1995; Treede et al. 1992). In the present study we observed a similar sensitization to heat in a proportion of both A and C fibers recorded in embryonic chick after brief exposure to a mixture of inflammatory mediators. This resulted in an increased slope of the stimulus response function relating intracutaneous temperature to neural discharge (Fig. 7). Moreover, ~20% of the afferents (2/10 C fibers and 5/18 A fibers) that did not exhibit an obvious direct excitation to the chemicals showed sensitization to the subsequent heat stimulus. In summary, the interaction of heat and chemical stimulus modalities in the embryonic chick is qualitatively identical to the situation in mature mammals (Reeh and Kress 1995).

DISCUSSION

In this study we have characterized the receptive properties of sensory neurons innervating the hindlimb skin of chick embryos and in posthatchling chicks up to 3 wk old. By using an in vitro preparation and a range of controlled, quantitative stimuli, we found that subpopulations of sensory neurons can readily be identified on functional grounds late in embryonic development. We also show that the response properties are not completely mature, because some response properties change during the early posthatching period.

The subtypes of afferents that emerged were polymodal nociceptive C fibers, mechanosensitive C fibers, polymodal nociceptive A fibers, and A-fiber mechanoreceptors with rapidly or slowly adapting properties. In mature animals it is usually possible to differentiate nociceptive and nonnociceptive primary afferents on the basis of their different stimulus response functions to mechanical stimuli and conduction velocity alone (Meyer et al. 1994). However, in the developing chick this is not easily accomplished because the distribution of mechanical thresholds and conduction velocities of all fibers spanned a narrow range. This difficulty in differentiating between neurons having different functions applies particularly to A fibers, which are known to contain several subpopulations of nonnociceptive receptors in the adult chicken (Gentle 1989). However, when nonmechanical stimuli, notably noxious heat and algesic chemicals, are used as diagnostic tools (Handwerker and Reeh 1991), it becomes clear that a large percentage of C fibers as well as A fibers have nociceptive properties. Furthermore, among the A-fiber population, it is possible to differentiate between fibers with rapidly or slowly adapting properties, but further subdivisions were not possible because skin appendages (different kinds of feathers) could not be stimulated selectively due partly to the ''inside-up'' position of the skin.

In similar preparations taken from adult mice or rats, we have previously shown that the afferent types previously described in vivo can also be easily distinguished using this in vitro inside-up preparation (Airaksinen et al. 1996; Koltzenburg et al. 1997; Kress et al. 1992). It is possible that in the present experiments more information could have been obtained regarding afferents selectively activated by mechanical displacement of the feathers. However, the range of stimuli that we have used here, especially those pertaining

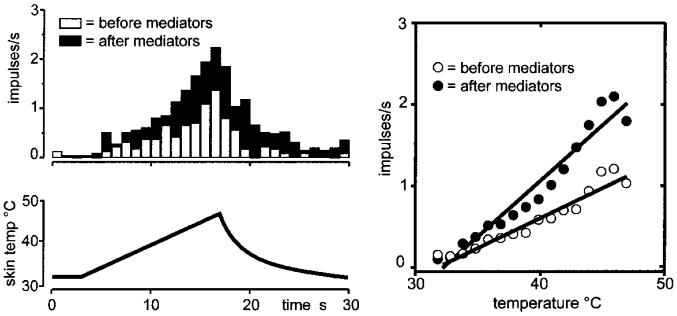

FIG. 7. Average heat response and stimulus response functions of fibers tested before and after exposure of the receptive field to inflammatory mediators in embryonic chick. Note the increased mean discharge and the increase slope of the stimulus response function indicating sensitization. In this case the responses of all the *C* fibers tested with heat and chemicals, including those with no initial heat response are plotted ($n = 32$).

to nociceptive responses, was most appropriate for the inside-up preparation. Thus the heat stimulus was applied from the epidermal side (from underneath the transparent chamber), and chemicals were applied to the inside face of the skin where such inflammatory mediators would normally accumulate. It has previously been shown in adult rat that the resulting temperature from our standard heat stimulus is ~5°C degree lower when it is measured on the corium side as compared with the epidermal side (Reeh 1986). This fully explains the seemingly lower heat thresholds of nociceptors in vitro compared with the values found in vitro where thresholds are usually measured as skin surface temperature. The quantitative mechanical stimuli used (calibrated von Frey hairs or feedback-controlled constant force stimuli) have also been shown in previous experiments to yield qualitatively identical responses with the exception that thresholds obtained in vitro are systematically lower and less variable than in vivo (Kress et al. 1992; Reeh 1986). This can probably be explained by the lack of a ''damping'' effect of the epidermis and the homogenous consistency of the silicone elastomer (Sylgard) compared with the various tissues (bone, fat, muscle) underlying the skin in vivo (Kress et al. 1992). In short the inside-up preparation offered the best compromise in allowing a wide range of different stimuli to be appropriately applied to the receptive surface in vitro.

In contrast to the postnatal development of mammals (Fitzgerald 1987b; Fulton 1987), changes in conduction velocity and growth of axons and their myelin sheaths are not prominent in the developing chick during the period investigated here (Saxod and Bouvet 1982). This means that the functional alterations observed in the present study probably reflect changes in the intrinsic receptive properties of the neurons rather than changes in the sampling of neurons due to increased axon growth and myelination. During the 3 wk after hatching, the mechanical thresholds of both A-fiber and C-fiber afferents increased, and median threshold doubled in both populations compared with the embryonic condition. In the adult chicken, the available evidence suggests that mechanical thresholds of nociceptors are even higher (Gentle 1989). Previous studies in rat indicated that thresholds of mechanical thresholds of nociceptors do not appear to increase during the fetal and early postnatal period (Fitzgerald 1987a,b). However, the values recorded in those studies are well below those of the adult rat (Kress et al. 1992; Lewin and Mendell 1994), implying that the receptor threshold rises slowly postnatally. This is in agreement with behavioral measurements that show a slow increase in the mechanical withdrawal thresholds of rats as they mature (Fitzgerald et al. 1988; Lewin et al. 1993). Although this increase in threshold is consistent with a maturation of the intrinsic properties of the afferents, changes in the physical properties of the skin cannot be completely ruled out. However, we think this less likely, first because tissue compliance in this preparation is primarily determined by the elasticity of the Sylgard underneath the skin, and second because changes of tissue compliance play only a minor part even in the response characteristics of low-threshold mechanoreceptors (Baumann et al. 1986; Pubols 1982).

Another functional maturation that we have observed is an increase in the discharge to suprathreshold noxious heat stimuli. This was indicated by an increased slope of the average response to a standard heat ramp in hatchling chicks compared with late embryonic chicks (Fig. 6). This occurred in the absence of a significant change in the proportion of unmyelinated afferents that responded to heat stimuli, indicating that the heat-sensitive apparatus within individual neurons is maturing as the animal ages. The increased heat response cannot be assigned to changes in the capacity of the skin to conduct the heat stimulus because the temperature was feedback-controlled at the receptive field (see METHODS). The characteristics of the heat response in chicks were different from those in most mammals. In mammals afferents usually stop discharging at the end of a linearly rising heat ramp when the temperature passes its zenith (Kress et al. 1992). In chick, however, afferents remained active after termination of the heat stimulus as the skin passively cooled. In spite of the marked increase of the nociceptor discharge in the posthatching period, these characteristics appeared not to be different between preparations taken from embryonic or hatchling chicks.

We have also shown that afferents from both embryonic and hatchling afferents can be activated by a mixture of algogenic chemicals and show an enhanced heat response after chemical stimulation (Fig. 3). This plastic process of nociceptor sensitization, which is an important cause of the increased pain sensations after tissue damage, was very similar to that observed in mature mammalian preparations (Kessler et al. 1992; Koltzenburg 1995; Meyer et al. 1994). In embryonic chicks, we found that 36% of the A fibers (Table 1) responded to noxious heat, but none in the hatchling chicks, which is in agreement with results from adult chickens where myelinated trigeminal nociceptors were found not to respond to noxious thermal stimuli (Gentle 1989). The lack of polymodal A-fiber nociceptors in the hatchling chicks is especially surprising because the total sample of these faster conducting afferents in the hatchling animals (50%) was not very different from that seen in embryos (53%). This could mean that a population of polymodal nociceptive A fibers disappeared in the immediate posthatching period. Since cell death is thought not to be predominant in the posthatching period (Hamburger et al. 1981), our data are consistent with the idea that a subpopulation of myelinated afferents lose their ability to respond to heat. This suggests that, at the time of hatching, there is still considerable plasticity in the capacity of neurons to respond to noxious stimuli. This is not to say that the A-fiber nociceptors do not exist in the hatchling because actually many of these afferents have relatively high von Frey thresholds (40% have thresholds >4 mN).

Much recent work indicates that during development as well as in adult animals the availability of nerve growth factor (NGF) can affect the expression of heat sensitivity in nociceptors. Application of exogenous NGF during the early postnatal period results in a permanent increase of heat sensitivity of nociceptive afferents found in the adult rat (Lewin and Mendell 1994). Moreover, removal of endogenous NGF in adult animals using a recombinant trkA-IgG fusion protein results in a differential decrease of the nociceptors' responsiveness to heat and chemicals, but not to mechanical stimuli (Koltzenburg et al. 1996). Furthermore, acute application of NGF to the receptive field of adult ro-

dents increases the heat response of a subpopulation of nociceptors (Rueff and Mendell 1995; Tal et al. 1996). In the present study we found a dramatically increased sensitivity of C-fiber nociceptors to heat, as the chick matured; however, A fibers exhibited a profound decrease in heat sensitivity. This differential change in heat sensitivity could be explained by a tight spatial regulation of NGF availability such that A fibers have less access to NGF. An alternative explanation is that differential expressions of neurotrophin receptors could be responsible. For example, high-threshold mechanoreceptors loose their heat sensitivity in transgenic mice lacking the low affinity neurotrophin receptor p75, whereas the heat sensitivity of unmyelinated nociceptors remains unimpaired (Stucky and Koltzenburg 1997). One advantage of our chick preparation is that these questions can now be functionally studied by perturbation of endogenous neurotrophin levels during defined time intervals of embryonic development (Lewin et al. 1994).

The developmental time point that we have first recorded afferent activity is 4 days before hatching. At this time the afferents have innervated both their peripheral and central targets, which for nociceptive afferents are established between *E11* and *E14* (Eide and Glover 1995; Mendelson et al. 1987). However, in the rodent it has been shown that even at this developmental stage the fine form and probably the central connections continue to be modified (Fitzgerald 1985; Fitzgerald et al. 1994). Furthermore, in the chick it has been demonstrated that neural excitation mediated via *N*-methyl-D-aspartate (NMDA) receptors might be important in the establishment of somatotopic organization even through to these late stages of embryonic development (Mendelson 1994). Thus, after chronic blockade of NMDA receptors in ovo, the central terminals of presumptive nociceptive afferents in lamina I and II form in a somatotopically inappropriate fashion. It is clear from our data that by *E17*, and probably earlier, the mechanical thresholds of putative nociceptors are sufficiently low to be activated by embryonic limb movements, which start around *E7* (Hamburger et al. 1965). Thus nociceptive afferents are likely to be capable of activating spinal cord NMDA receptors during the embryonic period from *E12* onwards, the time at which lamina II begins to be innervated in the chick (Eide and Glover 1995; Mendelson et al. 1987). Although it might be feasible to adapt the preparation to even younger embryos, we found that our present recording setup only allowed us to conveniently record single-unit afferent activity starting at *E17*. It has been shown that robust brush-evoked multiunit activity can first be seen between *E6* and *E8* (HH stage 29–34) (Scott 1982). From those studies it was not possible to ascertain whether prospective nociceptors or touch receptors were responsible for this activity. Our data from older embryos indicate that nociceptors (defined as such by their polymodal nature) have very low mechanical thresholds for activation and can easily be activated by brush stimuli. It is possible that these receptors have even lower mechanical thresholds earlier in development because their receptive thresholds continue to rise after *E17*. This issue should be addressed more specifically in future experiments.

In conclusion, by using an in vitro skin-nerve preparation we have been able to characterize in detail the development of the receptive properties of cutaneous chick sensory neurons both during late embryonic development and the post-hatching period. Our data indicate that several subtypes of afferents can be recognized within several days after they form their central connections. However, the receptive properties of nociceptors undergo gradual maturation in the post-hatching period. Thus the responses of C fibers to noxious heat become more dynamic and robust, and their mechanical thresholds for activation rise gradually as development proceeds. On the other hand A-fiber nociceptors appear to lose their capacity to respond to noxious heat during the same time period.

We thank C. Stucky for comments on the manuscript and Y.-A. Barde and K. Toyka for continuous support and encouragement.

This work was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 353. G. R. Lewin was supported during part of the study by a fellowship from the Alexander von Humboldt foundation.

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Received 2 December 1996; accepted in final form 2 July 1997.

REFERENCES

AIRAKSINEN, M. S., KOLTZENBURG, M., LEWIN, G. R., MASU, Y., HELBIG, C., WOLF, E., BREHM, G., TOYKA, K. V., THOENEN, H., AND MEYER, M. Specific sub-types of cutaneous mechanoreceptors require neurotrophin-3 following peripheral target innervation. *Neuron* 16: 287–295, 1996.

BAUMANN, K. I., HAMANN, W., AND LEUNG, M. S. Mechanical properties of skin and responsiveness of slowly adapting type I mechanoreceptors in rats at different ages. *J. Physiol. (Lond.)* 371: 329–337, 1986.

BROWN, A. G. *Organization in the Spinal Cord.* Berlin: Springer-Verlag, 1981.

DAVIES, A. M. The role of neurotrophins in the developing nervous system. *J. Neurobiol.* 25: 1334–1348, 1994.

EIDE, A. L. AND GLOVER, J. C. Development of the longitudinal projection patterns of lumbar primary sensory afferents in the chicken embryo. *J. Comp. Neurol.* 353: 247–259, 1995.

FERRINGTON, D. G., HORA, M. O., AND ROWE, M. J. Functional maturation of tactile sensory fibers in the kitten. *J. Neurophysiol.* 52: 74–85, 1984.

FERRINGTON, D. G. AND ROWE, M. J. Functional capacities of tactile afferent fibres in neonatal kittens. *J. Physiol. (Lond.)* 307: 335–353, 1980.

FITZGERALD, M. The post-natal development of cutaneous afferent fibre input and receptive field organization in the rat dorsal horn. *J. Physiol. (Lond.)* 364: 1–18, 1985.

FITZGERALD, M. Spontaneous and evoked activity of fetal primary afferents in vivo. *Nature* 326: 603–605, 1987a.

FITZGERALD, M. Cutaneous primary afferent properties in the hind limb of the neonatal rat. *J. Physiol. (Lond.)* 383: 79–92, 1987b.

FITZGERALD, M., BUTCHER, T., AND SHORTLAND, P. Developmental changes in the laminar termination of A fibre cutaneous sensory afferents in the rat spinal cord dorsal horn. *J. Comp. Neurol.* 348: 225–233, 1994.

FITZGERALD, M., MILLARD, C., AND MACINTOSH, N. Hyperalgesia in premature infants. *Lancet* 1: 292, 1988.

FORSTER, C. AND HANDWERKER, H. O. Automatic classification and analysis of microneurographic spike data using a PC/AT. *J. Neurosci. Methods* 31: 109–118, 1990.

FULTON, B. P. Postnatal changes in conduction velocity and soma action potential parameters of rat dorsal root ganglion neurones. *Neurosci. Lett.* 73: 125–130, 1987.

GENTLE, M. J. Cutaneous sensory afferents recorded from the nervus intramandibularis of *Gallus gallus var domesticus. J. Comp. Physiol.* [*A*] 164: 763–774, 1989.

GOTTMANN, K., DIETZEL, I. D., LUX, H. D., HUCK, S., AND ROHRER, H. Development of inward currents in chick sensory and autonomic neuronal precursor cells in culture. *J. Neurosci.* 8: 3722–3732, 1988.

HAMBURGER, V. Trophic interactions in neurogenesis: a personal historical account. *Annu. Rev. Neurosci.* 3: 269–278, 1980.

HAMBURGER, V., BALABAN, M., OPPENHEIM, R., AND WENGER, E. Periodic motility of normal and spinal chick embryos between 8 and 17 days of incubation. *J. Exp. Zool.* 159: 1–13, 1965.

HAMBURGER, V., BRUNSO-BECHTOLD, J. K., AND YIP, J. W. Neuronal death in the spinal ganglia of the chick embryo and its reduction by nerve growth factor. *J. Neurosci.* 1: 60–71, 1981.

HAMBURGER, V. AND HAMILTON, H. C. A series of normal stages in the development of the chick embryo. *J. Morphol.* 88: 49–92, 1951.

HANDWERKER, H. O. AND REEH, P. W. Pain and inflammation. In: *Proceedings of the VIth World Congress on Pain,* edited by M. R. Bond, J. E. Charlton, and C. J. Woolf. Amsterdam: Elsevier, 1991, p. 59–70.

IGGO, A. The electrophysiological identification of single nerve fibres, with particular reference to the slowest-conducting vagal afferent fibres in the cat. *J. Physiol. (Lond.)* 142: 110–126, 1958.

KESSLER, W., KIRCHHOFF, C., REEH, P. W., AND HANDWERKER, H. O. Excitation of cutaneous afferent nerve endings in vitro by a combination of inflammatory mediators and conditioning effect of substance P. *Exp. Brain Res.* 91: 467–476, 1992.

KOERBER, H. R. AND MENDELL, L. M. Functional heterogeneity of dorsal root ganglion cells. In: *Sensory Neurons: Diversity, Development and Plasticity,* edited by S. A. Scott. New York: Oxford Univ. Press, 1992, p. 77–96.

KOLTZENBURG, M. The stability and plasticity of the encoding properties of peripheral nerve fibres and their relationship to provoked and ongoing pain. *Semin. Neurosci.* 7: 199–210, 1995.

KOLTZENBURG, M., BENNETT, D.L.H., MCMAHON, S. B., SHELTON, D. L., AND TOYKA, K. V. Sequestration of endogenous nerve growth factor (NGF) reduces the sensitivity of nociceptors. *Abstr. 8th World Congr. Pain* 120, 1996.

KOLTZENBURG, M., KRESS, M., AND REEH, P. W. The nociceptor sensitization by bradykinin does not depend on sympathetic neurones. *Neuroscience* 46: 465–473, 1992.

KOLTZENBURG, M., STUCKY, C. L., AND LEWIN, G. R. Receptive properties of mouse cutaneous sensory neurons. *J. Neurophysiol.* 78: 1841–1856, 1997.

KRESS, M., KOLTZENBURG, M., REEH, P. W., AND HANDWERKER, H. O. Responsiveness and functional attributes of electrically localized terminals of cutaneous C-fibers in vivo and in vitro. *J. Neurophysiol.* 68: 581–595, 1992.

LANG, E., NOVAK, A., REEH, P. W., AND HANDWERKER, H. O. Chemosensitivity of fine afferents from rat skin in vitro. *J. Neurophysiol.* 63: 887–901, 1990.

LAWSON, S. N. Morphological and biochemical cell types of sensory neurons. In: *Sensory Neurons: Diversity, Development, and Plasticity,* edited by S. A. Scott. New York: Oxford Univ. Press, 1992, p. 27–59.

LE DOUARIN, N. M. *The Neural Crest.* Cambridge, UK: Cambridge Univ. Press, 1982.

LEVI-MONTALCINI, R. AND HAMBURGER, V. Selective growth-stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. *J. Exp. Zool.* 116: 321–361, 1951.

LEWIN, G. R. AND BARDE, Y.-A. Physiology of the neurotrophins. *Annu. Rev. Neurosci.* 19: 289–317, 1996.

LEWIN, G. R., KOLTZENBURG, M., TOYKA, K. V., AND BARDE, Y.-A. Involvement of neurotrophins in the phenotypic specification of chick cutaneous afferents. *Soc. Neurosci. Abstr.* 20: 1094, 1994.

LEWIN, G. R. AND MENDELL, L. M. Regulation of cutaneous C-fiber heat nociceptors by nerve growth factor in the developing rat. *J. Neurophysiol.* 71: 941–949, 1994.

LEWIN, G. R., RITTER, A. M., AND MENDELL, L. M. Nerve growth factor–induced hyperalgesia in the neonatal and adult rat. *J. Neurosci.* 13: 2136–2148, 1993.

MENDELSON, B. Chronic embryonic MK-801 exposure disrupts the somatotopic organization of cutaneous nerve projections in the chick spinal cord. *Dev. Brain Res.* 82: 152–166, 1994.

MENDELSON, B., KOERBER, H. R., AND FRANK, E. Development of cutaneous and proprioceptive afferent projections in the chick spinal cord. *Neurosci. Lett.* 138: 72–76, 1987.

MEYER, R. A., CAMPBELL, J. N., AND RAJA, S. N. Peripheral neural mechanisms of nociception. In: *Textbook of Pain,* edited by P. D. Wall and R. Melzack. Edinburgh: Churchill Livingstone, 1994, p. 13–44.

NOVICKI, M. Volatge-gated ion channels in DRG neurons. In: *Sensory Neurons: Diversity, Development and Plasticity,* edited S. A. Scott. New York: Oxford Univ. Press, 1992, p. 97–115.

PERL, E. R. Function of dorsal root ganglion neurons: an overview. In: *Sensory Neurons: Diversity, Development, and Plasticity,* edited by S. A. Scott. New York: Oxford Univ. Press, 1992, p. 3–23.

PERL, E. R. AND BURGESS, P. R. Cutaneous mechanoreceptors and nociceptors. In: *Somatosensory System,* edited by A. Iggo. Berlin: Springer-Verlag, 1973, p. 29–79.

PUBOLS, B. H. Factors affecting cutaneous mechanoreceptor response. II. Changes in mechanical properties of skin with repeated stimulation. *J. Neurophysiol.* 47: 530–542, 1982.

REEH, P. W. Sensory receptors in mammalian skin in an in vitro preparation. *Neurosci. Lett.* 66: 141–147, 1986.

REEH, P. W. AND KRESS, M. Effect of classic algogens. *Semin. Neurosci.* 7: 221–226, 1995.

RUEFF, A. AND MENDELL, L. M. NGF-induced increase in thermal sensitivity of nociceptive fibers in vitro. *Soc. Neurosci. Abstr.* 21: 1537, 1995.

SACHS, L. *Angewandte Statistik* (5th ed.). Heidelberg, Germany: Springer-Verlag, 1978.

SAXOD, R. AND BOUVET, J. Quantitative analysis of growth and myelination of cutaneous nerve fibers in the chick. *Dev. Neurosci.* 5: 143–155, 1982.

SCHMELZ, M., FORSTER, C., SCHMIDT, R., RINGKAMP, M., HANDWERKER, H. O., AND TOREBJÖRK, H. E. Delayed responses to electrical stimuli reflect C-fiber responsiveness in human microneurography. *Exp. Brain Res.* 104: 331–336, 1995.

SCOTT, S. A. The development of the segmental pattern of skin sensory innervation in embryonic chick hind limb. *J. Physiol. (Lond.)* 330: 203–220, 1982.

SMITH, C. L. AND FRANK, E. Peripheral specification of sensory connections in the spinal cord. *Brain Behav. Evol.* 31: 227–242, 1988.

STUCKY, C. L. AND KOLTZENBURG, M. The low affinity neurotrophin receptor p75 regulates the function but not the selective survival of specific subpopulations of sensory neurons. *J. Neurosci.* 17: 4398–4405, 1997.

TAL, M., SCHNEIDER, M., TOYKA, K. V., AND KOLTZENBURG, M. Heat hyperalgesia and nociceptor excitation after application of nerve growth factor (NGF) or inflammatory mediators. *Abstr. 8th World Congr. Pain* 119–120, 1996.

TOREBJÖRK, H. E., VALLBO, Å. B., AND OCHOA, J. L. Intraneural microstimulation in man. Its relation to specificity of tactile sensations. *Brain* 110: 1509–1529, 1987.

TREEDE, R.-D., MEYER, R. A., RAJA, S. N., AND CAMPBELL, J. N. Peripheral and central mechanisms of cutaneous hyperalgesia. *Prog. Neurobiol.* 38: 397–421, 1992.