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Development of cutaneous and proprioceptive afferent projections in the chick spinal cord

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Muscle and cutaneous nerves were individually labeled with DiI in chick embryos to examine the development of sensory afferent arborizations in the spinal cord. Initially, cutaneous and muscle arbors were similar; both types first entered the spinal gray matter at stage 28–29 (embryonic day (E) 6). Differences in projections were first observed by late stage 34 (E8.5): muscle afferent collaterals extended almost unbranched to the level of motoneuronal dendrites while cutaneous afferents branched frequently and remained within the dorsal horn. Projections of putative small caliber axons into laminae 1 and 2, located laterally in the chick, did not develop until E13–14.

In the mature nervous system, individual sensory neurons have central arborizations in the spinal cord that are specific for their sensory modality. Cutaneous afferent axons terminate within the dorsal horn whereas large caliber muscle afferents have an additional projection to more ventral laminae [2]. Although a number of studies have examined the general development of sensory neurons [6, 13] and their central projections by labeling multiple populations of cells [3, 11, 18] it has been difficult to analyze the development of identified subpopulations of sensory afferents. For example, earlier suggestions that muscle and cutaneous afferents might project into the spinal cord at different times could not be tested directly. Taking advantage of the fact that processes of sensory neurons grow to specific targets before contacts are formed centrally in the spinal cord [9, 14, 17, 19] we labeled central projections of specific afferent types by applying lipophilic dyes [8] to individual peripheral nerves. Using embryos fixed at different stages, we have been able to trace the development of the central projections of identified groups of sensory afferents.

Embryos were perfused transcardially with 20 ml of chick Tyrodes solution [10] followed by 20 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) and the spinal cords were dissected with peripheral nerves intact. After fixation, isolated muscle (sartorius (Sart) or adductor (Add)) or cutaneous (lateral femoral cutaneous (LFC)) nerves were labeled with 10% DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Molecular Probes, Inc.) in ethanol. The ethanol was allowed to evaporate leaving crystals of DiI on the nerves, which were kept from touching other tissue. The spinal cords were stored in 4% formaldehyde in the dark at 37°C for 1–6 weeks. Vibratome sections were cut at 50 or 100 μm and mounted in serial order in phosphate buffer. To analyze the distribution of labelled collaterals with respect to spinal cord laminae, in selected sections, the DiI fluorescence was photo-converted to a permanent reaction product [16]. Briefly, the sections were placed in a depression slide in a solution of 150 mg diaminobenzidine in 100 ml Tris buffer (pH 8.2) at 0°C for 30 min. A selected region of the section was then illuminated by a 100 W mercury light source using a rhodamine filter set and an Olympus D-Plan Apo 20X objective (N.A. 0.7) for 40 min. The photo-converted sections were counterstained with Neutral red, dehydrated and coverslipped with Permount.

The adequacy and specificity of DiI labeling were confirmed in two ways. Positions of individual sensory and motor pools were mapped and found to be within the previously published locations [7, 9, 10]. In addition, the DiI labeling was generally of uniform brightness

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throughout the rostrocaudal extent of each labeled pool and individual collaterals ended in structures resembling synaptic terminals or, in some cases, growth cones. It is therefore likely that DiI labeled the total extent of the sensory arbors. To minimize possible errors resulting from incomplete labeling, however, we have limited our analysis to the region of spinal cord within 150 μm of the dorsal root entry zone. Preparations with non-uniform DiI brightness or in which DiI had leaked into adjacent peripheral nerves or muscles were not used. The findings presented here are based on the results of at least 5 successful fills of the LFC and Sart or Add nerves at each stage (St 28–29, 32–33, 34, 36, 38 and 40).

Initially, cutaneous and muscle afferents have similar projection patterns within the spinal cord. Their axons are present within the primordium of the dorsal funiculus (the oval bundle of His) and extend for at least two segments rostral and caudal to their point of entry by stage 28–29. At about this time *both* cutaneous and muscle afferents begin to penetrate the gray matter. In the adult chicken, laminae 1 and 2 are located lateral to lamina 3 [1, 12]. At these early stages the growing fibers enter the gray matter near the bundle of His (Fig. 1A); as development proceeds the cord grows such that these fibers come to occupy a more medial position within lamina 3 (compare Fig. 1A with Fig. 1B,C). Muscle and cutaneous fiber collaterals continue to grow in a similar manner within the dorsal horn, giving rise to few major branches, through stage 33.

The patterns of muscle and cutaneous collateral arborizations begin to diverge at late stage 34 (E8.5). Muscle afferents grow ventrally through the intermediate laminae (5–7) to terminate within the dorsal part of the ventral horn. These collaterals do not branch extensively within the dorsal horn, but have some branches and structures resembling terminals in intermediate laminae. At this stage, overlap of muscle afferent collaterals with motoneuronal dendrites is observed. Cutaneous collaterals, in contrast, remain confined to the dorsal horn where they now begin to branch profusely. At these stages (St 32–38), almost all cutaneous collaterals are restricted to the medial portion of the dorsal horn: very few penetrate into laminae 1 and 2 (Fig. 1A,B). By stage 36 (E10), muscle afferent collaterals have increased numbers of branches and terminals in laminae 4–7 and some processes extend medially into Clarke's column where bouton-like swellings are observed. The most ventral muscle collaterals project to the lateral motor column, ending in close proximity to homonymous motoneuronal somata. The number of collateral branches and boutons continues to increase in all laminae ventral to 4 through stage 40 (E14, Fig. 2B) when the pattern of muscle afferent arborizations resembles the 'mature' pattern observed by Martin [12] in silver-stained preparations of late-stage embryos and hatchlings.

Cutaneous afferents continue to branch and form boutons within laminae 3–5 through stage 35 (E9). At stage 38 (E12), a few collaterals project into laminae 1 and 2 and two days later, a well developed lateral projection is observed, anatomically distinct from the earlier projection to lamina 3 (Fig. 2A). Cutaneous collaterals rarely reach more ventral laminae. The cutaneous afferent projections thus develop in two stages: an early projection confined to the medial part of the dorsal horn and a later projection into the lateral dorsal horn. A recent physiological investigation [20] has shown that in adults, afferents innervating laminae 1 and 2 mainly consist of small caliber fibers whereas larger fibers supply laminae 3 and 4. In addition, single unit recordings revealed a segregation of sensory modalities with light tactile inputs confined to laminae 3–4 and firm pressure and pinch inputs in laminae 1–2. Although neither the fiber diameters nor the sensory modalities of the afferents were determined in this study, the separate terminal fields demonstrated here suggest that in the chick, collaterals destined to be large diameter non-nociceptive cutaneous fibers develop central projections before those destined to be small caliber and nociceptive. This conclusion confirms a similar temporal sequence of afferent development in rats [4].

Earlier studies of the anatomical development of sensory afferent projections in the spinal cord did not differentiate between muscle and cutaneous afferents [3, 11, 18]. In our study, however, individual peripheral nerves were labeled so the development of identified subpopulations of afferents could be examined. Central projections of muscle and cutaneous afferent collaterals develop contemporaneously and at no stage is there evidence of terminations within inappropriate laminae.

In a study of GAP-43 immunoreactivity in the developing rat limb and spinal cord, Fitzgerald and colleagues found that at a given proximo-distal level in the limb, cutaneous innervation developed before muscle innervation [15] and they suggested that the central collateral projections of these afferents were triggered by peripheral innervation [5]. Because the peripheral targets of the LFC, Sart and Add nerves are all located at a similar proximo-distal level [17], our finding that muscle and cutaneous afferent collaterals develop at the same time suggests that, in the chick, either muscle and cutaneous innervation of the periphery occur simultaneously or that growth of central collaterals is not tightly correlated to the time of peripheral innervation.

The patterns of cutaneous and muscle afferent projections begin to diverge around stage 34 (E8.5) when muscle afferents extend ventrally into the intermediate and ventral laminae while cutaneous collaterals remain and

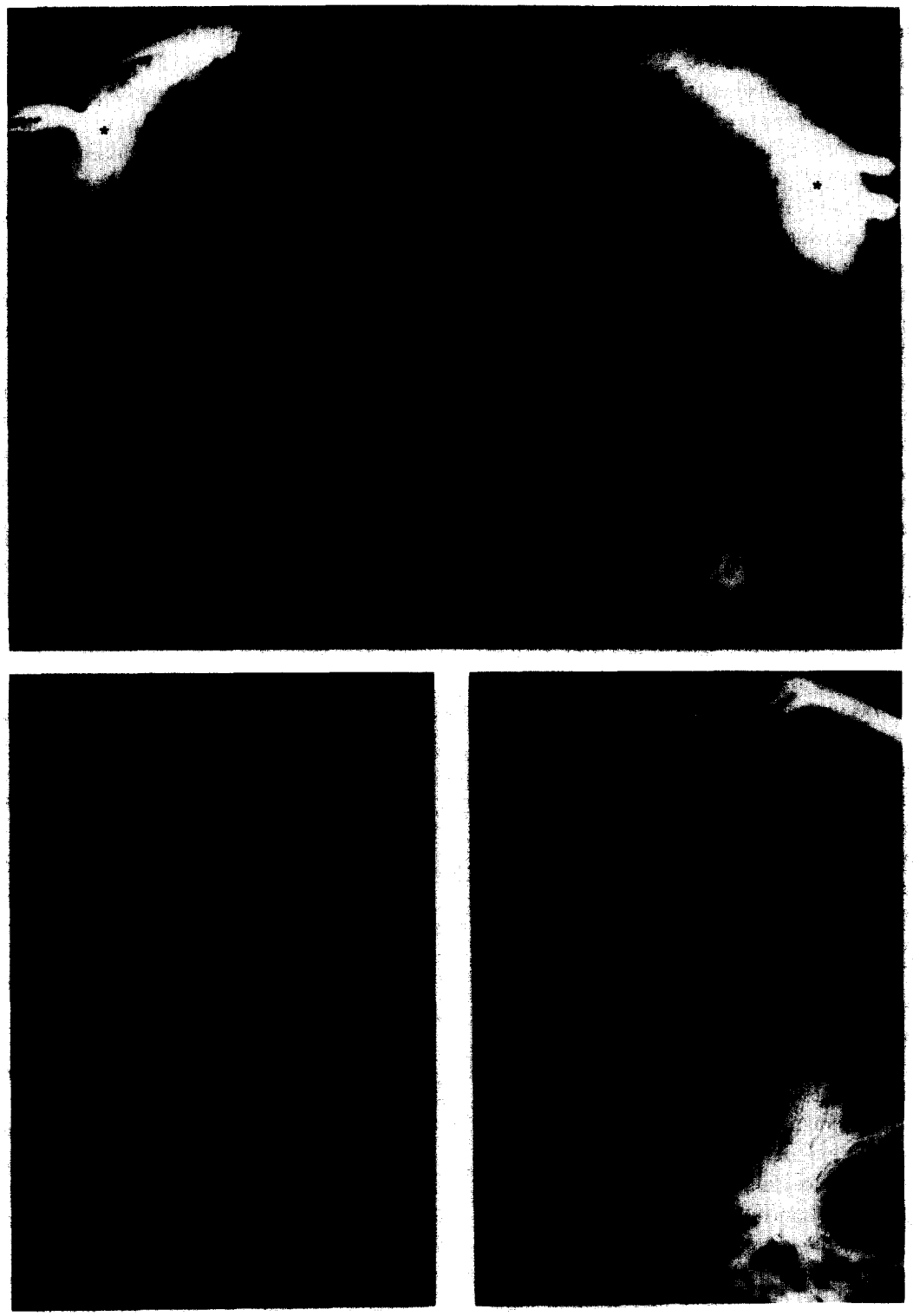

Fig. 1. A: transverse section of the lumbar spinal cord taken from an E8 (Stage 33.5) embryo in which the left LFC and the right Sart nerves were labeled with DiI. Cutaneous and muscle afferent fibers are both at similar depths within the dorsal horn. The brightly stained bundles of fibers at the dorsolateral margins of both sides of the cord represent the oval bundles of His (indicated by *, cc indicates the central canal). B,C: transverse sections from E12, Stage 38 animals. B: a transverse section of the left half of the lumbar spinal cord where the LFC nerve was labeled. C: a transverse section of the right half of the lumbar spinal cord where the adductor nerve has been labeled. At this later stage, muscle afferent collaterals extend into the ventral horn whereas cutaneous fibers are confined to the medial dorsal horn (laminae 3–4). Bars = 100 μm.

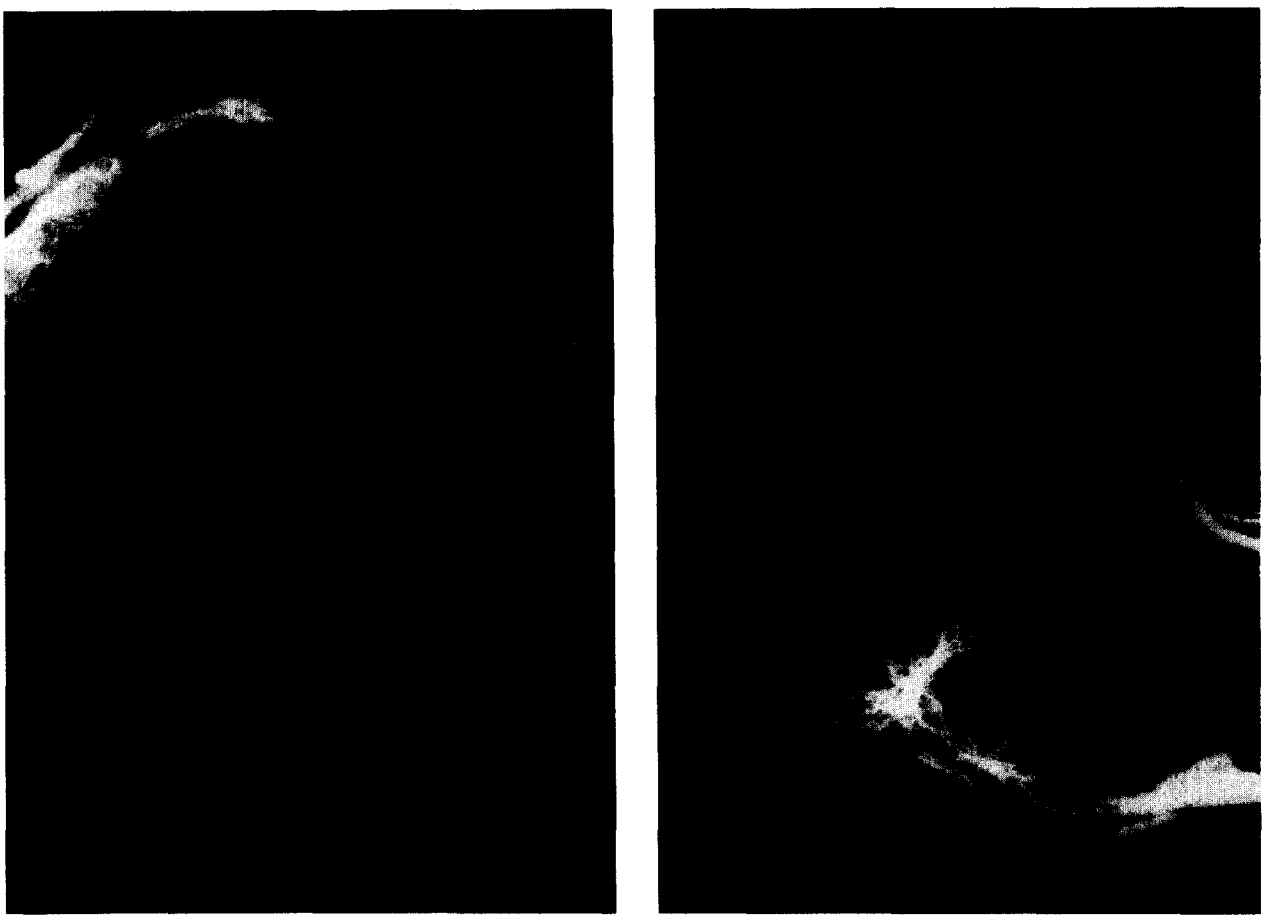

Fig. 2. A: transverse section of the left half of the spinal cord from an E14 embryo in which the LFC nerve was labeled (M indicates medial). At this stage there are two distinct populations of cutaneous fibers, one where the fibers enter the gray matter laterally and arborize in laminae 1–2 and a separate population that enters more medially and branches within laminae 3–4. The fiber-free area between the two populations of fibers is indicated by an arrowhead. B: similar section from an E14 embryo in which the Add nerve was labeled. Bars = 100 μm.

arborize within the dorsal horn. Because both types of afferents grow directly to appropriate laminae during the same developmental stages, differences in the times that these two types of afferent collaterals enter the gray matter cannot be involved in guiding these processes to correct target regions. Rather, the two types of collaterals must interpret signals within the extracellular environment differentially, and subsequently use these signals to grow directly to particular target areas in the spinal cord. It will be of interest to determine the specific molecular signals that guide particular types of sensory afferents to their appropriate target regions.

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