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The Dependence of Motoneurons on Their Target Muscle During Postnatal Development of the Mouse

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Motoneurons seem to require contact with their target muscle even after embryogenesis is complete, but the consequences of target-deprivation during postnatal development are poorly understood. To examine the fate of motoneurons separated from their targets postnatally, we labeled the motoneurons that innervate the biceps brachii muscle with the retrograde tracer Fluorogold and then separated them from their muscle by amputating the forelimb. Fluorogold was subsequently found within motoneurons, as well as within much smaller cells that were identified as microglia. The number of labeled microglial cells steadily increased with time following limb amputation, while the number of labeled motoneurons declined. The magnitude of this response depended on the age of the animal: the younger the animal at the time of the amputation, the greater the number of labeled microglia and the more extensive the neuronal loss.

To ensure that the response to amputation was caused by target deprivation, rather than by the injury itself, the nerve to the biceps muscle was cut or crushed. In this way, axons were transected but target access was only temporarily denied. After the nerve was cut, motoneurons began to reinnervate the muscle within 3 weeks but, just as after amputation, the spinal cord subsequently contained labeled microglia and a reduced number of motoneurons. In contrast, after nerve crush, reinnervation began within 4 d and there was no evidence of motoneuron death. Our results demonstrate that target-deprivation causes motoneurons to be lost in an age- and time-dependent manner, and indicate a critical period after axotomy during which motoneurons must reinnervate their target in order to survive. Further, we provide evidence that microglial cells may phagocytose dying motoneurons. The approach we used would provide a convenient assay for testing candidate motoneuron growth factors in animals where *in vivo* studies of the embryo are difficult.

The vertebrate nervous system is generated by a series of complex events that, paradoxically, includes the death of a large fraction of the neurons initially generated. This cell loss occurs in many diverse regions of the developing nervous system and is often extensive. For example, almost 70% of the motoneurons generated within the lumbar region of the neural tube are lost before embryogenesis is complete (Lance-Jones, 1982). Determining the mechanisms that regulate cell death would therefore greatly contribute to our understanding of how the nervous system acquires and maintains its form.

The phenomenon of neuronal death, as well as clues about its regulation, first became apparent in studies of the relationship between avian motoneurons and their developing target muscle. These studies established a strong correlation between the number of neurons that survive the period of naturally occurring cell death and the size of their target organ. If, for example, the peripheral target is enlarged by grafting an extra limb bud to the embryo, then more motoneurons survive (Hollyday and Hamburger, 1976). Conversely, reducing the size of the peripheral target exacerbates neuronal death (Hamburger, 1958; see also Lanser et al., 1986; Tanaka and Landmesser, 1986; Comans et al., 1988). One explanation for these observations is that developing motoneurons compete for a target-derived trophic factor and that only those cells obtaining sufficient quantities of the substance are able to survive (Hamburger and Levi-Montalcini, 1949; Purves, 1980, 1988; Oppenheim, 1989; but see Lamb, 1980).

It is possible that the principles governing cell survival during embryogenesis are similar to those that maintain neurons as they mature. In particular, the target organ may continue to be important for the survival and maintenance of neurons during postnatal development. The contribution of the target to neuronal survival has been assessed in neonatal rats by examining axotomized motoneurons that were either allowed to reinnervate their former target or deliberately prevented from doing so (Kashihara et al., 1987). When target access was prevented, a substantial number of motoneurons died. This suggests that target contact ensures motoneuron survival during *early* postnatal development, but it is difficult to reconcile other studies that examine target-dependence at different times during postnatal life (Romanes, 1946; Schmalbruch, 1984; Heath et al., 1986; Lowrie et al., 1987; Comans et al., 1988; McBride et al., 1988; Snider and Thanedar, 1989). Part of this difficulty might arise from differences between these studies in several important parameters such as the means of target deprivation, duration of the survival period, and the animal species used. Therefore, even though the target clearly influences motoneuron survival during early postnatal development, how long this continues as motoneurons mature, the pattern and time course of cell loss within a motor pool, and the fate of target-deprived neurons remain unclear.

In the present study, the fate of motoneurons deprived of their target is examined throughout postnatal development in the mouse. To accomplish this, motoneurons that innervate the biceps brachii muscle were retrogradely labeled and then sep-

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arated from their target either by cutting or crushing the nerve, or by amputating the forelimb. These injuries damaged the axons in a similar way, but the resultant target deprivation varied in duration. The effects of the injury itself could, therefore, be distinguished from those caused by separation from the target. The operations were performed on mice at various stages of postnatal development, from birth to adulthood, and the motoneurons were examined up to 6 weeks later. In response to prolonged target deprivation, the number of labeled motoneurons declined in an age- and time-dependent manner; the younger the animal at the time of the injury and the longer the survival time, the more extensive the neuronal loss. Further, labeling a discrete population of motoneurons *before* they were separated from their target muscle revealed a potential role for microglial cells in the events associated with neuronal death: microglia were able to accumulate the tracer that was formerly contained within motoneurons, suggesting that these cells phagocytose degenerating motoneurons. These results help to establish that access to the target is important for neuronal survival throughout postnatal development, are compatible with the idea that motoneurons are maintained by a target-derived trophic substance, and indicate a potential role for microglial cells in postnatal motoneuron death. Portions of this study have been reported in abstract form (Crews and Wigston, 1987, 1988).

Materials and Methods

Animals. Experiments were performed on randomly sexed C57BL/6J mice obtained from Jackson Laboratories (Bar Harbor, ME) or bred on-site. They were anesthetized with chloral hydrate (350 mg/kg, i.p.) for all surgical procedures.

Motoneuron labeling. Motoneurons that project to the biceps brachii muscle were labeled with the fluorescent retrograde tracer Fluorogold (Fluorochrome Inc.). To accomplish this, the skin of the forelimb was incised parallel to the humerus, exposing the biceps brachii muscle. Approximately 1 μl Fluorogold (2% in distilled water) was pressure-injected into the muscle mass from a micropipette. The incision was sutured with 7-0 silk, and the procedure was repeatedly contralaterally. To determine the time required for the biceps motoneurons to become labeled, the spinal cord was examined 12, 24, 48, or 72 hr after the Fluorogold injection. The number of labeled cells increased as the survival times were extended from 12 to 48 hr, but there was no difference in the average number of labeled cells between 48 and 72 hr, indicating that 48 hr is sufficient time for the motoneurons innervating the biceps muscle to become labeled with Fluorogold. Bilateral injections were routinely made when the mice were either 12, 19, or >60 d old.

Surgery. After allowing 2 d for retrograde transport of Fluorogold to the neuronal cell bodies, the animals were reanesthetized, and the axons of the labeled cells were transected either by amputating the limb or by cutting or crushing the nerve branch to the biceps muscle. To amputate the limb, the skin was incised around the elbow and retracted to the shoulder, the brachial artery was ligated, the limb was severed just distal to the shoulder joint, and the skin was trimmed and sutured over the stump. The proximal tendon of biceps, and sometimes a short segment of the biceps muscle itself, remained in the stump, but the great majority of the muscle mass was removed. Alternatively, the incision made previously for the dye injection was reopened, and the branch of the musculocutaneous nerve that innervates the biceps was cut or crushed. When the nerve was cut, its proximal end retracted somewhat but remained just beneath the biceps muscle. When the nerve was crushed repeatedly with forceps instead of cut, it became transparent, but the proximal segment did not retract because the continuity of the perineuronal connective tissue was not disrupted. While motoneurons were labeled bilaterally, only one forelimb was injured. The animals recovered without apparent distress and there were no signs of infection.

Histology. Unless otherwise stated, mice were allowed to survive for either 2, 3, or 6 weeks following limb amputation. After the chosen survival time, anesthetized animals were perfused transcardially with normal saline followed by a mixed aldehyde fixative (4% paraformaldehyde, 0.2% glutaraldehyde, and 4% sucrose in 0.1 M phosphate buffer). The spinal cord, from the high cervical to the midthoracic region, was removed, embedded in gelatin–albumin, frozen, sectioned transversely at 50 μm, and mounted on gelatin-coated slides. The tissue was dehydrated in a graded series of ethanol, cleared (Histoclear; National Diagnostics), and coverslipped in DPX (Gallard-Schlesenger). Each section was examined with an epifluorescence microscope (340–380 nm excitation) using a 50× objective lens. The Fluorogold-labeled cells on the injured side were compared with those on the contralateral side of the spinal cord that were labeled but not injured. All of the labeled cells were counted. Since the plane of section may pass through some cells, we tried to avoid counting these cells twice by considering the size of the labeled cellular profile, the position of the cell within the spinal cord, the intensity of the labeling, and whether there were labeled processes. It was usually not difficult to distinguish an intact cell from a fragment; most cells had multiple labeled primary, and, frequently, secondary, dendritic processes, and even lightly labeled cells were clearly delineated. In ambiguous cases, fragments of cells were probably counted as cells as often as cells were mistaken for fragments. We attempted to minimize errors of this kind by cutting the tissue in relatively thick sections and basing our conclusions on differences between control and experimental cell populations, rather than on the absolute number of cells.

Morphometry. The size of randomly selected cells was measured using a video image-analysis system. Control motoneurons, injured motoneurons, and microglial cells were measured. Ten representatives of each of these 3 cell types were randomly selected in each of 50 animals, for a total sample of 1500 cells. Cells were chosen from along the entire rostrocaudal extent of the biceps motor pool. To measure the cells, the microscope image was projected to a video camera (Dage 67 with Newvicon tube) interfaced with a digital image processor. The cross-sectional area of the fluorescently labeled cells was measured from digitally stored images using software from Analytical Imaging Concepts. The area of each cell soma was taken as the average of 3 independent measurements.

Intracellular recording. To determine the time course of reinnervation after the nerve was cut or crushed, intracellular potentials were recorded from biceps muscle fibers. The biceps muscle was removed with its nerve intact at various times ranging from immediately to 42 d after nerve injuries performed on postnatal day 21 (P21). Muscles were pinned in a Sylgard-lined dish and bathed with mammalian Ringer's containing 6 mM Ca^{2+}. The nerve was drawn into a suction electrode and muscle fibers were impaled with a KCl-filled microelectrode. For fibers with resting potentials more negative than −40 mV, the presence or absence of miniature endplate potentials (mepps) was noted. The nerve was then stimulated, and evoked synaptic potentials were recorded.

Immunocytochemistry. Following Fluorogold labeling and limb amputation, sections of the spinal cord were incubated with antibodies to identify neurons, astrocytes, or macrophages. The tissue was prepared as described above except that glutaraldehyde was omitted from the fixative. The neuron-specific antibodies included several that were directed against neurofilaments (NFs), as well as those that recognize choline acetyltransferase, neuron-specific enolase, and an endogenous lactose-binding lectin, RL-14.5 (Regan et al., 1986). The specific monoclonal antibodies used to identify NFs included those directed against the nonphosphorylated 200 and 150 kD NF protein subunits (SMI 32, Sternberger-Meyer), the same NF proteins regardless of their state of phosphorylation (SMI 33), and antibody RT-97 (Wood and Anderton, 1981) that recognizes phosphorylated NF subunits (Developmental Studies Hybridoma Bank). The marker used for astrocytes was a polyclonal antiserum directed against glial fibrillary acidic protein (GFAP; Dako Corp.). The macrophage-specific marker Mac-1 was used in an attempt to identify microglia, which are thought to be the resident macrophages of CNS tissue.

Unless otherwise noted, free-floating 50 μm sections were preincubated with 0.5% BSA in PBS containing 0.3% Triton (BSA/PBS/Triton) for 30 min. The sections were incubated overnight at 4°C with the primary antibody (anti-NFs, 1:1000 in BSA/PBS/Triton; anti-GFAP, 1:100 in BSA/PBS/Triton; anti-Mac-1, 1:1 or 1:20 in BSA/PBS; anti-RL-14.5, 1:100 in BSA/PBS). Tissue-bound anti-NF and anti-RL-14.5 were visualized with fluorescein-conjugated goat anti-mouse IgG (Cappel), GFAP was visualized with fluorescein goat anti-rabbit IgG (Cappel) and anti-Mac-1 with fluorescein-conjugated goat anti-rat IgG, all at 1:100 for 45 min. Stock solutions of the secondary antibodies were made according to the manufacturers' recommendation. After further washing in 0.1 M phosphate buffer, sections were mounted on gelatin-

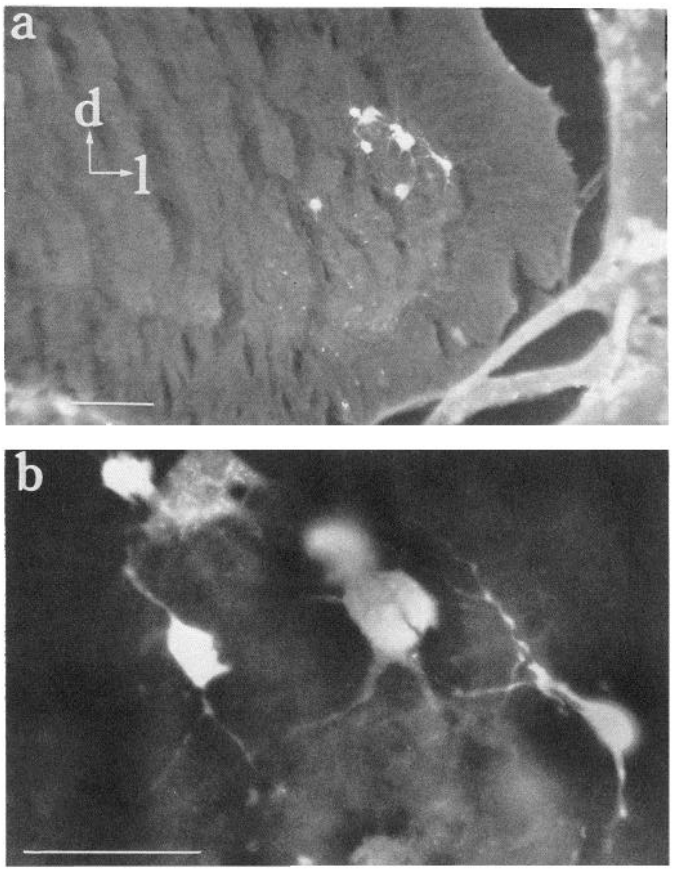


Figure 1. Photomicrographs of Fluorogold-labeled motoneurons within the spinal cord of an adult mouse. These control motoneurons were labeled by injecting Fluorogold into the biceps brachii muscle. The motoneurons that innervate the biceps muscle are invariably found in the dorsolateral region of the ventral horn (as in *a*). At higher magnification (*b*), note that the cell bodies and processes are extensively filled with Fluorogold. Dorsal is up, lateral to the right. Scale bars, 50 μm.

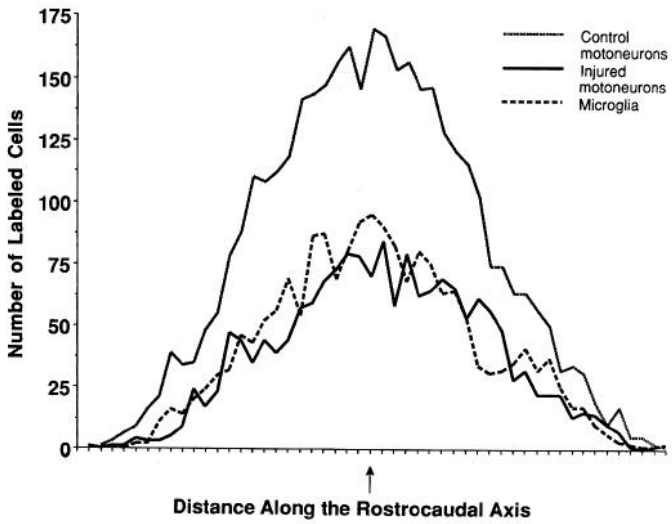


Figure 2. Distribution of Fluorogold-labeled cells along the rostrocaudal axis of the spinal cord. Displayed are the number of motoneurons on the control side of the animal, motoneurons on the injured side of the spinal cord, and microglial cells on the injured side of the spinal cord. The total number of cells counted from all groups of animals ($n = 48$) is shown for adjacent 50 μm sections of the spinal cord after limb amputation. The most rostral sections are on the extreme left. Data from adult and juvenile animals have been combined from every survival time, and the motor pools were brought into register by aligning their centers (*arrow*). Note that there is no strong preferential loss of motoneurons from any particular region of the motor pool and that the total number of labeled cells on the injured side of the spinal cord (motoneurons and ramified microglia) is approximately equal to the number of motoneurons that normally innervate the biceps muscle.

coated slides, dehydrated, cleared, coverslipped, and examined with appropriate filters.

Thiaminepyrophosphatase histochemistry. The plasma membrane-associated enzyme, thiaminepyrophosphatase (TPPase) can be used to identify microglial cells that have extended processes (ramified) within nervous tissue (Murabe and Sano, 1981). To visualize the TPPase associated with microglial cells histochemically, mice were anesthetized and perfused with 0.9% NaCl followed by 4% paraformaldehyde and 8% sucrose in 0.1 M cacodylate buffer. The spinal cord, from C1–T5, was removed, fixed for a further 4.5 hr at 4°C, and sectioned on a Vibratome at 50 μm. Sections were stored overnight in cacodylate buffer with 8% sucrose at 4°C then washed briefly in 0.2 M Tris-maleate buffer and incubated for 1 hr at 37°C in a solution consisting of 20% 0.01 M cocarboxylase (thiamine pyrophosphate chloride; Sigma), 40% 0.2 M Tris-maleate buffer, 12% 0.03 M lead nitrate, 20% 25 mM manganese chloride, and 8% distilled water (Novikoff and Goldfischer, 1961). After a brief rinse in distilled water, the sections were exposed to a 2% solution of ammonium sulfide for approximately 1 min and then rinsed again in distilled water. Sections were then mounted on gelatin-coated slides, coverslipped with an aqueous mounting medium (Chemicon), and examined with bright-field optics.

Results

The biceps brachii motor pool

To assess the response of motoneurons to target deprivation during postnatal development, labeled motoneurons that were separated from the biceps muscle were compared with those of the same age that remained in contact with the muscle. To establish a baseline for comparison, the location, number and size of the labeled cells in the contralateral *control* biceps brachii motor pool were determined. Motoneurons that project to the biceps brachii muscle were consistently located in the dorsolateral region of the ventral horn (Fig. 1), and their distribution along the rostrocaudal axis of the spinal cord followed a bell-shaped curve (Fig. 2).

The number of labeled motoneurons was highly consistent *within* groups of animals that were of the same age and survival time, but there was more variation *between* the groups (Table 1). The number of cells labeled in the youngest animals, averaged over all survival times, was 83 ± 11 (mean ± SEM; $n = 17$). The equivalent value from the adult groups was 61 ± 15 (mean ± SEM; $n = 17$). Although more cells were labeled in younger animals than in adults, the difference was not significant ($p > 0.05$), and it is likely that this reflects difficulties with restricting the dye injection to the biceps muscle in younger animals and filling the entire muscle in adults. There was also a decline in the number of labeled control motoneurons as time progressed after the Fluorogold injection. This difference was significant for each age group (Table 1; $p < 0.005$ for cells injured on P14, $p < 0.05$ for cells injured on P21, and $p < 0.01$ for cells injured during adulthood). Despite the relative stability of Fluorogold labeling (Schmued and Fallon, 1986), these declining numbers probably reflect the gradual loss of Fluorogold from motoneurons over time. It is possible, however, that Fluorogold is toxic and that it causes the death of motoneurons either directly or indirectly by compromising the muscle fibers they innervate. The variation in the number of labeled motoneurons

Table 1. Average number of Fluorogold-labeled cells in the spinal cord after limb amputation

Age when injured	Cell type	Number of Fluorogold-filled cells at survival times of: 2 weeks	3 weeks	6 weeks
2 weeks	$MN_{control}$[a]	130 ± 19[d,e]	64 ± 8[f]	52 ± 4
	MN_{exp}[b]	48 ± 10	20 ± 7	7 ± 1
	mg_{exp}[c]	66 ± 6	51 ± 7	35 ± 9
3 weeks	$MN_{control}$	94 ± 11[f]	77 ± 4[g]	65 ± 6
	MN_{exp}	47 ± 4	41 ± 9	18 ± 5
	mg_{exp}	43 ± 23	52 ± 16	30 ± 6
Adult	$MN_{control}$	80 ± 7[g]	64 ± 8	48 ± 6
	MN_{exp}	69 ± 2	57 ± 6	41 ± 5
	mg_{exp}	13 ± 5	8 ± 3	24 ± 6

[a] Fluorogold-labeled motoneurons on the side of the spinal cord contralateral to a limb amputation.

[b] Fluorogold-labeled motoneurons on the side of the spinal cord ipsilateral to a limb amputation.

[c] Fluorogold-labeled microglial cells on the side of the spinal cord ipsilateral to a limb amputation.

[d] Means ± SEM.

[e] $n = 6$; six animals were used at each age and survival time unless otherwise noted.

[f] $n = 4$.

[g] $n = 5$.

Table 2. Average cross-sectional area of Fluorogold-labeled cells in the spinal cord after limb amputation

Age when injured	Cell type	Area (μm^2) of Fluorogold-filled cells at survival times of: 2 weeks	3 weeks	6 weeks
2 weeks	$MN_{control}$[a]	395 ± 29[d,e]	322 ± 42	277 ± 26
	MN_{exp}[b]	405 ± 37	285 ± 30	149 ± 9
	mg_{exp}[c]	32 ± 4	28 ± 2	31 ± 4
3 weeks	$MN_{control}$	346 ± 23	321 ± 29	325 ± 28
	MN_{exp}	351 ± 49	348 ± 39	157 ± 21
	mg_{exp}	28 ± 2	34 ± 9	27 ± 3
Adult	$MN_{control}$	536 ± 45	603 ± 47	400 ± 25
	MN_{exp}	492 ± 39	588 ± 59	303 ± 24
	mg_{exp}	26 ± 3	25 ± 3	35 ± 4

[a] Fluorogold-labeled motoneurons on the side of the spinal cord contralateral to a limb amputation.

[b] Fluorogold-labeled motoneurons on the side of the spinal cord ipsilateral to a limb amputation.

[c] Fluorogold-labeled microglial cells on the side of the spinal cord ipsilateral to a limb amputation.

[d] Means ± SEM.

[e] $n = 50$ cells of each type, measured at each age and survival time.

on the control side of the animal makes it difficult to determine the true size of the biceps motor pool. A conservative estimate, based on the average number of cells labeled in adult animals after the shortest survival time, is that the biceps muscle is innervated by at least 80 motoneurons.

The cross-sectional area of Fluorogold-filled motoneuron cell bodies was also determined. Cell size varied between the 9 groups of animals but was consistent within each group. By 2 weeks after dye injection, the area of labeled cells in adult animals was 536 ± 45 μm^2, whereas cells in animals injected on either P14 or P21 measured 395 ± 29 and 346 ± 23 μm^2, respectively (mean ± SEM). These control values declined by 6–30% between 2 and 6 weeks following the Fluorogold injection (Table 2). It is understandable that the labeled cell bodies in adults were larger than those in juveniles, but it is not clear why the average size of the labeled cells declined with time.

The location of the biceps motor pool we observed in the mouse is the same as that reported for the rat (Rootman et al., 1981; Bennett et al., 1983), and the size of the labeled cells is generally consistent with a previous description of spinal motoneurons in the mouse (Baulac and Meninger, 1983). The variation observed with age and survival time was controlled for by comparing control and experimental values only within, not between, each group. The findings outlined in this section therefore established control values against which any changes induced when motoneurons were separated from their target could be assessed.

Two labeled cell types are apparent within the spinal cord following limb amputation

The response to target deprivation during postnatal development was determined by examining labeled biceps motoneurons after limb amputation. When sections of the spinal cord were examined, 2 distinct types of labeled cells were present within the spinal cord. Three observations indicate that one of these cell types was neuronal: (1) the cells were morphologically indistinguishable from control motoneurons, (2) their position within both the dorsolateral and rostrocaudal regions of the spinal cord was similar to the position of control motoneurons, and (3) they were recognized by several different antibodies directed against neurofilaments (Fig. 3). The second cell type was also well labeled with Fluorogold but was much smaller with crenulated processes (Fig. 3).

Antibodies to cell type-specific markers were used to determine the identity of these small cells. Several antibodies were used to identify neurons, an antiserum against GFAP to identify astrocytes and a histochemical reaction specific for the plasma membrane-associated enzyme thiaminepyrophosphatase (TPPase) to identify microglial cells. The small cells did not express neuron-specific antigens (Fig. 4). Further, they were morphologically distinct from GFAP-positive astrocytes (Fig. 5) and were not labeled with antibodies to GFAP (Fig. 4). However, histochemical staining for TPPase provided evidence that the small cells were ramified microglia. First, the small cells labeled with Fluorogold and those stained for TPPase were morphologically similar (Figs. 4, 5). Second, although Fluorogold-labeled cells are difficult to visualize following the histochemical procedure for demonstrating TPPase, the dye could still be detected in the cell bodies of a few TPPase-positive cells (Fig. 4). Thus, after limb amputation, microglial cells, as well as the motoneurons that were initially labeled, contained Fluorogold.

As an independent test of the identity of the small, Fluorogold-filled cells, the biceps muscle was injected with Fluorogold 1 d after the mice were born, 2 d later the forelimb was amputated, and the spinal cord was examined after 4, 7, or 18 d survival. Normally, during the first few weeks of postnatal life, ameboid microglia are thought to extend processes to become the ramified microglia of CNS tissue (Ling, 1981; Murabe and Sano, 1982; Perry and Gordon, 1988; Schnitzer, 1989). The small Fluorogold-filled cells that we observed after limb amputation in neonates showed similar changes in morphology; their shape became increasingly complex with time after amputation (Fig. 6). On P7, most of the Fluorogold-filled cells were

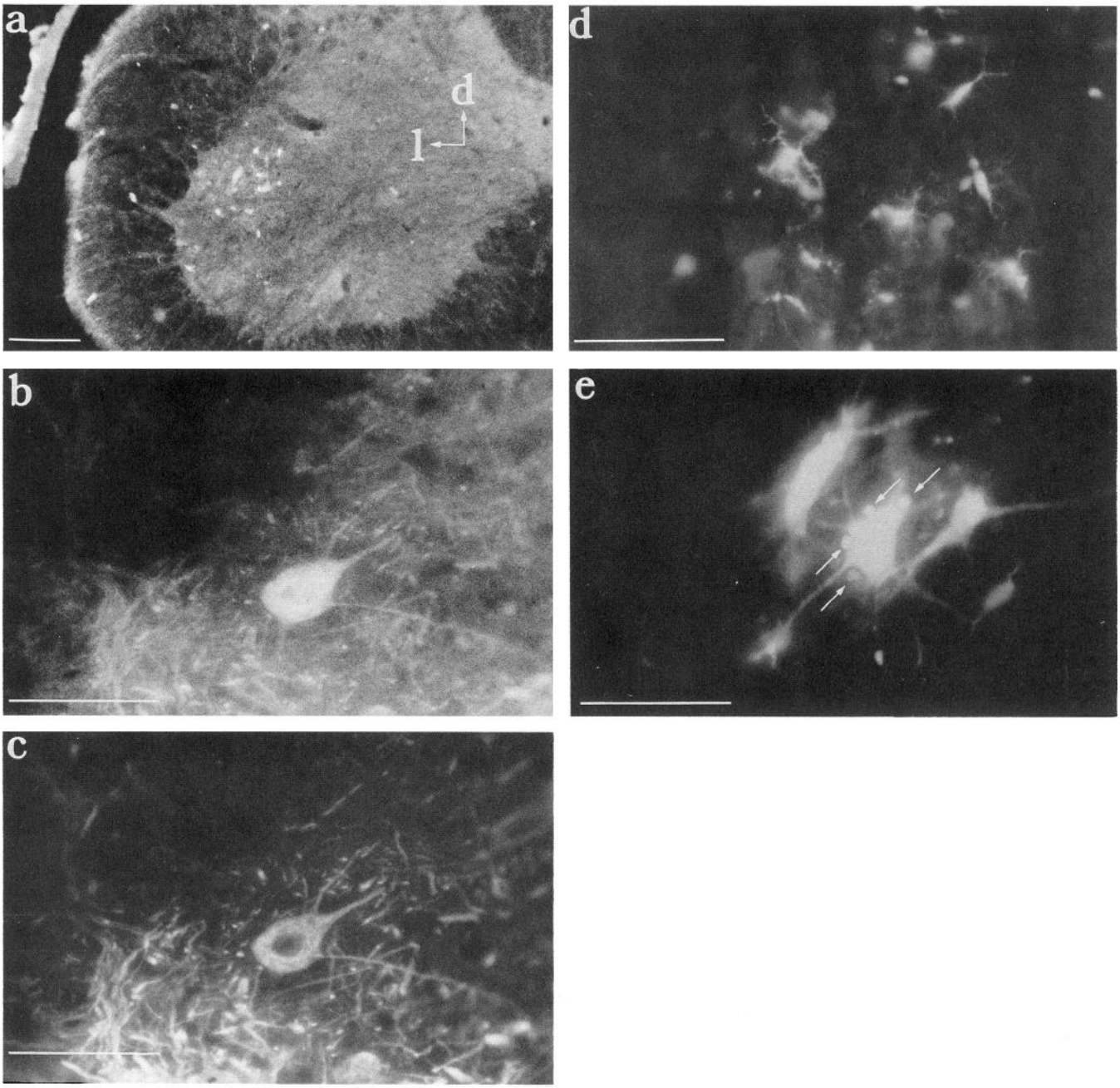
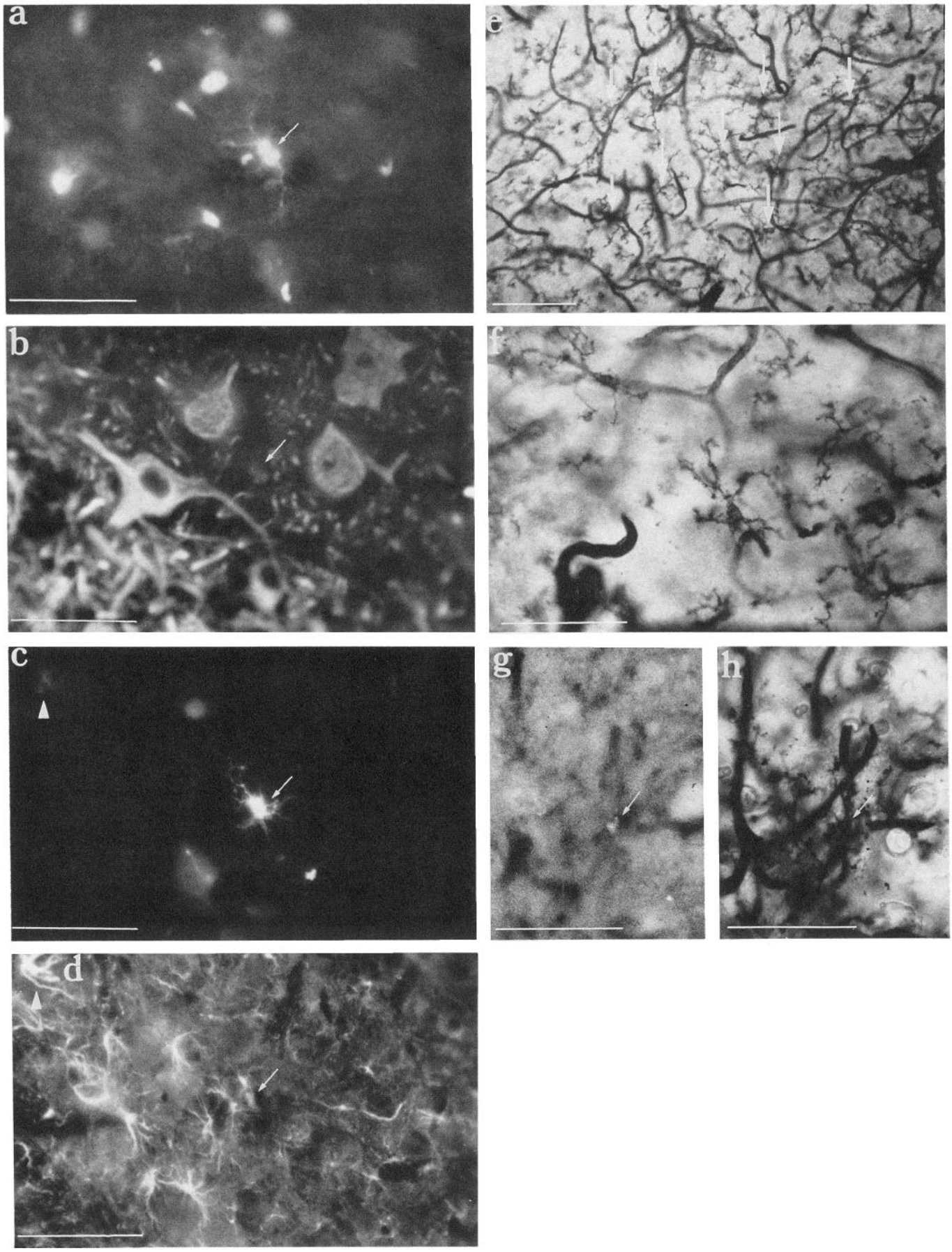


Figure 3. Photomicrographs of Fluorogold-filled cells in the spinal cord following limb amputation. In *a*, labeled cells can be seen in the dorsolateral region of the ventral horn, which is the same region where control cells are located (Fig. 1). This section contains both labeled motoneurons and labeled microglial cells. An adjacent section at higher magnification (*b*) contains a single Fluorogold-labeled motoneuron which is clearly labeled by neurofilament antibodies (*c*). The smaller cells, shown in *d* were subsequently identified as ramified microglia. Their cell bodies are intensely labeled with Fluorogold, as are their fine, crenulated processes. Small cells without processes, as in *e* (*arrows*), may be ameboid microglial cells. These cells could be labeled following injection of Fluorogold or a number of other retrograde tracers. In this instance (*e*), the motoneurons were labeled with True blue. In contrast, ramified microglia were apparent only when motoneurons were labeled with Fluorogold. Scale bars, 50 μm.

ameboid but some had a single process. The cells appeared more complex on P10, often with 2 or more processes. By P21 many cells were extensively ramified and were indistinguishable from microglial cells in the adult. This parallel change in morphology between differentiating microglia and the small cells in question here further suggests that the small Fluorogold-filled cells apparent after limb amputation were microglia.

Fluorogold-filled cells that resembled ameboid microglia were also seen within the spinal cord of juvenile and adult animals (Fig. 3). However, they were present in the vicinity of both experimental and control motoneurons. These small, round cells could often be seen in close apposition to labeled motoneurons, but there did not seem to be any consistency in when, or how frequently, they appeared. These cells were not positively identified: ameboid microglia are not stained by TPPase histochemistry and the ameboid cells we observed were not recognized by the macrophage-specific antibodies tested. Thus, we suspect that ameboid microglia also obtain Fluorogold from the motoneu-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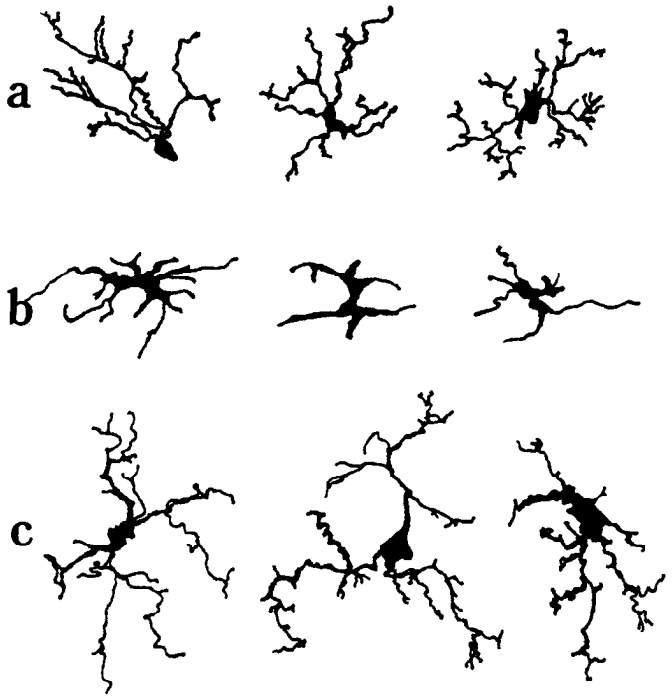

Figure 5. Camera lucida drawings of (*a*) the small Fluorogold-filled cells that appear after the nerve to the biceps muscle is cut or the limb is amputated, (*b*) astrocytes recognized by antibodies directed against GFAP, and (*c*) microglial cells stained by a histochemical reaction for TPPase. All drawings were made at the same magnification. Note that the fine processes of the Fluorogold-filled cells in *a* and the microglial cells in *c* emerge abruptly from triangular-shaped cell bodies, while the astrocytes have thicker processes that taper gradually away from the cell body.

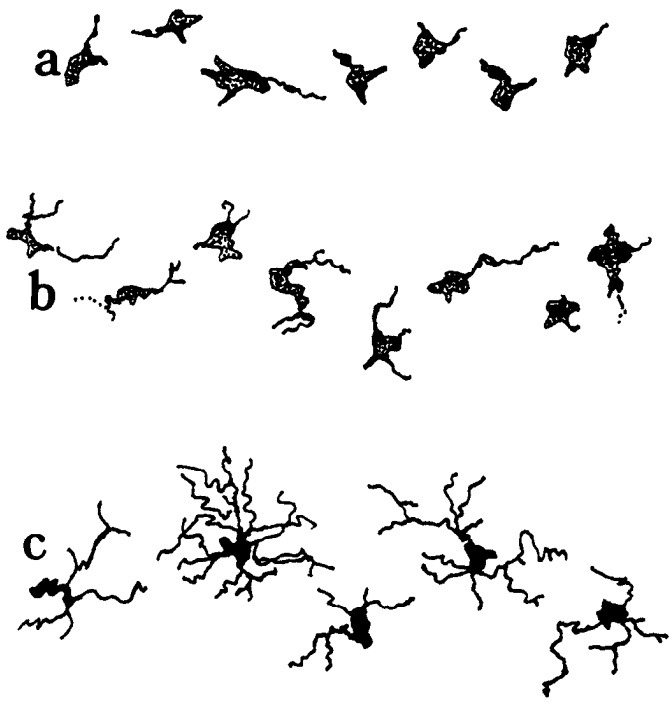

Figure 6. Camera lucida drawings of small Fluorogold-filled cells 4 d (*a*), 7 d (*b*), and 18 d (*c*) after the forelimb was amputated on postnatal day 3. The morphology of the cells becomes progressively more complex with time after amputation. This progression is consistent with previous reports on the differentiation of ramified microglial cells during postnatal development.

rons that were originally labeled, but only ramified microglial cells were positively identified and restricted to the vicinity of injured motoneurons.

Ramified microglia become labeled following limb amputation as the number of labeled motoneurons declines

To examine the potential extension of target dependency through postnatal development, labeled motoneurons were counted following limb amputation on P14, P21, or in adulthood. These animals were allowed to survive for either 2, 3, or 6 weeks so the time course of the response could be followed for a relatively long period. The principal finding was that there were generally fewer labeled cells that were morphologically recognizable as motoneurons ipsilateral to the amputation than on the contralateral, uninjured side of the spinal cord. The number of motoneurons that were lost depended on the age of the animal and its survival time: the younger the animal at the time of the injury, the more extensive the neuronal loss. For example, after 2 weeks, a population of labeled cells that were injured by amputation on P14 had only 37% as many cells as were labeled on the control side. However, 51% of the neurons that were deprived of their target a week later were maintained through the same survival time. The oldest animals, those injured as adults, lost few, if any, motoneurons in the 2 weeks following amputation. Therefore, more motoneurons are lost by younger animals than by older animals after any given survival time (Table 1, Fig. 7). Furthermore, within each age group, the number of labeled motoneurons steadily declined as the survival times were lengthened. The number of labeled motoneurons in the juvenile mice (injured on P14 or P21) gradually declined until, by 6 weeks after the amputation, the number of labeled motoneurons on the injured side was only about 20–30% of the

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Figure 4. Identification of Fluorogold-labeled cells. Photomicrographs of a spinal cord section containing a small Fluorogold-filled cell (*a*; *arrow*). This same section was also exposed to antibodies directed against *neurofilaments* (*b*). *c* and *d*, Photomicrographs of another spinal cord section containing a small Fluorogold-filled cell (*c*; *arrow*) that was exposed to antibodies directed against *GFAP* (*d*). The intensely labeled astrocyte at the upper left in *d* is also visible in *c* (*arrowheads*), confirming that these photographs are of identical regions. The antibodies to neurofilaments or GFAP did not bind to the small Fluorogold-filled cells. The small Fluorogold-filled cells, at the *arrows* in *a* and *c* are just visible at the same positions in *b* and *d* only because Fluorogold is excited weakly at the wavelengths used to visualize the fluorescein-conjugated secondary antibodies to neurofilaments or GFAP (Fluorogold fluorescence could easily be distinguished from that of fluorescein by its color). *e*, A photomicrograph of a spinal cord section after TPPase histochemistry shows the even distribution of microglia within the ventral horn of the mouse spinal cord. The vascular endothelium is also heavily stained by TPPase histochemistry (*arrows* indicate microglial somata). *f*, At higher magnification, note that the fine processes emerge abruptly from the microglial cell body. Although Fluorogold labeling and TPPase histochemistry are not compatible, small cells that are weakly labeled with Fluorogold can still be seen (*g*; *arrow*) following TPPase histochemistry. When a section containing such a cell (as in *g*) is viewed with bright-field optics, the Fluorogold-containing cell appears to be a microglial cell (*h*). Scale bars, 50 μm.

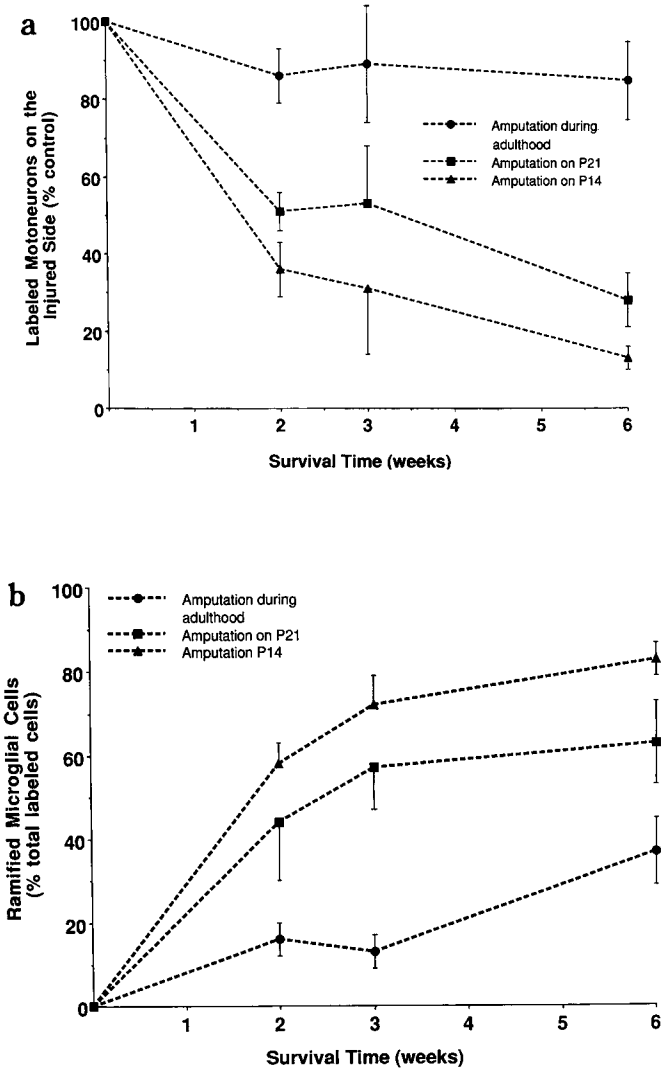

Figure 7. Average number of labeled cells within the spinal cord following limb amputation at various ages. Animals were examined either 2, 3, or 6 weeks after target deprivation on P14, P21, or in adulthood. *a,* Average number of labeled *motoneurons* in juvenile mice; Those injured on P14 (*triangles*) or P21 (*squares*), fell sharply after amputation; by 6 weeks, there were less than 40% as many motoneurons on the injured side as there were on the control side of the spinal cord. The average number of labeled motoneurons also declined in adult animals (*circles*), but not as dramatically; after 6 weeks, adults still maintained 85% of their target-deprived motoneurons. *b,* Average number of labeled *microglial cells* within the spinal cord following limb amputation. As the number of labeled motoneurons declined, the number of labeled microglial cells increased. In juvenile animals (*squares, triangles*), microglial cells continued to become labeled with time; in the youngest animals, more than 80% of the labeled cells present 6 weeks after amputation were microglial cells. The average number of labeled microglia also increased with time in adult animals. After 6 weeks, more than 30% of the labeled cells on the injured side were ramified microglia. Means ± SEM are presented.

control value (Table 1, Fig. 7). A gradual loss of neurons after axotomy has been found in other studies (see, for example, Laiwand et al., 1987) and indicates that individual neurons within any given population differ in their ability to survive separation from their target.

The ramified microglial cells that contained Fluorogold reflected the population of degenerating motoneurons in 2 ways.

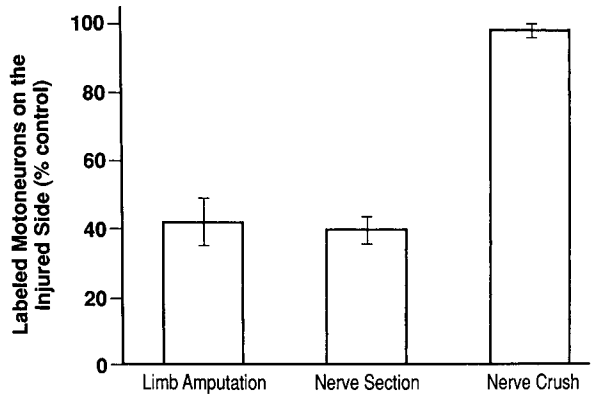

Figure 8. Comparison of the percentage of labeled motoneurons that remained after target deprivation by cutting the nerve, crushing the nerve, or amputating the limb. Five animals were injured on P21 by each method of target deprivation and allowed to survive for 3 weeks. The cell loss following nerve section was as extensive as that seen after limb amputation, but there was no decline in the number of labeled motoneurons following nerve crush. Means ± SEM are presented.

First, the time course of their appearance complements the disappearance of labeled motoneurons. Just as the number of labeled motoneurons declined with time following amputation, the number of labeled microglial cells increased (Fig. 7). Within every group, the total number of labeled cells on the injured side, motoneurons and ramified microglia, was similar to the number of labeled motoneurons on the contralateral, control side of the spinal cord (Table 1). Second, the distribution of ramified microglial cells that contained Fluorogold followed the distribution of labeled motoneurons within the spinal cord. The ramified microglia were restricted to the same rostrocaudal location as the normal biceps motor pool, and, along this axis, their number was proportional to the number of labeled motoneurons that were lost (Fig. 2). Fluorogold-filled, ramified microglial cells were more abundant in the center of the pool, where a greater absolute number of neurons died, than at its rostral and caudal extents. These relationships, in conjunction with the likelihood that only motoneurons are labeled by injecting Fluorogold into the biceps, led us to suspect initially that these cells were atrophied motoneurons. However, their subsequent identification as ramified microglia shows instead that microglial cells become labeled as motoneurons are lost following target deprivation by limb amputation.

The size of the labeled motoneurons also declines following limb amputation

The cross-sectional area of the labeled cell bodies in animals of each age and survival time was measured after limb amputation. Injured motoneurons and microglial cells as well as control motoneurons on the contralateral side of the spinal cord were measured. The area of the labeled motoneurons declined following amputation (Table 2). In animals of all ages, injured motoneurons did not differ in size from control motoneurons for at least 3 weeks following limb amputation ($0.99 > p > 0.50$). By 6 weeks, however, the injured adult motoneurons were 25% smaller than control neurons ($p < 0.02$), and those in juvenile mice were reduced to approximately half of the control size ($p < 0.001$; Table 2). The ramified microglial cells that became labeled with Fluorogold were about one-tenth the size

of motoneurons and, in contrast to neurons, their cross-sectional area remained the same in the animals of different ages as well as with time following amputation ($0.90 > p > 0.1$; Table 2).

Comparison of response to amputation, nerve section, and nerve crush

To determine whether the response seen following limb amputation was caused directly by the axonal injury or by the resulting target deprivation, the biceps motoneurons of juvenile mice were retrogradely labeled as before but then injured either by cutting or crushing the nerve. These injuries transected axons, just as amputation did, but the target muscle remained intact. Motoneurons reinnervated the muscle more quickly after nerve crush than after the nerve was cut. Recording synaptic potentials from the biceps muscle fibers showed that reinnervation began within 4 d after nerve crush, but almost 3 weeks elapsed before motoneurons whose axons were cut began to reinnervate their target. When the nerve was cut, motoneuron loss was as extensive as that seen after limb amputation, but no cell death was evident following nerve crush (Fig. 8). Likewise, after cutting the nerve, ramified microglia became labeled in proportion to the degree of neuronal loss, but when no cell death was apparent, as after nerve crush, ramified microglia were not labeled. It appears, therefore, that the interval between loss of target innervation and its reestablishment is an important determinant of neuronal fate following axotomy.

Discussion

This study examines the consequences of depriving motoneurons of contact with their target muscle during postnatal development. To address this, motoneurons that innervate the biceps brachii muscle were retrogradely labeled in mice of different ages and then examined at various times after target deprivation. The number, as well as the size, of labeled motoneurons declined progressively with time after amputation, and concurrently, ramified microglial cells became labeled. Postnatal motoneurons are therefore dependent on contact with their target for survival.

To determine whether motoneuron death was caused by the loss of the target muscle or by the injury itself, the axons of labeled motoneurons were cut or crushed. The target deprivation that resulted from these injuries varied in its duration; motoneurons that were cut took almost 5 times longer to begin reinnervating the biceps muscle than motoneurons that were crushed. The most likely explanation for this difference is that the perineural connective tissue sheath, which remains intact after nerve crush, guided regenerating axons directly back to their muscle. After nerve crush, little if any neuronal death was evident. However, when the period of deprivation was more extensive, as it was when the nerve was cut, almost half of the biceps' motoneurons were lost and microglial cells became labeled. Our findings support the results of Kashihara et al. (1987) in that contact with the target is required for motoneuron survival and that there is a critical period after axotomy during which these motoneurons must regain target access in order to avert their death.

Postnatal motoneurons become less dependent on contact with their target as they mature

In agreement with previous studies, we found that the extent of cell death following limb amputation depended on the age of the animal at the time of the injury. Cell death was apparent in mice of all ages, but motoneurons in younger animals were lost much more readily than those in adults. A difference could be detected even in animals aged only 1 week apart; mice injured on P14 lost significantly more cells after 6 weeks (87%) than those injured on P21 (72%). It is more difficult to say with certainty that motoneurons in adult animals are dependent on their target since, on average, only 15% of these motoneurons were lost following limb amputation. However, in conjunction with our findings that adult motoneurons became progressively atrophic after target separation, and ramified microglial cells became labeled with Fluorogold (as was the case only when motoneurons died in response to prolonged target deprivation), this suggests that the target continues to influence motoneuronal survival well into adulthood. This is consistent with the findings reported by Snider and Thanedar (1989) for the hypoglossal motor nucleus, which, even in maturity, showed neuronal atrophy and apparent cell loss following target-deprivation. Likewise, several other classes of neurons, such as the cholinergic neurons in the basal forebrain nuclei (Hefti, 1986) and cells in sympathetic and sensory ganglia, degenerate in adulthood when axotomized or exposed to antibodies against the target-derived growth factor NGF (Gorin and Johnson, 1980; Johnson et al., 1982).

It is not clear, however, why younger motoneurons are more susceptible to target deprivation. It could be that the inherent properties of younger motoneurons make them almost completely dependent on their target, as they are during embryogenesis, but that the changes that occur within the neurons as they mature make them less target-dependent. Alternatively, younger motoneurons may die more readily than older cells because potential sources of trophic support other than the target, such as afferent input (Okado and Oppenheim, 1984; Furber et al., 1987) or contact with glial cells (Johnson et al., 1988), are not fully available during the early postnatal period. Similarly, it remains uncertain why some of the cells within a given motor pool are able to survive longer than others. It is possible that other sources of trophic support such as glial cells or afferent inputs may be able to maintain these cells transiently during that time. The availability and sufficiency of several sources of support may therefore explain the differential response of younger and older motoneurons and could also account for how some neurons in a given population survive longer than others after target deprivation.

Even though some motoneurons die more readily than others, they are not selectively lost from any particular region of the motor pool (Fig. 2). This pattern of loss differs from observations made during embryogenesis, during which naturally occurring cell death is greater within the most rostral and caudal regions of the lumbar motor column (Flanagan, 1969; Lance-Jones, 1982). Motoneurons developing at the extremes of the column (L1 and L6) probably take slightly longer to enter a ventral root that projects to the hindlimb muscles. They may then reach the target later than cells projecting from more central regions of the lumbar column and, thereby, be at a disadvantage in competing for a trophic substance. In contrast, postnatal motoneurons have all established contact with their target muscle, and their axons are all in an equivalent position at the time of target deprivation. This difference between loss from the motor column in a developing system and from a motor pool after birth is therefore consistent with the idea that cell death is regulated by a competitive interaction at the level of the target.

Motoneurons atrophy following limb amputation

Structural changes in the neuronal cell body in response to axotomy have been described at both the light and electron microscopic level (for example, see Ramón y Cajal, 1904; Kerns and Hinsman, 1974a, b). These changes are somewhat variable, but swelling, displacement of the nucleus to an eccentric position, and the dissolution of Nissl substance (chromatolysis) are among those most consistently reported (Cragg, 1970; Lieberman, 1971). In the present study, the only such change that was quantified was that in the cross-sectional area of the cell body following target deprivation by limb amputation. There was no indication of the swelling that is a common response to axonal injury, but this may have occurred prior to the earliest measurements, which were not made until 2 weeks after the amputation. The target-deprived biceps motoneurons were the same size as control motoneurons after 2 or 3 weeks, but between 3–6 weeks following amputation the motoneurons ipsilateral to the amputated limb became atrophic. Just as the change in the number of motoneurons was more dramatic in younger animals, their cell size was also more severely affected. The average area of the motoneuron cell bodies of juvenile animals declined to about 50% of their former size, while cells in adults were only 25% smaller than control motoneurons. The decline in the average cell size could result if all of the labeled motoneurons experienced about the same degree of atrophy or if only larger, α-motoneurons were selectively lost. We could not distinguish between these possibilities, since a clear bimodal distribution of motoneuron size, indicative of differently sized populations of α- and γ-motoneurons, was not apparent. Further exploration of the relationship between cell size and cell death is warranted in that it may determine whether different classes of motoneurons vary in their susceptibility to target deprivation.

Potential involvement of microglial cells in motoneuron death

Our finding that microglia were able to obtain the fluorescent tracer formerly restricted to motoneurons provides evidence that microglia phagocytose the debris created when neuronal death is induced by axotomy during postnatal development. Ramified microglia must have obtained the tracer from degenerating motoneurons because they became labeled only when motoneurons died. After nerve crush, and on the control side of animals injured by unilateral amputation or axotomy, motoneurons did not die; in these cases, ramified microglial cells were not labeled.

The cells that were positively identified as microglia and counted in this study had extensive processes. These cells are referred to as ramified, or resting, microglial cells. It is thought that these cells arise from macrophages that invade the developing nervous system and then differentiate into ramified microglia (Ling, 1981; Murabe and Sano, 1982; Perry and Gordon, 1988; Schnitzer, 1989). The morphological transition that occurs as ameboid cells extend processes and become ramified microglial cells was evident in this study when motoneurons were labeled and injured very early in development (P3): the morphology of the Fluorogold-filled cells became increasingly complex as the survival time increased. The similarity between these changing cellular profiles and those reported by others helps to confirm that the small labeled cells seen after motoneuron death were microglia.

Although not positively identified, cells that resembled macrophages or ameboid microglial cells in both size and morphology were also frequently labeled with Fluorogold. In contrast to the ramified microglia, these cells were often seen in close apposition to labeled motoneurons (Fig. 3). It is therefore possible that ameboid microglia obtain Fluorogold from dying motoneurons and subsequently extend processes (see also Springer and Wilson, 1989). It is curious why there was virtually a 1:1 correspondence between the number of motoneurons that are lost and the number of ramified microglial cells that become labeled. It does not seem likely that a single microglial cell would be assigned to a single motoneuron or, from their disparate sizes, that one microglial cell could phagocytose an entire neuron. However, a striking feature of ramified microglial cells is their uniform distribution within the spinal cord (see Fig. 4). The rules that establish this spatial arrangement might therefore place strict limits on the number of microglial cells that could remain in the vicinity of each dying motoneuron following target deprivation. The experimental design used here, whereby Fluorogold is transferred from degenerating motoneurons to microglial cells may facilitate further studies of the interactions between these 2 cell types.

From our study, it was clear that microglia somehow obtained their Fluorogold labeling from motoneurons, but whether they achieved this by engulfing the motoneurons was not determined. This does, indeed, seem likely in light of the known phagocytic capabilities of microglia (Perry and Gordon, 1988). Microglia have been implicated in phagocytosis of degenerating motoneurons during the normal period of neuronal death in the chick embryo (Chu-Wang and Oppenheim, 1978). Likewise in the developing visual system, macrophages have been shown to migrate from the vasculature to phagocytose retinal ganglion cells during naturally-occurring cell death, and subsequently to differentiate into ramified microglia (Hume et al., 1983) which lack phagocytic activity (Giulian and Baker, 1986; Bocchini et al. 1988). Ultrastructural analysis of microglia in the human optic nerve between 8 and 10 weeks postconception also suggests that they are actively engaged in the phagocytosis of degenerating axons (Sturrock, 1988).

A number of diverse functions have been proposed for microglial cells in normal development, in addition to the phagocytic role outlined above, but there is still considerable uncertainty about the function of ramified microglia in adult animals (Schnitzer, 1989; for review, Streit et al., 1988). The apparent involvement of ramified microglial cells in postnatal neuronal death indicates that these cells may mediate immunological processes by acting as brain macrophages.

In summary, the findings reported here are consistent with previous suggestions that motoneurons continue to be maintained by a target-derived trophic substance throughout postnatal development and indicate that motoneurons are lost in an age- and time-dependent manner following a period of target deprivation. In addition our findings indicate a role for microglial cells in postnatal neuronal death. However, the entire scheme by which motoneurons are supported cannot be fully understood until the trophic substances involved have been identified. While a great deal of effort has been directed towards the identification of putative motoneuron growth factors, few of these substances have been evaluated *in vivo* (but see Oppenheim et al., 1988). The procedure used here to study the survival of a discrete population of prelabeled motoneurons could aid such studies in that it offers an attractive *in vivo* assay for testing candidate motoneuron growth factors in mammalian animals.

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