Neurite Outgrowth on Cryostat Sections of Innervated and Denervated Skeletal Muscle

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Abstract. To localize factors that guide axons reinnervating skeletal muscle, we cultured ciliary ganglion neurons on cryostat sections of innervated and denervated adult muscle. Neurons extended neurites on sections of muscle (and several other tissues), generally in close apposition to sectioned cell surfaces. Average neurite length was greater on sections of denervated than on sections of innervated muscle, supporting the existence of functionally important differences between innervated and denervated muscle fiber surfaces. Furthermore, outgrowth was greater on sections of denervated muscle cut from endplate-rich regions than on sections from endplate-free regions, suggesting that a neurite outgrowth–promoting factor is concentrated near synapses. Finally, 80% of the neurites that contacted original synaptic sites (which are known to be preferentially reinnervated by regenerating axons in vivo) terminated precisely at those contacts, thereby demonstrating a specific response to components concentrated at endplates. Together, these results support the hypothesis that denervated muscles use cell surface (membrane and matrix) molecules to inform regenerating axons of their state of innervation and proximity to synaptic sites.

WHEN motor axons reinnervate skeletal muscle fibers after nerve injury, their behavior is influenced by cues that muscles provide. Thus, while innervated muscles are refractory to hyperinnervation, denervated muscles readily accept synapses, demonstrating that muscles regulate their susceptibility to synapse formation in accordance with their current state of innervation. Furthermore, while new ("ectopic") synapses sometimes form on extra-synaptic membrane, neuromuscular junctions are preferentially established at original synaptic sites, suggesting that axons can find and recognize cues concentrated at these sites. Finally, axons differentiate into nerve terminals only in regions where they contact muscle fibers, implying the existence of cues that trigger or organize this differentiation (reviewed in Sanes and Covault, 1985). A variety of cell membrane and extracellular matrix molecules have been suggested as mediators of these interactions, based largely on immunohistochemical studies of innervated and denervated muscle (e.g., Sanes and Chiu, 1983; Covault and Sanes, 1985; Rieger et al., 1985; Sanes et al., 1986; Moore and Walsh, 1986; Chiu et al., 1986). Antibodies to some of these molecules have been shown to perturb axonal behavior in vitro (e.g., Matthew and Patterson, 1983; Rutishauser et al., 1983; Bixby et al., 1987). However, which, if any, of these molecules are active in vivo remains unknown, in large part due to the difficulty of carrying out or interpreting experiments with antibodies in whole animals.

To bridge the gap between studies of axon guidance in intact animals and in culture, we have developed a technique which exploits the advantages of in vitro methods to examine axon guidance by tissue elements. Our approach has been to culture dissociated neurons on cryostat sections of rat tissues. In initial experiments, we found that neurons extend neurites on sections of several adult tissues including muscle, and that patterns of outgrowth reflect interactions of neurites with sectioned cell surfaces. Thus, our method may be useful for examining factors that guide axon outgrowth in a variety of tissues. Subsequently, we focused on the behavior of neurons cultured on sections of skeletal muscle. We found that neurite outgrowth is greater on sections of denervated muscle than on sections of innervated muscle. Additionally, outgrowth was greater on sections of denervated muscle taken near synaptic sites than on sections taken far from synaptic sites. Finally, neurites generally ceased to grow upon contact with a synaptic site. These results provide both new data on the localization of factors that denervated muscles could use to guide regenerating axons, and a bioassay that can be used to identify specific molecules involved in axonal guidance.

Materials and Methods

Cryostat Sections

Tissues from adult Sprague-Dawley rats (Sasco, Omaha, NE) were mounted in Tissue-Tek (Miles Laboratories, Inc., Naperville, IL), frozen in liquid N2-cooled isopentane and sectioned at 6 μm in a cryostat (Hacker Instruments, Fairfield, NJ). Ribbons of 2-4 sections were collected on ethanol-
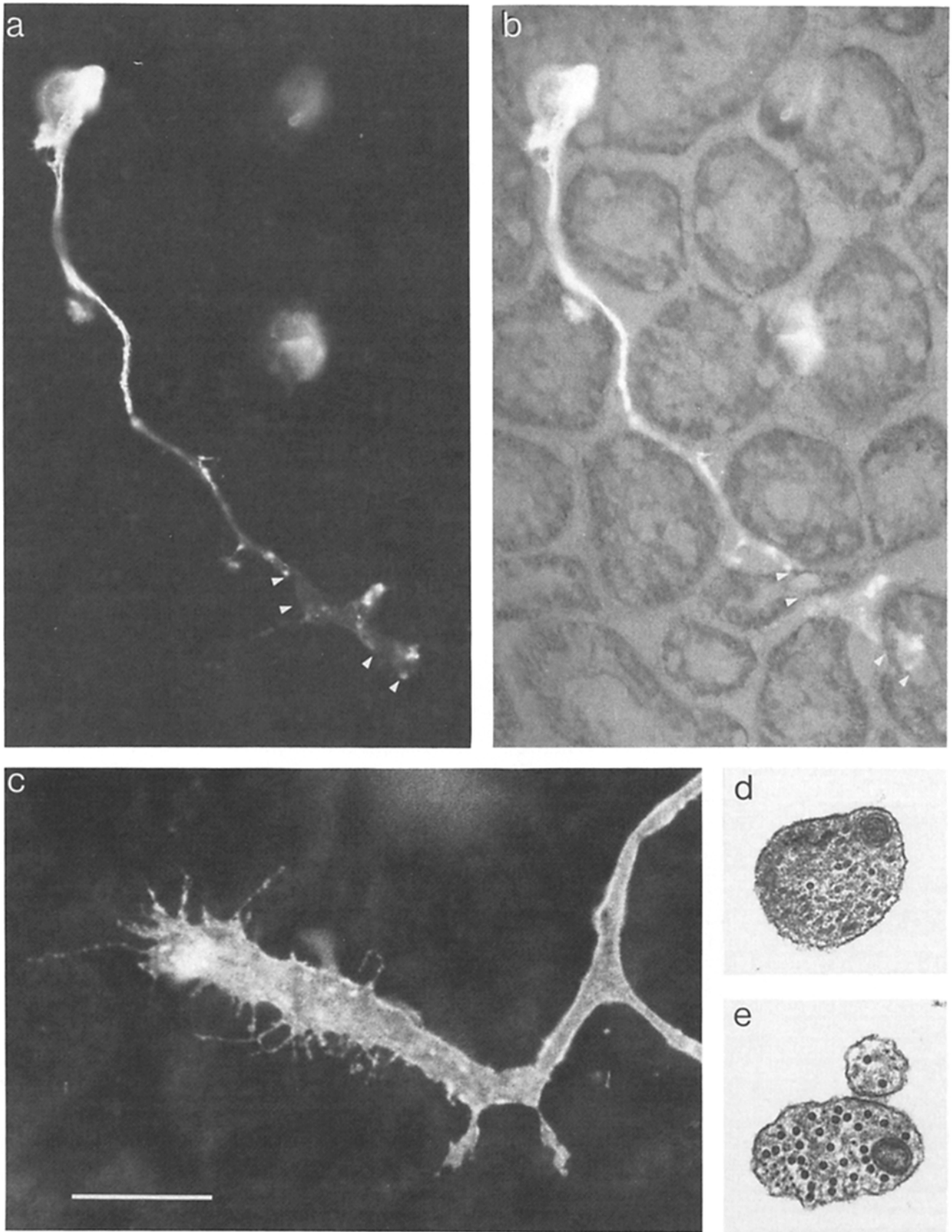
Figure 1. Neurons extend neurites on cryostat sections. (*a*) A ciliary ganglion neuron, cultured for 3 d on a cryostat section of kidney, then fixed, stained with anti–NCAM, and photographed using fluorescein optics. (*b*) Same field counterstained with Coomassie Blue and photographed with both bright-field and fluorescence illumination, to show that the neurite follows the surface contours of cross-sectioned tubules. Arrows in *a* and *b* indicate stretches of the advancing growth cone that have extended over intracellular areas. (*c*) Higher magnifica-

sterilized 12-mm–diameter glass coverslips. Sections were used within 6 h or stored for up to 1 wk at −70°C. In some experiments, strips of innervated and denervated rat diaphragm were frozen as a composite tissue block containing alternating layers of innervated and denervated muscle. Muscle blocks were prepared either from endplate-rich areas, immediately lateral to the main intramuscular nerve, or from endplate-free areas, far from the nerve (diagrammed in Merlie and Sanes, 1985). The presence or absence of endplates was confirmed at the time of sectioning by a histochemical stain for cholinesterase (Karnovsky, 1969). Left hemi-diaphragms were denervated by cutting the phrenic nerve intrathoracically (Miledi and Slater, 1968).

Cell Cultures

Ciliary ganglia from 11-d-old chick embryos were dissociated by incubation for 25 min at 37°C with 0.01% porcine trypsin (type IX; Sigma Chemical Co., St. Louis, MO), followed by trituration, as described by Nishi and Berg (1977). Dissociated neurons were pipetted onto cryostat section-bearing cover slips, which had been placed into the 16-mm diameter wells of 24-well tissue culture plates. Approximately 0.5–1 ganglion equivalent was added to each well, in 0.25 ml of MEM containing Earle's salts, 10% heat-inactivated FBS, 5% chick embryo extract, 10 U/ml penicillin, and 10 μg/ml streptomycin. Cultures were incubated at 37°C in a humidified atmosphere containing 7% CO_2.

Histology

For staining, cultures were fixed in methanol at −20°C, then rehydrated with PBS (150 mM NaCl, 15 mM sodium phosphate, pH 7.5), and incubated for 30–60 min at room temperature with antibodies diluted in PBS containing 10 mg/ml BSA. Neurons were stained with polyclonal (Covault and Sanes, 1985) or monoclonal (Lemmon et al., 1982; Cole and Glaser, 1986) antibodies to chicken neural cell adhesion molecule (NCAM)[1]. In some experiments, anti-laminin was also included, to stain basal lamina in the cryostat sections. Cultures were then washed in PBS–BSA, and incubated with fluorescein goat anti-mouse IgG + IgM and/or rhodamine goat anti-rabbit IgG (Cooper Biomedical, Malvern, PA; Jackson Immunoresearch, Avondale, PA; Atlantic Antibodies, Scarborough, ME). To identify synaptic sites in muscle sections, rhodamine-α-bungarotoxin (Ravdin and Axelrod, 1977), which binds to acetylcholine receptors in the postsynaptic membrane, was mixed with the second antibody. Rhodamine-α-bungarotoxin unambiguously marks synaptic sites in denervated as well as in innervated muscles, because the extrajunctional receptors that appear after denervation are insufficiently dense to be readily detected with this stain (<10^3/μm²) while junctional accumulations persist (>10^4/μm²) (Salpeter and Loring, 1985; see Covault and Sanes, 1985, and Sanes et al., 1986, for examples). Stained cultures were mounted in glycerol containing phenylenediamine (Johnson and Araujo, 1981) and examined with filters selective for fluorescein or rhodamine.

Images of neurons were collected on video tape using a Silicon Intensifier Target (SIT) camera (Dage, Michigan City, IN) and a video recorder. Later, images were traced from the video monitor onto acetate sheets and neurite lengths measured from tracings using the morphometry subroutine of the IMAGR program (Voyvodic, 1986). Neurons whose cell body or processes contacted other neurons, blood vessels, or nerve trunks were excluded from analysis.

For electron microscopy, cultures were fixed overnight in 2% glutaraldehyde plus 2% paraformaldehyde, refixed in 1% OsO_4, and embedded in Araldite.

Results

Neurite Outgrowth on Cryostat Sections

In initial experiments, neurons dissociated from embryonic chick ciliary ganglia were plated on cryostat sections of sev-

eral adult rat tissues, including brain, spinal cord, sciatic nerve, and kidney, as well as muscle. From 8 h to 7 d later, the cultures were fixed, stained with antibodies to chick NCAM, and viewed with fluorescence optics. Neurons settled and extended processes on sections of all tissues tested. Both neuronal somata and neurites were brightly stained by anti–chicken NCAM (Fig. 1 a). Neurites terminated in growth cones, which frequently bore broad lamellopodia and long filopodial extensions (Fig. 1 c). Electron microscopy revealed microtubule- and neurofilament-rich neurites growing either singly (Fig. 1 d) or in groups of two or three (Fig. 1 e). Neurite outgrowth was observed within 8 h of plating, and average neurite length increased for at least 2 d. Neurons remained viable for up to 1 wk.

On each tissue tested, most neurites were closely associated with sectioned cell membranes and/or adjacent areas of extracellular matrix. Because membrane and matrix are not distinguishable in the cryostat sections, we refer to them together as the "cell surface." For example, the gently undulating course of the long, unbranched neurite seen growing on a section of a kidney in Fig. 1 a is revealed by counterstaining to reflect precisely the surface of cross-sectioned renal tubules (Fig. 1 b). Similarly, on sections of muscle, neurites were generally associated with cell surfaces and avoided intracellular regions of sectioned muscle fibers (Fig. 2 a). In a group of 110 neurons from two cultures, an average of 86% of the neurites' length (±15%, SD) was within 2 μm of a muscle fiber surface. Another striking demonstration of the tendency of neurites to grow on cell surfaces was provided by comparing sciatic nerves sectioned in different planes. On longitudinal sections, neurites were long and oriented parallel to each other and to the underlying nerve fibers (Fig. 2 c). On cross-sections, in contrast, neurites grew in nearly circular arcs that corresponded to individual axon-Schwann cell units (Fig. 2 b). Neurons plated on brain sections had highly branched neurites which exhibited no regular orientation (Fig. 2 d), as expected for a tissue in which cell surfaces are closely packed and intricately intertwined.

These differences in the patterns of neurite outgrowth on cryostat sections from different tissues suggest that the advancing growth cone interacts with and makes choices among tissue elements present in the sections. A trivial alternative, that tissues mask portions of a uniformly attractive, underlying substrate can be dismissed: neurons that settled on the glass coverslip rather than on a section never extended neurites (Fig. 2 e). It is also unlikely that neurites are physically restricted to areas abutting cell surfaces: while neuritic shafts remained close to the cell surface, advancing growth cones and their filopodia frequently extended across sectioned intracellular regions (e.g., Fig. 1, a and b). This pattern suggests that the growth cones had access to the entire section but "chose" to follow cellular contours. To further distinguish between chemical and mechanical sources of neurite guidance, we assessed outgrowth on sections of muscle and kidney that had been irradiated with ultraviolet light (1–2 h at 30 cm from a germicidal lamp) to denature proteins;

1. *Abbreviation used in this paper:* NCAM, neural cell adhesion molecule.

tion micrograph of a growth cone terminating a neurite in the same culture, to show NCAM-rich filopodia emanating from the central, lamellopodial area. (d and e) Electron micrographs of neurites from a similar culture. Wisps of extracellular matrix from the cryostat section are not visible here, but were more prominent and clearly associated with neurites in other sections. Bar: (a and b) 40 μm; (c) 20 μm; (d and e) 0.5 μm.
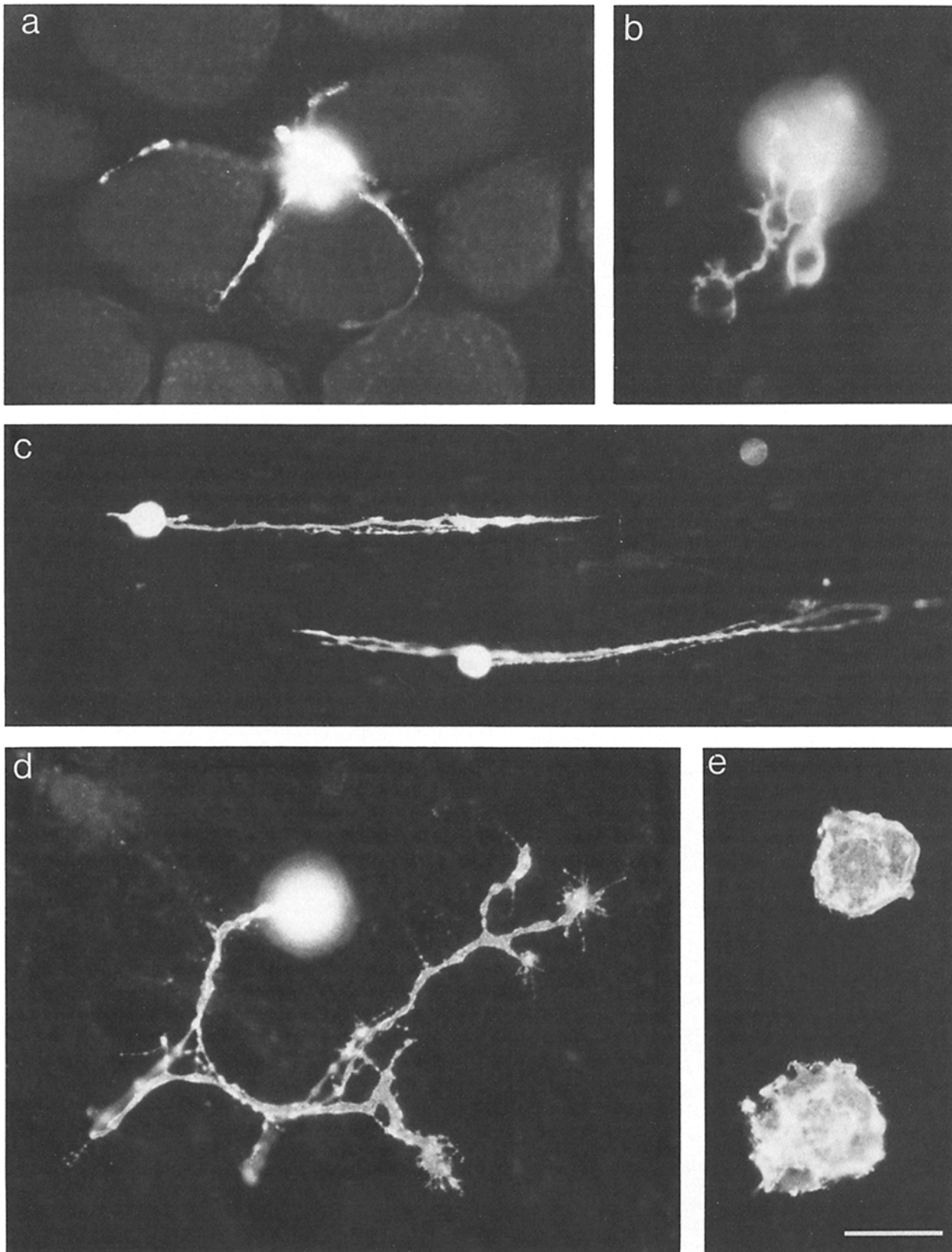
Figure 2. Neurite outgrowth reflects the architecture of the sectioned tissue. NCAM-stained neurons grown on cryostat sections of cross-sectioned muscle (*a*), cross-sectioned sciatic nerve (*b*), longitudinally sectioned sciatic nerve (*c*), cerebral cortex (*d*), or on uncoated glass (*e*). Outlines of muscle fibers are visible in *a*; neurites appose fiber surfaces. Counterstaining of sciatic nerve (not shown) revealed that neurites followed longitudinally sectioned and encircled cross-sectioned endoneurial tubes. Bar: (*a*, *b*, *d*, and *e*) 25 μm; (*c*) 50 μm.
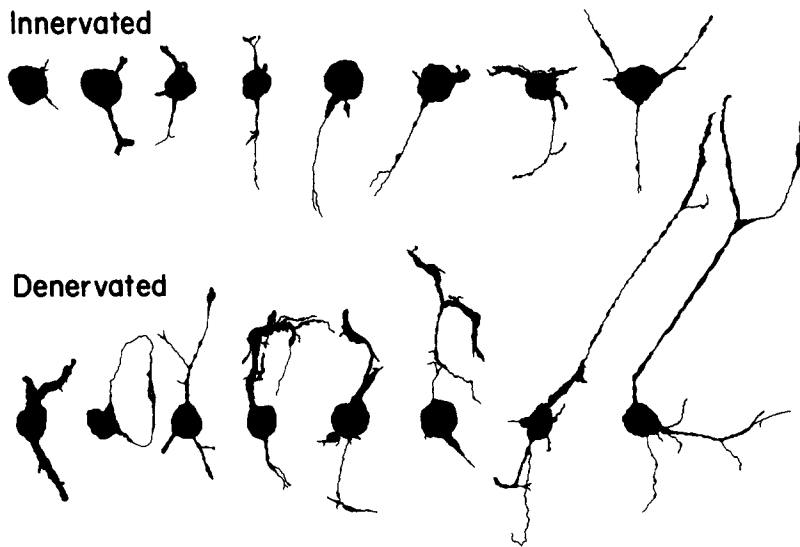
2482

Innervated

Denervated

Figure 3. Neurons growing on cryostat sections of innervated and denervated muscle in a single culture, which was fixed and stained 40 h after neurons were plated. 25 neurons on each section were photographed and arranged in order of total neurite length; every third neuron in the series was then traced. On average, neurites are longer, broader, and more highly branched on sections of denervated muscle.

Hammarback et al. (1985) have shown that irradiation of laminin substrata by this protocol reduces their adhesivity for neurons without obvious mechanical description of the surface. In both kidney and muscle, neurite outgrowth was markedly reduced by irradiation (not shown). Finally, in preliminary experiments, we have found that antibodies to adhesive macromolecules inhibit neurite outgrowth on cryostat sections in a tissue specific manner: anti-rat NCAM inhibits growth on brain but not on sciatic nerve, while an antibody to a laminin–heparan sulfate proteoglycan complex (Chiu et al., 1986) inhibits outgrowth on nerve but not on brain (Covault and Sanes, 1987, and manuscript in preparation). Together, these results argue strongly that neurite outgrowth on cryostat sections is organized in large part by specific chemical interactions rather than by physical irregularities in the sections.

Length of Neurites on Sections of Innervated and Denervated Muscle

Innervated skeletal muscle fibers are refractory to hyperinnervation, while denervated fibers readily accept synapses. It has been suggested that the increased susceptibility of denervated fibers to innervation is due, in part, to an increased attractiveness of their surface to axons (Brown et al., 1981; Covault and Sanes, 1985; Sanes and Covault, 1985; Chiu et al., 1986). To ask whether an effect of this sort could be detected in our culture system, we compared outgrowth from neurons plated on cryostat sections of innervated and denervated rat muscle. Strips from hemidiaphragms that had been denervated 7–10 d previously were mounted, frozen, and cross-sectioned together with strips of the innervated, contralateral hemidiaphragms, allowing us to assess neurite outgrowth on innervated and denervated muscle from the same animal in a single culture.

Fig. 3 shows representative neurons growing on cryostat sections of innervated and denervated muscles in the same culture, while Fig. 4 compares the distribution of neurite lengths for an entire population of neurons in another experiment. On average, neurites on denervated muscle were longer, broader, and more highly branched than those on innervated muscle. Fig. 5 *a* summarizes results from 29 ex-

periments. Average neurite length was greater on denervated than on innervated muscle in 27 of the 29 experiments. Overall, the ratio of average neurite lengths on denervated and innervated muscle was 1.71 ± 0.15. Thus, as measured by neurite length, neurons prefer denervated to innervated muscle.

The increased length of neurites on denervated as compared to innervated muscle could result from poor attach-
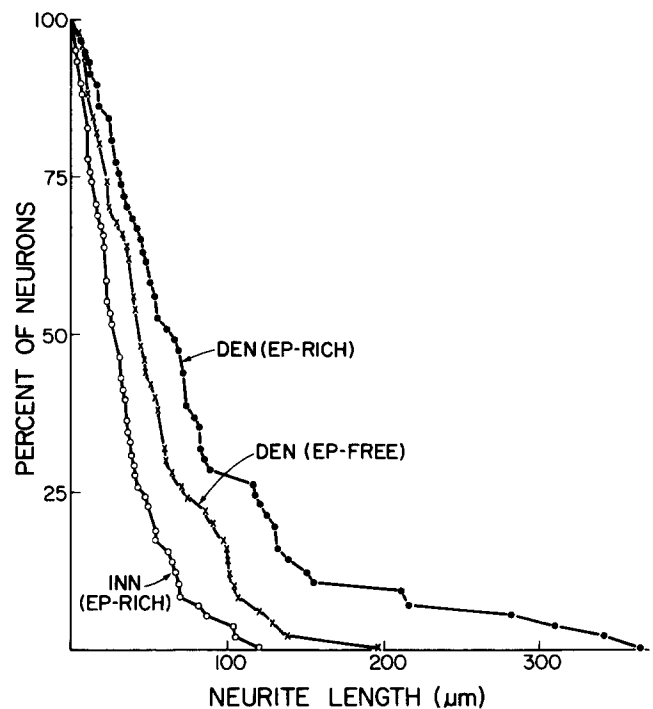

Figure 4. Neurite outgrowth on sections of innervated muscle near endplates (*INN* [*EP-RICH*]) and of denervated muscle near to (*DEN* [*EP-RICH*]) and far from (*DEN* [*EP-FREE*]) endplates, all in a single culture. The culture was fixed and stained 35 h after neurons were plated. Distributions of neurite lengths are plotted by the method of Chang et al. (1987), in which the ordinate shows the percentage of neurons with neurites longer than the values on the abscissa. About 50 neurons on each section were measured.
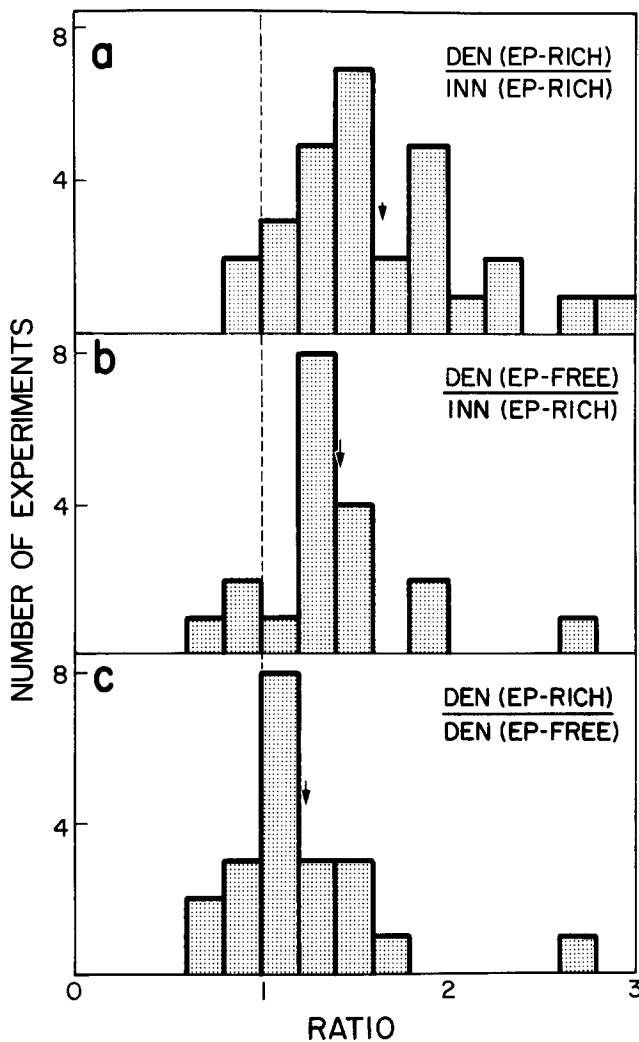
Figure 5. Ratios of average neurite lengths on endplate-rich sections of innervated muscle (*INN [EP-RICH]*), endplate-rich sections of denervated muscle (*DEN [EP-RICH]*) and endplate-free sections of denervated muscle (*DEN [EP-FREE]*). Each experiment represents a single culture. Cultures were fixed and stained from 11 to 40 h (average 30 h) after neurons were plated, and 20–50 neurons per section were measured. Mean ratios (indicated by *arrows*) are 1.71 ± 0.15, 1.41 ± 0.10, and 1.23 ± 0.09 (± SEM) in *a–c*, respectively.

ment of neurons to innervated muscle, with consequent difficulty in initiating outgrowth, or from an increased net rate of outgrowth on denervated muscle. Indirect evidence against the first alternative is that similar numbers of neurons remained attached to sections of denervated and innervated muscle throughout the culture period (ratio of 1.16 ± 0.1, n = 7; ratio of neurite lengths was 1.9 ± 0.2 in these cultures). Evidence in favor of the second alternative was obtained by measuring neurites in cultures fixed 8–42 h after neurons were plated (Fig. 6). Neurite outgrowth proceeded at a faster rate on denervated than on innervated muscle throughout this period, suggesting that increased neurite length on denervated muscle is due, at least in part, to an increased net rate of outgrowth.

In the experiments described above, neurite outgrowth was assessed on cryostat sections taken from the central, endplate-rich region of the diaphragm. Previously, we found that

several adhesive macromolecules preferentially accumulate in interstitial spaces near denervated synaptic sites (Sanes et al., 1986). It was therefore possible that increased neurite outgrowth on denervated muscle reflected either differences between innervated and denervated muscle in general, or some special property of endplate-rich areas. To distinguish these alternatives, we assessed neurite outgrowth on sections from endplate-free areas of denervated diaphragm. Average neurite length was greater on endplate-free sections of denervated muscle than on either endplate-rich or endplate-free sections of innervated muscle (ratios of 1.4 ± 0.1, n = 19, and 1.5 ± 0.2, n = 4, respectively; Figs. 4 and 5 *b*). However, while neurite outgrowth on endplate-free sections of denervated muscle was greater than that on innervated muscle, it was significantly less than that on endplate-rich areas of denervated muscle (ratio of 0.89 ± 0.05, with a ratio of <1 in 16 of 21 experiments; Fig. 5 *c*). Thus, the increased response of neurites to endplate-rich regions of denervated diaphragm can be divided into two components: a difference between innervated and denervated muscles, and a difference between perisynaptic and nonsynaptic areas of denervated muscles.

Interaction of Neurites with Original Synaptic Sites

When axons reinnervate skeletal muscles, they preferentially reinnervate original synaptic sites, a selectivity mediated at least in part by components of the basal lamina (Sanes et al., 1978). Synaptic sites are easily recognized in cryostat sections, in that they can be specifically stained with rhodamine-α-bungarotoxin; however, they occupy <1% of the muscle fiber surface even in sections cut from endplate-rich regions.
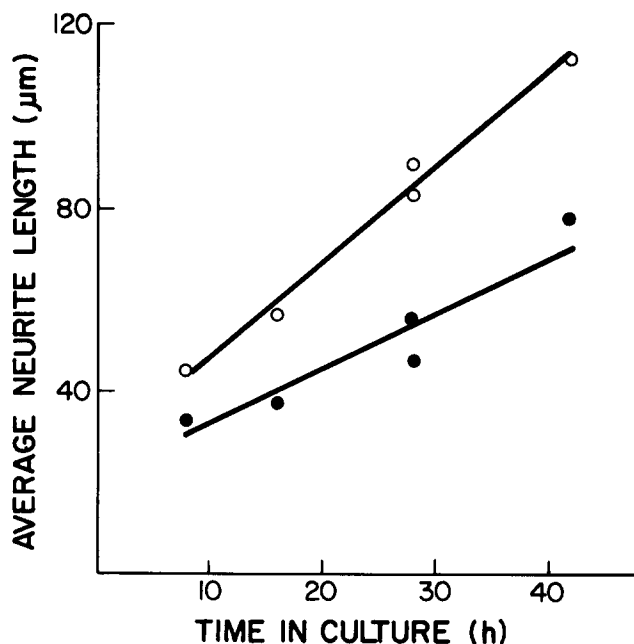

Figure 6. Average neurite length on endplate-rich sections of innervated (●) and denervated (○) muscle, at various times after neurons were plated. Each point represents 20–40 neurons from a single culture, all from a single plating. The net rate of neurite outgrowth was greater on denervated than on innervated muscle during this period.
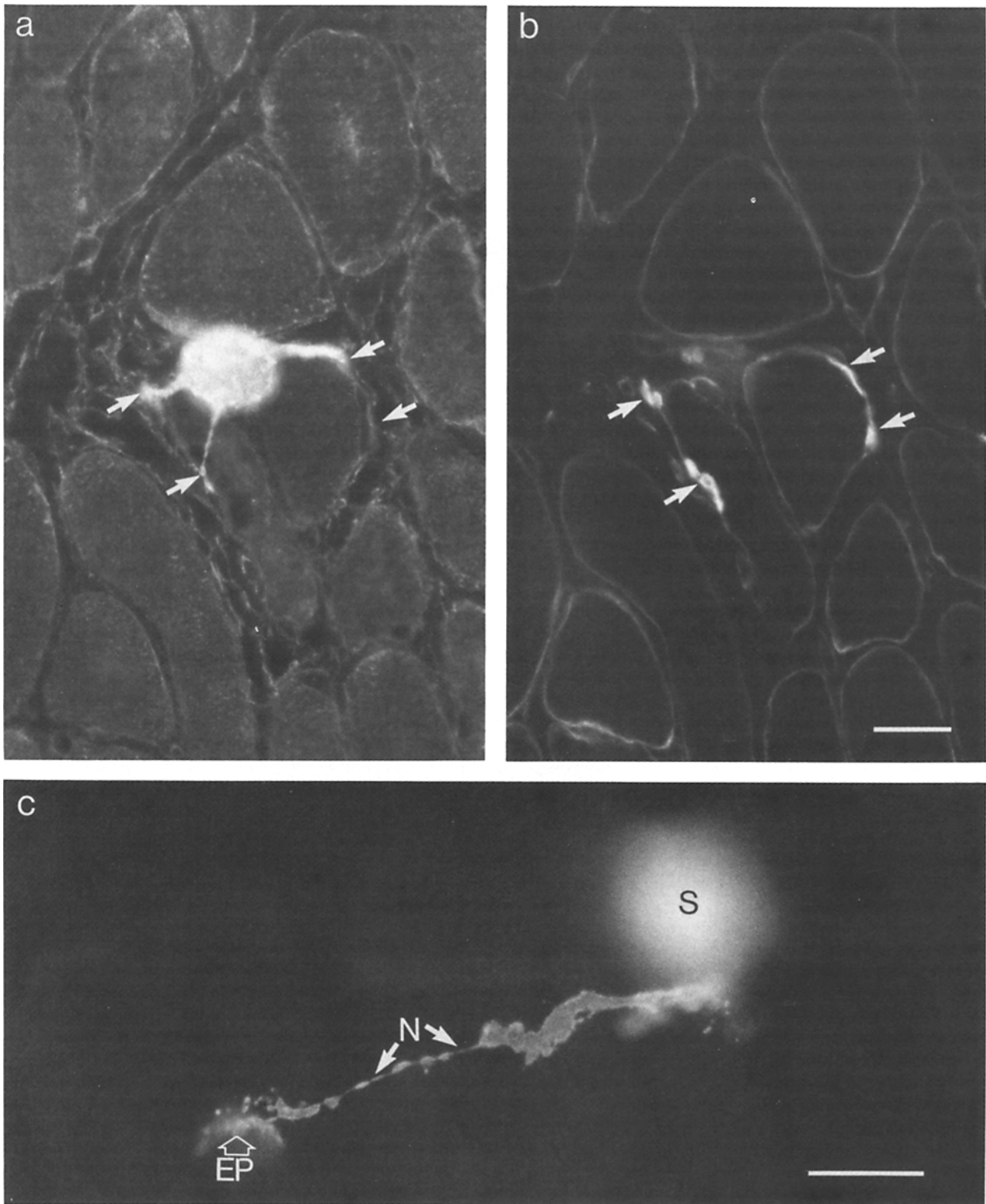
Figure 7. Neurites terminate at synaptic sites on cryostat sections of muscle. (*a* and *b*) This culture was triply stained with rabbit anti–NCAM plus fluorescein goat anti–rabbit IgG to show the neuron and the underlying denervated muscle fibers; with rhodamine-α-bungarotoxin to mark synaptic sites (*arrows*); and with mouse anti–laminin plus rhodamine goat anti-mouse IgG to outline muscle fibers. Fluorescein optics (*a*) shows a neuron that has extended three neurites. Rhodamine optics (*b*) shows that each neurite has terminated within 1 μm of a synaptic site. (*c*) Another culture was doubly stained with anti–NCAM, rhodamine-second antibody, and rhodamine-α-bungarotoxin to show, in one micrograph, the termination of an anti–NCAM-stained neurite (*N*) at an α-bungarotoxin-stained denervated endplate (*EP*). The neuronal soma (*S*) is out of the plane of focus. Bars, 20 μm.

There were therefore few cases in which neurites contacted synaptic sites. Nonetheless, in a series of eight cultures, we found 30 examples of such contacts. In 24, or 80% of these cases, neurites terminated within 1 μm of the α-bungarotoxin-stained postsynaptic membrane (Fig. 7). Since neurite length averaged 40 μm per neuron in these experiments, the probability of these terminations occurring by chance in such close apposition to the synaptic cleft is very low. Thus, neurites presumably recognize components of synaptic sites in cryostat sections of muscle.

Discussion

We describe a method using cryostat sections that may be generally useful for studying axonal guidance by tissue constituents. Using this "cryoculture" technique we have shown that neurites respond to innervated and denervated muscle and to synaptic and extrasynaptic areas of muscle fibers in ways that reflect their behavior in vivo.

Neurite Outgrowth on Cryostat Sections

Our method is based on the work of Stamper and Woodruff (1976), who showed that lymphocytes bind selectively to venules in cryostat sections of lymph nodes. Use of this binding as a short-term bioassay has facilitated isolation of cell surface molecules that mediate adhesion of lymphocytes to endothelium (Woodruff et al., 1987). We adapted the approach by using neurons instead of lymphocytes, monitoring outgrowth rather than attachment, and studying events occurring over hours instead of minutes. We found that neurons extended processes on sections of many tissues and that cultures remained uncontaminated (even though sections were not cut under sterile conditions) and viable for up to a week. Recently, Sandrock and Matthew (1987) and Carbonetto et al. (1987) have also reported the use of cryostat sections as substrates for neurite outgrowth.

We had several reasons for using neurons from chick ciliary ganglia in these studies. First, the ganglion contains a pure population of cholinergic neurons whose development has been extensively studied in vivo (Landmesser and Pilar, 1978). Second, methods for culturing chick ciliary neurons are well established (Nishi and Berg, 1977). Third, the pairing of chick neurons with rat tissue sections permits the use of species-specific antibodies for selective staining or immunological blockade of either neurons or substrate. Finally, and of particular importance for studies on muscle, ∼50% of chick ciliary neurons form conventional, cholinergic neuromuscular junctions on striated muscle fibers in vivo (Pilar et al., 1980), and are also known to do so in vitro (Nishi and Berg, 1977). These neurons have therefore been used as a substitute for spinal motoneurons, which are extremely difficult to isolate, in studies of nerve–muscle interactions in vitro (e.g., Role et al., 1985; Bixby and Reichardt, 1987). In a few experiments, we have successfully cultured neurons from chick dorsal root ganglia and rat spinal cord on cryostat sections (data not shown); these or other sources may prove to be useful in other experiments.

Patterns of neurite outgrowth on cryostat sections were tissue specific, in large part reflecting association of neurites with sectioned cell surfaces. While we have focused on muscle, the cryoculture system may be useful for studies of factors that guide axonal outgrowth or differentiation in a variety of developing and adult tissues (e.g., Keynes and Stern, 1984; Tosney and Landmesser, 1985; Wigston and Sanes, 1985). However, the use of cryocultures for investigating neurite–tissue interactions depends on knowing that tissue-specific patterns of outgrowth reflect specific molecular interactions rather than purely mechanical factors. Several lines of evidence support this conclusion. First, since neurons extended no axons on glass, the association does not arise from variable masking of an underlying attractive substrate by sectioned cells. Additionally, advancing growth cones frequently extended across cells while the trailing neurites generally apposed cell surfaces, demonstrating that growth cones were not mechanically constrained within the section and suggesting that neuritic patterns resulted from active choices among potential pathways. Furthermore, outgrowth was nearly abolished by UV irradiation of the sections, a treatment that denatures proteins without disturbing tissue architecture (Hammarback et al., 1985). Finally, antibodies to two adhesive cell surface components, NCAM and a laminin–heparan sulfate proteoglycan complex, inhibited neurite extension on cryostat sections in a tissue-specific manner (Covault and Sanes, 1987, and manuscript in preparation). Thus, it seems most likely that preferential association of neurites with cell membranes and/or extracellular matrix results from specific interactions of neurites with cell surface molecules.

Neurite Outgrowth on Innervated and Denervated Muscle

Evidence from a variety of studies suggests that in vivo: (a) denervated muscle fibers are more attractive to axons than innervated muscle fibers, (b) denervated muscles are more attractive in regions near synaptic sites than in regions distant from synaptic sites, and (c) original synaptic sites themselves provide the most attractive substrate (reviewed in Sanes and Covault, 1985; Sanes et al., 1987; and see below). Phenomena related to all three of these distinctions are demonstrable in the cryoculture system, providing new evidence that denervated muscles use cell surface and/or extracellular matrix components to modulate the behavior of axons.

When motor neurons are implanted in innervated muscle, axons show limited outgrowth and form no close contacts with muscle fibers. However, if the muscle is subsequently denervated, the axons readily sprout and form synapses (Elsberg, 1917; Aitken, 1950; Korneliussen and Sommerschild, 1976). It has been shown that denervated muscles release higher levels than do innervated muscles of soluble factors that stimulate neurite outgrowth from cultured neurons, and these factors may influence axonal growth in vivo (Henderson et al., 1983; Nurcombe et al., 1984; see Slack et al., 1983, for review). However, it has been suggested that the muscle cell surface also changes upon denervation in ways that axons recognize (Brown et al., 1981; Covault and Sanes, 1985). Candidate molecules for this surface-associated signal are NCAM and a laminin–heparan sulfate proteoglycan complex, both of which increase throughout muscle after denervation (Covault and Sanes, 1985; Rieger et al., 1985; Moore and Walsh, 1986; Chiu et al., 1986). We observed increased outgrowth of neurites on denervated compared to innervated muscle sections. Since large pools of soluble factors
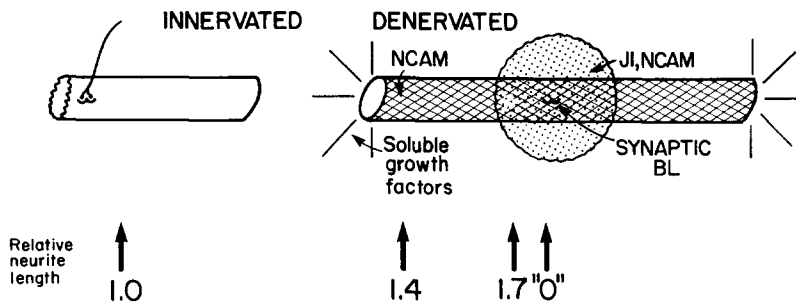
INNERVATED   DENERVATED

Relative neurite length

1.0   1.4   1.7 "0"

Figure 8. Correlation of neurite outgrowth on sections with molecular topography of innervated and denervated muscle. Some of the molecules that denervated muscles might use to influence regenerating axons are shown in the top part of the figure. Soluble neurite growth-promoting factors are released in greater amounts from denervated than from innervated muscle. NCAM appears throughout the muscle fiber surface after denervation. J1 and NCAM accumulate interstitially near denervated endplates. Antigens concentrated in the basal lamina (*BL*) of the synaptic cleft of innervated muscle persist after denervation. (See Discussion, Covault and Sanes, 1985, and Sanes et al., 1986 and 1987, for references.) This diagram is not meant to be all-inclusive, but rather to indicate that muscle-derived molecules to which neurites might respond are differentially distributed within muscles. For simplicity, some known neuroactive molecules (e.g., fibronectin, laminin, and heparan sulfate proteoglycan) are not illustrated, and we do not mean to imply that molecules already discovered account completely for axonal guidance (see, for example, Bixby et al., 1987). The bottom panel, summarizing results from Figs. 3–7, indicates that neurons extend relatively short neurites on cryostat sections of innervated muscle (arbitrarily assigned a value of 1.0), longer neurites on endplate-free sections of denervated muscle (on average 1.4 times as long as on innervated muscle), and still longer neurites on endplate-rich sections of denervated muscle (on average, 1.7 times as long as on innervated muscle); however, neurites generally terminate (indicated as "0" further growth) upon contact with an original synaptic site.

are unlikely to be maintained in these sections, our results provide evidence that differences between innervated and denervated muscle fiber surfaces are, in fact, recognized by growing axons.

Within denervated muscles, interstitial spaces between muscle fibers near synaptic sites accumulate deposits of several adhesive molecules including NCAM, J1, fibronectin, and a heparan sulfate proteoglycan (Sanes et al., 1986). (J1 is a neuron–astrocyte adhesion molecule recently isolated from mouse brain, which appears to be similar, if not identical, to "myotendinous antigen," "cytotactin," and "tenascin" in chick; Kruse et al., 1985; Chiquet and Fambrough, 1983; Grumet et al., 1985; Chiquet-Ehrismann et al., 1986.) These molecules appear to be synthesized by fibroblasts that proliferate in perisynaptic areas after denervation (Gatchalian and Sanes, 1987). We have suggested that these accumulations play a role in guiding regenerating axons to original sites which they preferentially reinnervate (Sanes et al., 1986). In the cryoculture system, neurite extension is greater on sections of denervated muscle cut from synapse-rich areas than on those cut from synapse-free areas; both types of denervated sections, however, support more outgrowth than do sections of innervated muscle. Thus, our experiments support the idea that cell or matrix-bound factors enriched in perisynaptic areas stimulate axonal growth in these regions.

A particularly striking behavior of axons reinnervating muscle is their selective reinnervation of denervated endplates: in some cases, over 95% of new neuromuscular junctions form at original synaptic sites, even though these sites occupy less then 0.1% of the muscle fiber surface. Experiments on "reinnervation" of basal lamina sheaths from which muscle fibers had been removed showed that at least some factors that axons recognize at original synaptic sites are contained in, or tightly attached to, the basal lamina of the synaptic cleft (Sanes et al., 1978; Glicksman and Sanes, 1983). The scarcity of these sites makes them difficult to study as axonal targets. Nonetheless, we noted that neurites displayed a specific response—cessation of growth—upon contacting such sites on cryostat sections. When regenerating axons

contact original synaptic sites in vivo, they differentiate into nerve terminals (e.g., accumulate synaptic vesicles). Our result raises the possibility that cessation of growth might be related to differentiation, either as prerequisite or consequence, an issue that could be addressed by ultrastructural or analysis of neurites that contact synaptic sites. Furthermore, several antibodies have been obtained that selectively stain synaptic basal lamina (Sanes and Hall, 1979; Sanes and Chiu, 1983; Anderson and Fambrough, 1983; Fallon et al., 1985), but in no case has the antigen been shown to interact with axons. The cryoculture system might permit us to assess the function of these synaptic antigens.

In conclusion, analysis of neurite outgrowth on cryostat sections of muscle reveals differences in neurite outgrowth-regulating activities between innervated and denervated muscles, synapse-rich and synapse-poor regions of denervated muscle, and synaptic and extrasynaptic portions of muscle fiber surfaces. Fig. 8 combines these data with previous immunohistochemical results (discussed above) to show that regional variations in neurite outgrowth parallel known features of the molecular topography of denervated muscle. Together, these results support the idea that denervated muscles use cell surface and extracellular matrix molecules to influence the behavior of regenerating axons, and provide a basis for immunological tests of several candidate molecules that may play a role in mediating these interactions.

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