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Evidence that endogenous β nerve growth factor is responsible for the collateral sprouting, but not the regeneration, of nociceptive axons in adult rats

(growth factors/plasticity/neurotrophism/sensory fields)

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ABSTRACT **A key role has not yet been identified for β nerve growth factor (NGF) in the growth responses that continue to be expressed in the sensory neurons of adult animals. We have now examined the effects of daily administration to adult rats (and in a few experiments, mice) of antiserum to NGF on (*i*) the collateral sprouting of undamaged nociceptive nerves that occurs into denervated adjacent skin and (*ii*) the regeneration of cutaneous sensory axons that occurs after they are damaged. The results were unexpected. All collateral sprouting was prevented and that already in progress was halted; sprouting resumed when treatment was discontinued. In contrast, the reestablishment, and even enlargement, of cutaneous nerve fields by regenerating axons was unaffected by anti-NGF treatment, even after dorsal rhizotomy was done to eliminate any central trophic support. In denervated skin, regenerating and collaterally sprouting axons utilized the same cellular pathways to establish functionally identical fields, thus displaying apparently identical growth behaviors, yet anti-NGF treatment clearly distinguished between them. We suggest that endogenous NGF is responsible for the collateral sprouting of nociceptive axons, probably reflecting an ongoing function of NGF in the regulation of their fields. This demonstration in the adult sensory system of a defined role for NGF in nerve growth could apply to nerve growth factors generally in the adult nervous system. The regeneration, however, of nociceptive axons (and nonnociceptive ones) is not dependent on NGF.**

Although β nerve growth factor (NGF) is essential for the development and survival of neuronal populations in the autonomic and sensory nervous systems (1–4), by birth or shortly thereafter, sensory neurons will survive largely independent of it (1, 5). Nevertheless in adult animals NGF continues to be synthesized in peripheral target tissues (6, 7), sensory axons can take up and transport it retrogradely (8, 9), while maintained NGF deprivation leads to a lowering of both substance P levels (10), and even neuronal cell size (11), in dorsal root ganglia. Significantly, two striking *growth* behaviors of sensory neurons also continue to be demonstrable in adult animals: these are axonal elongation and collateral sprouting. There are more than morphological distinctions between these two. Whereas collateral sprouting, both during development and later, is a characteristic of normal undamaged nerves, and is essentially confined to target tissues (12, 13), elongation is seen in adults particularly in the form of regeneration of an axon after peripheral nerve *damage*. In adult mammals, large myelinated mechanosensory axons readily regenerate after they are crushed, but unlike both myelinated (14) and unmyelinated (15) nociceptive axons, they fail to sprout collaterals into denervated skin when they are intact (16); in contrast, within the adult mammalian central nervous system (CNS) collateral sprouting of undamaged axons is the more readily evoked behavior (17), and axonal regeneration either fails to occur or occurs only under special conditions (18, 19). Of particular interest, regenerating axons are almost uniformly successful when in competition with collaterally sprouting axons for occupancy of common target tissues (reviewed in ref. 20).

In the present study we compared the effects of anti-NGF treatment on collateral sprouting and axonal regeneration of cutaneous nociceptive nerves in adult rats. Surprisingly, though the cutaneous pathways normally followed by each were identical, sprouting was prevented, while regeneration was unaffected. The findings suggest a possible role for growth factors generally in adult nervous systems and may have implications for the design of strategies to initiate regrowth of nerves—e.g., after damage to the brain or spinal cord.

METHODS

Nociceptive Nerve Sprouting. Nerve sprouting in adult rat skin was evoked, identified, and measured as described previously (14, 15). Briefly, the nerve supply to an entire area of the back skin of Wistar rats (150–250 g) anesthetized with sodium pentobarbital (35–45 mg/kg) was permanently eliminated except for the medial branch of the dorsal cutaneous nerve (DCN) of thoracic segment 13 (mDCN-T13), whose sensory field was thus "isolated." Its mechanonociceptive ("pinch") and heat-nociceptive ("heat") fields were mapped, respectively, by fine forceps pinching and by brief application of a 60°C probe, both of which evoke the visible reflex response of the underlying cutaneus trunci muscle (Fig. 1). The "touch" field of the large myelinated Aα axons in the mDCN-T13 was mapped directly by recording the afferent impulses evoked in the nerve by stroking the skin with a fine bristle (16).

Axonal Regeneration. Regeneration was produced after similarly isolating the mDCN-T13 field, by crushing this remaining nerve; regeneration was evaluated by measuring the time to onset and the extent of recovery of pinch, heat, and light touch sensitivity in the skin. In one group of animals, in addition to crushing mDCN-T13, we cut the ipsilateral dorsal roots of segments T11–L2 and excised the segments. Regeneration of sensory C fibers to the skin was then detected (*i*) by antidromic electrical excitation of mDCN-T13 after the animal had been injected with Evans blue dye, to evoke the characteristic (visible) blue extravasation in the skin (15, 21); and (*ii*) by their reappearance in the denervated skin when viewed in the electron microscope; regeneration of "touch" fibers was evaluated electrophysiologically as before.

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Abbreviations: NGF, β nerve growth factor; CNS, central nervous system; DCN, dorsal cutaneous nerve.

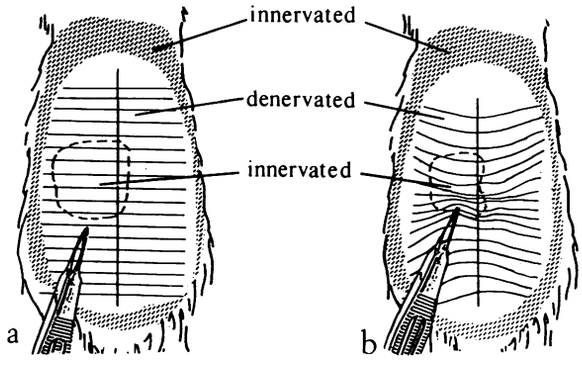

FIG. 1. Mapping of nociceptive fields. The dashed outline within each of these sketches of the rat's back represents the border of the field of the selected nerve (mDCN-T13), "isolated" by elimination of the surrounding nerves. The transverse lines are drawn across the (shaved) skin for clarity. The vertical line represents the midline. In *a*, pinching (as shown) or focal heat has no effect when applied to the denervated skin just outside the innervated area; in *b* the pinch is applied just *inside* the field, evoking the bilateral reflex contraction of the underlying cutaneus trunci muscle; by systematic exploration with the stimulus the border of the isolated field is determined (14, 15).

Antiserum to Mouse NGF, Its Potency, and Its Use. Antiserum to the 2.5S NGF was raised in rabbits according to methods described by Mobley *et al.* (22, 23). Activity was determined by means of the dissociated cell assay (24), using neonatal mouse sympathetic neurons (25): a 1:10,000 dilution of the antiserum (anti-NGF) totally inhibited the activity of 7S NGF at 10 ng/ml. Approximately 2.5 μl of antiserum per g of body weight was injected subcutaneously in the groin or dorsal cervical regions of the rats; the appropriateness of the dosage was confirmed empirically, after noting that an antiserum whose potency had reduced to about ⅓ of its initial level failed to prevent (though it reduced) sprouting.

Morphology. Dermal perineurial tubes and their contained axons were revealed by silver staining 30-μm-thick sections of frozen skin (14); unmyelinated fibers within these tubes, and running in the subepidermal horizontal network, were identified by electron microscopy after conventional fixation and staining (15).

Pilot Studies in Mice. We also investigated a few mice of the strain used to obtain NGF; because of the large production of salivary gland NGF by the male, females were used as more appropriate for comparative purposes. Three to five sequential DCNs were crushed on one side to evoke regeneration, and the border of the resulting discrete pinch-insensitive area was determined. In half of the animals, daily anti-NGF treatment was begun at the time of operation. Recovery of nociceptive function to denervated skin was evaluated by the reappearance of reflex responsiveness to pinch; collateral sprouting of the surrounding intact DCNs (when it occurred) was evidenced by the progressive shrinkage of the initially insensitive area.

Effects on Sympathetic Ganglia. No systematic study was done, but an independent indicator of the effectiveness of the standard anti-NGF treatment came from measurements of the blotted wet weights of the superior cervical ganglia; the mean ganglion weight for seven rats after 4 weeks of anti-NGF treatment (0.95 ± 0.07 mg, SEM) was significantly less ($P < 0.001$, Mann–Whitney U test) than that of eight weight-matched control animals (1.48 ± 0.08 mg, SEM).

RESULTS

Sprouting and the Effects of Anti-NGF. Fig. 2*a* shows examples of the expansion of the pinch and heat fields of

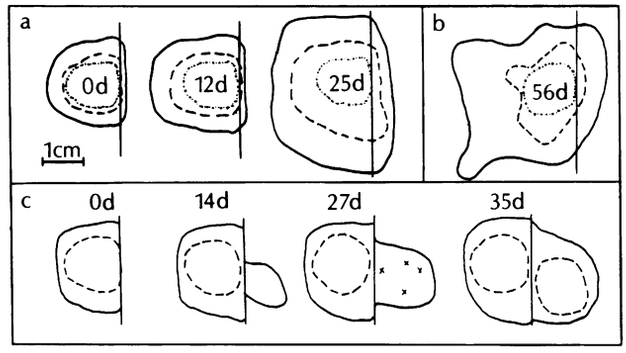

FIG. 2. The light touch and nociceptive fields of the mDCN-T13, from three different experiments (*a*, *b*, and *c*). In each case the midline is indicated by the vertical straight line, the touch fields (the smallest) by dotted lines, heat fields by dashed lines, and pinch fields (the largest) by continuous lines; the calibration applies to all the figures. (*a*) Fields on the left side, measured at the indicated times (d, days) after their isolation. Note that the touch field did not change (16), while the two nociceptive ones expanded (14, 15). The incision line for the operation (not shown) was always to the right of the midline. (*b*) Sensory recovery achieved by *regeneration*, 56 days after the left mDCN-T13 was crushed, and daily anti-NGF administration was begun; the initial fields prior to crushing resembled those shown at day 0 in *a*; usually (though not in this instance) the touch fields would also have regenerated to a greater-than-normal size by 56 days (cf. ref. 16). (*c*) In this animal a *midline* incision was used; both the left and right mDCN-T13 were isolated initially, but the right nerve was immediately crushed to evoke its regeneration; daily anti-NGF treatment began at the same time. The first signs of regeneration, on the right side, were a limited recovery to pinching on day 12 (not shown), improving to that illustrated by day 14; on day 27 a few sites (crosses) were also heat sensitive, and by day 35, when the experiment ended, the original fields were essentially restored, including touch (not shown). Note that the surviving *left* mDCN-T13 nociceptive fields failed to expand significantly over the entire period of treatment.

mDCN-T13 (and lack of expansion of the touch field) that occurred after their "isolation"; Fig. 3 (open histograms) gives the quantitative description of this expansion. These expansions are attributable, respectively, to the collateral sprouting of intact Aδ fibers (14) and C fibers (15). Daily injections of anti-NGF serum begun at the same time as the denervations dramatically affected these results; expansions of both pinch and heat fields were prevented (Fig. 3, filled histograms), and in the surrounding skin axons remained absent from both the dermal perineurial tubes (Fig. 4) and the subepidermal Schwann tubes (Fig. 5). When the injections were delayed until field expansion had already begun, this appeared to have been quickly arrested (Fig. 3, hatched histograms; the apparent reversal of the heat field expansion requires further examination); fields were always observed to expand after cessation of anti-NGF treatment (Fig. 3, stippled histograms). We noted (cf. ref. 26) that rats on maintained anti-NGF treatment tended to develop apparently atrophic skin lesions in the neck region.

None of the effects of anti-NGF serum noted above, including the prevention of sprouting, were observed in a group of five operated animals that received daily injections of nonimmune serum for 4 weeks.

Ineffectiveness of anti-NGF Treatment on Regeneration. The regeneration of nociceptive fibers along a peripheral nerve has already been shown to occur during anti-NGF treatment (11). However, it was surprising to find that daily anti-NGF injections failed to affect the ability of regenerating mDCN-T13 fibers not only to reestablish functional nociceptive fields in denervated skin but also to produce *expanded* fields that encroached on territory formerly supplied by neighboring DCNs (Fig. 2*b*). An "internal" control was provided by studying axonal regeneration and collateral

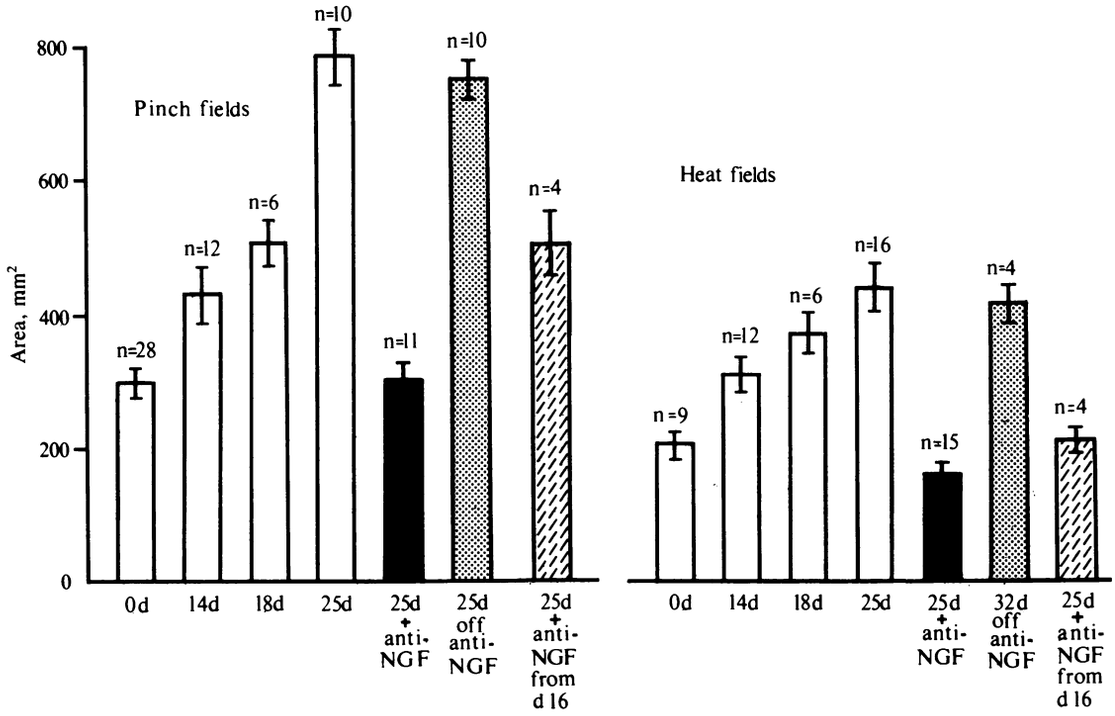

FIG. 3. Histograms of the left mDCN-T13 pinch and heat fields in various groups of rats. Abscissae are not scaled, but shown below are the various times (in days) when the measurements were done after the initial field isolation. The number of animals in each group is indicated by the *n* values. Error bars indicate ±SEM. The normal rate of field expansion is seen from the first four histograms (open) to the left for both the pinch and heat series. Expansion was maximum at about 24–25 days. The filled histogram (25 days + anti-NGF) came from animals that were treated daily for the entire period; clearly there had been no field expansions. The treatment was then discontinued, and 25 days later for the pinch fields (the 25 days off anti-NGF histogram, stippled) and somewhat longer, 32 days later, for the heat fields (32 days off anti-NGF) the normal maximum expansion of the fields had occurred. The hatched histograms on the far right of each series are from animals in which the anti-NGF treatment was not begun until 16 days after the fields were isolated. Comparison with the four normal groups indicates that in these rats the field expansions already in progress must have been rapidly terminated by the treatment; the significance of the apparent reversal of the heat expansion requires further examination.

sprouting in the same animal (Fig. 2*c*); the mDCN-T13 fields were first isolated on both sides (by utilizing a midline skin incision), then the nerve to the left was crushed to evoke regeneration. As expected, anti-NGF treatment prevented expansion of the isolated (right) field, but normal sensory function was restored by nerves regenerating to the left side. Even a 3-fold increase in anti-NGF dosage failed to affect regeneration (Table 1).

Results from Mice. This preliminary study gave results essentially identical to those from the rats. The 3-fold increased dosage of anti-NGF serum (the most stringent testing regime used on the rats) totally prevented collateral sprouting of pinch-sensitive nerves, but in the same animals axonal regeneration was unaffected, as compared to untreated controls.

Pathways Followed by Regenerating Axons. Fibers regenerating within rat skin, with or without anti-NGF treatment, were observed to grow along the identical pathways that sprouting fibers follow in the absence of treatment (14, 15)—namely, the dermal perineurial tubes (Fig. 4), and for unmyelinated fibers exclusively, the subepidermal horizontal Schwann tubes also (Fig. 5); the reoccupancy of the Schwann tubes by regenerated fibers was only partial, just as found previously for normal collateral sprouting (15).

Regeneration after Elimination of Possible Central Sources of Trophic Support. Nerves continued to regenerate successfully after their central projections were eliminated by dorsal rhizotomy (cf. ref. 27), and this seemed not to be affected by anti-NGF treatment. In the rhizotomized rats sensory C fiber

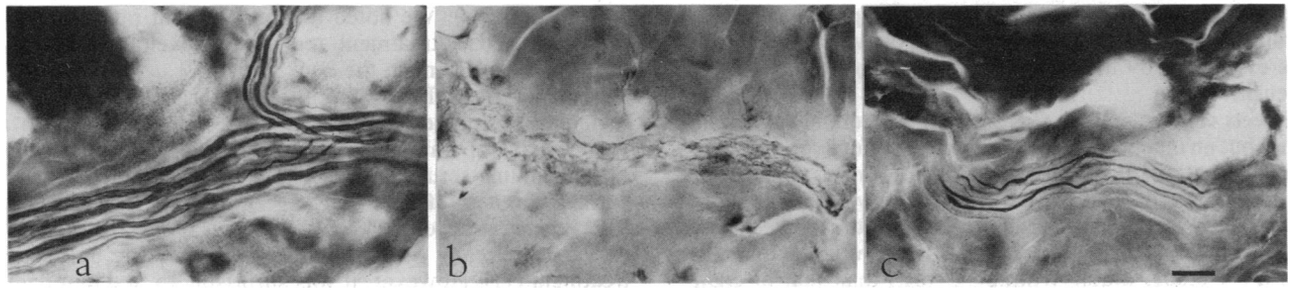

FIG. 4. Thirty-micrometer-thick silver-stained sections of rat skin, all showing perineurial tubes within the dermis. (*a*) Normal skin, with typical parallel bundles of axons. (*b*) Insensitive skin immediately surrounding a mDCN-T13 field that had been isolated 34 days earlier but prevented from expanding by daily anti-NGF treatment; in the absence of such treatment such "empty" perineurial tubes (which were typical of denervated, insensitive skin), would have contained sprouted fibers (14). (*c*) Another skin sample from the same rat as in *b*, but in this instance from an area on the opposite side that had been reinnervated by *regenerating* axons from the opposite, crushed, mDCN-T13, and displayed good pinch and moderate heat sensitivity. The calibration (20 μm) refers to *a* and *b* also.

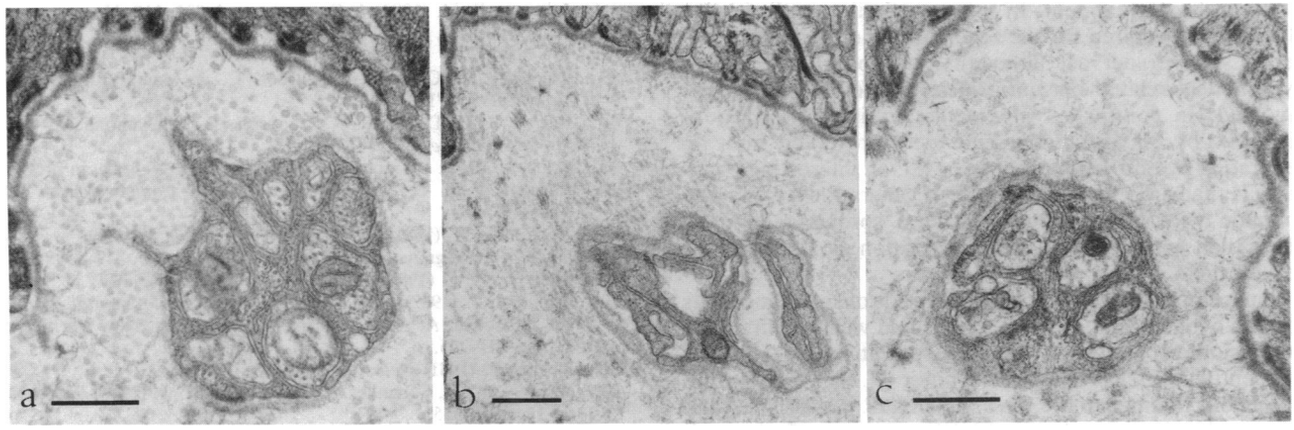

FIG. 5. Electron micrographs of portions of the subepidermal horizontal fiber network in rat skin; each shows a transverse section across an epidermal "gutter" containing a Schwann tube surrounded by a basal lamina. In *a* and *c* these tubes contain a number of unmyelinated axon profiles; in *b* only an "empty" (axon-free) Schwann tube is present. (*a*) Normal skin. (*b*) The same insensitive skin shown in Fig. 4*b*, which was adjacent to a mDCN-T13 field whose expansion was prevented by daily anti-NGF treatment. Had expansion been allowed the electron microscopic examination would have revealed C fibers both in the dermal perineurial tubes and in some of the subepidermal Schwann tubes (15). (*c*) Region of skin on the opposite side of the same animal as *b* that had been reinnervated by regenerating axons (compare Fig. 4*c*) and had recovered both pinch and heat sensitivity; unmyelinated axons are present within this Schwann tube. Calibrations = 0.5 μm.

reinnervation was revealed by the characteristic Evans blue extravasation when the peripheral nerve was electrically excited (Fig. 6; cf. ref. 15), and that of Aα axons was revealed by the recording of mechanosensory impulses; presumably the Aδ axons (not examined) regenerated similarly.

DISCUSSION

Collateral Sprouting and NGF. Our anti-NGF serum seemed comparable in potency to that used effectively in other investigations (e.g., refs. 4 and 28); we suggest then that collateral sprouting of nociceptive axons is normally evoked by endogenous NGF operating in adult rats at levels similar to those that during development regulate the differentiation and maintenance of autonomic and sensory neurons. We do not yet know the source of this NGF (cf. ref. 29), but in the denervated iris NGF has been localized to degenerating nerve pathways, probably to the Schwann cells (ref. 30 and see below). Nor do we know if NGF production increases after denervation or if its effective level in skin is determined, e.g., by uptake in local axons, as suggested for the iris (refs. 31–33, cf. refs. 34 and 35), in which *in vivo* levels of mRNA for NGF are unchanged after denervation (36).

Table 1. Return of nociceptive function by regenerating nerves unaffected by anti-NGF treatment

Group	Time, days		
	To first pinch response	To first heat response	To restoration of original heat and pinch fields
Normal (n = 4)	>8, <14	13–25	25–35
Anti-NGF	>8, <14 (n = 2)	25 (n = 2)	28–35 (n = 5)
3× anti-NGF (n = 4)	>8, <20	14–27	30–35

Times for recovery of pinch and heat sensitivity by *regenerating* nerves in normal animals (top row), animals receiving a daily anti-NGF treatment adequate to prevent sprouting (middle row), and animals receiving a 3 times larger dosage than this (bottom row). Examinations were done only at the end of the first and second weeks after nerve crush, and then every 3–5 days thereafter. Since the regeneration distances varied by as much as 5 mm among the animals, the various times to recovery are only approximate. There were no obvious differences between the three groups, however, either in the times when pinch and heat responses were first detected or the times to restore the originally measured pinch and heat fields.

Regeneration Is Independent of NGF. The redevelopment, and even *expansion*, of functional nociceptive fields by nerves regenerating after crush was unaffected by an anti-NGF regime even 3 times that which totally blocked collateral sprouting of intact nerves, both in rats (Table 1) and in mice. Could the regenerating axons have been inaccessible to the circulating antibodies? This seems unlikely. First, the normal perineurial hindrance to macromolecules is strikingly disrupted in regenerating peripheral nerves (reviewed in ref. 37). Second, within the skin itself regenerating fibers utilized the same cellular pathways as did collaterally sprouting ones, and the latter were certainly accessible to the anti-NGF treatment. This also argues against the possibility that the regeneration was evoked by a transfer of NGF [perhaps of Schwann cell origin (38, 39)] from the low-affinity NGF receptors that appear on Schwann cells in degenerating pathways (40) to the high-affinity receptors of the axolemma (41). Nor can the regeneration depend on a central source of NGF (42), since it occurred after dorsal rhizotomy combined

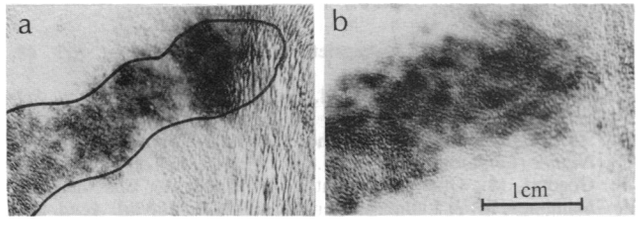

FIG. 6. Evans blue extravasation test for sensory C fibers. In animals preloaded with the dye, the entire DCN-T13 on one side was excited electrically by a stimulus selected to evoke the long-latency cutaneous trunci muscle reflex [caused by activity in the unmyelinated heat-nociceptive axons (14, 15)]. (*a*) Typical area of dye extravasation; the subsequently determined heat-nociceptive field of the same nerve is outlined. (*b*) The central projections of the stimulated nerve (and also of DCNs T11–L2) had been eliminated by dorsal rhizotomy 31 days earlier, at which time the DCN-T13 was crushed to evoke its regeneration, and daily anti-NGF treatment was begun. Although the cutaneous trunci muscle reflex could not be utilized, unmyelinated sensory axons were clearly shown to have regenerated by the positive Evans blue test; the large touch-sensitive axons had also regenerated, and their endings, revealed electrophysiologically, were distributed over approximately the same area. Such regeneration, in the absence of dorsal roots, was essentially similar to that obtained when no anti-NGF treatment was given.

with anti-NGF treatment. In sum, our findings indicate that NGF is not required for the regeneration of damaged cutaneous sensory nerves, although it is conceivable that a hypothetical related but antigenically different molecule is involved. In contrast, *sympathetic* terminal arborizations disrupted by 6-hydroxydopamine were prevented from regenerating by anti-NGF treatment (43); an interesting possibility is that such "arborizational" regeneration has more in common with target-related collateral sprouting of intact fibers than with conventional regeneration along peripheral nerve trunks.

Implications of Findings. The proposed role of endogenous NGF in evoked collateral sprouting could well be a dramatic expression of a normal ongoing regulation by NGF of nociceptive terminal fields in the adult (cf. ref. 44). Extrapolating our findings to the CNS, where mRNA for NGF and NGF itself (45, 46), and axonal NGF receptors (47), have all been demonstrated, an analogous ongoing regulation of axonal terminal fields by growth factors can be postulated as one mechanism of plasticity *in the adult CNS*. An implication of this reasoning could be that the terminals involved are continually turning over (cf. refs. 48–51); in the electron microscope we often observe "degenerated" terminal profiles in the subepidermal sensory C fiber network, not far from perfectly normal ones (unpublished data).

The *functional* implications of collateral sprouting imply a degree of selectivity in the mechanisms evoking it. However, the readiness with which virtually all nerves, peripheral (52) and central (18), will regenerate along accessible peripheral nerve pathways suggests the operation of relatively nonselective growth-promoting influences (cf. ref. 53). Both NGF-like activity (54) and non-NGF neurotrophic substances (55) appear in degenerating peripheral nerve pathways, with time courses (56) appropriate to support both the collateral sprouting and the NGF-independent regeneration we studied. The respective availability and effectiveness of different growth stimuli could also underlie other of the apparent differences (see the introduction) between the axonal elongation, or regeneration, of severed axons and the collateral sprouting of intact ones.

Finally, nerve growth factors are often identified by techniques (especially *in vitro*) that do not distinguish between axonal regeneration and collateral sprouting. This could be an important distinction to make for any prospective therapeutic use of growth factors to promote recovery of function within the injured nervous system.

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