Neuron, Vol. 28, 967–978, December, 2000, Copyright ©2000 by Cell Press

Spinal Cord Atrophy and Reorganization of Motoneuron Connections Following Long-Standing Limb Loss in Primates

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Summary

Primates with long-standing therapeutic amputations of a limb at a young age were used to investigate the possibility that deefferented motor nerves sprout to new muscle targets. Injections of anatomical tracers into the muscles proximal to the amputated stump labeled a larger extent of motoneurons than matched injections on the intact side or in normal animals, including motoneurons that would normally supply only the missing limb muscles. Although the total numbers of distal limb motoneurons remained normal, some distal limb motoneurons on the amputated side were smaller in size and simpler in form. These results suggest that deprived motoneurons survive and retain function by reinnervating new muscle targets. The sprouted motor efferents may account for some of the reorganization of primary motor cortex that follows long-standing amputation.

Introduction

After amputation of a limb, M1 reorganizes so that movements of the remaining proximal portion of the limb can be evoked from cortical sites that were formally devoted to movements of the distal limb (Donoghue and Sanes, 1988; Hall et al., 1990; Sanes et al., 1990; Cohen et al., 1991; Ojemann and Silbergeld, 1995; Pascual-Leone et al., 1996; Schieber and Deuel, 1997; Wu and Kaas, 1999; Qi et al., 2000). In monkeys, the minimal levels of current necessary to evoke such movements in deefferented M1 varied from those in the normal range to those of higher levels (Schieber and Deuel, 1997; Wu and Kaas, 1999; Qi et al., 2000). Explanations for the causes of such changes in motor maps in M1 have focused on possible modifications in the strengths of intracortical connections and changes in corticospinal terminations (see Cohen et al., 1991; Ramachandran, 1993; Chen et al., 1998). Surprisingly, changes in the connectivity of motoneurons, the motor outputs to the muscle fibers, have not been considered in most explanations.

This study was designed to examine if cortical reorganization depends, at least in part, on changes in the spinal motor efferents to muscles. We were fortunate to be able to obtain several primates with previous therapeutic amputations of a limb, and several tracers are now available for labeling motoneurons after restricted injections into muscles. In normal animals, motor units controlling different groups of muscles are organized into medial and lateral longitudinal aggregates in the ventral horn, and these motor columns supplying individual limb muscles are somatotopically organized (Goering, 1928; Reed, 1940; Browne, 1950; Romanes, 1951; Burke et al., 1977; Brown et al., 1981; Kuypers, 1982; Jenny and Inukai, 1983; Rivero-Melian, 1996). Since shoulder and stump movements were elicited from a major portion of the deefferented M1 of the primates in our parallel study (Wu and Kaas, 1999), we looked for possible changes in the origin of motor efferents supplying shoulder and stump muscles following long-standing limb loss. Thus, we injected tracers into the muscles of stump or shoulder of New World monkeys and prosimian galagos years after the therapeutic amputation of a forelimb or hindlimb. These and other injections allowed us to identify normal and altered patterns of motoneuron connections with limb muscles. Moreover, since severe atrophy was observed in somatosensory relay nuclei following body injuries (Jones and Pons, 1998; Woods et al., 1999), we also examined the spinal cord for possible motoneuron atrophy as a result of deefferentiation. Spinal motoneurons were counted and measured on the side ipsilateral to the amputation and compared to those from the contralateral intact side. Results from these findings were related to the results of microstimulation in M1 of the same animals (Wu and Kaas, 1999) in order to directly correlate the patterns of peripheral connections to the changes in central function triggered by amputation.

Results

The present study describes the structural and connectional changes in motoneurons supplying amputated limb muscles after a long-standing loss of a limb. We first describe the somatotopic pattern of the connections of motoneurons innervating the forelimb and adjacent body parts in the normal controls, and compare it to the pattern of motoneuron connections that innervate the stump and shoulder muscles proximal to the missing forelimb in amputated animals. Second, we compare the somatotopic organizations of motoneuron columns innervating intact hindlimb and adjacent body parts with those formerly innervating the missing limb and adjacent muscles in hindlimb-amputated animals. Finally, we examine the evidence for possible changes in the numbers and sizes of motoneurons formerly controlling the missing limbs of the amputated animals.

Changes in the Anatomical Connections of the Motoneurons Formerly Supplying the Missing Limb

Organization of Motoneurons Innervating the Forelimb, Shoulder, and Upper Trunk in the Normal Animals

The ventral horn of the spinal cord is traditionally subdivided into medial and lateral motoneuron pools, also

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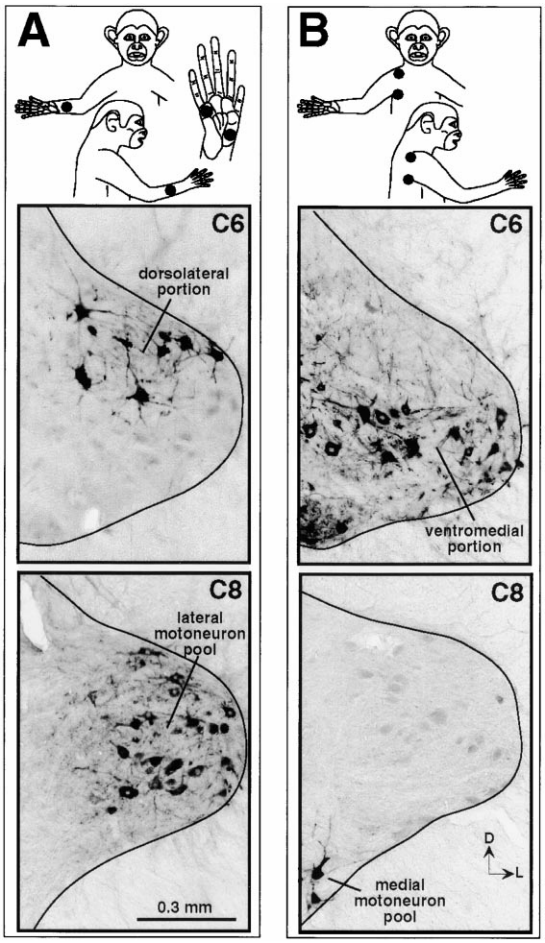

Figure 1. Distribution of Labeled Motoneurons in the Lateral Motoneuron Pools in the C6 and C8 Segments of the Spinal Cord in Normal Squirrel Monkeys

Photomicrographs are taken from spinal cord cross-sections. Motoneurons were retrogradely labeled following injections of CTB into (A) distal forelimb muscles or (B) shoulder muscles of a normal monkey. The level in the spinal cord is shown at the upper-right corner for each section. Within the lateral motoneuron groups (i.e., Rexed's lamina IX), the ventromedial portion projects to the proximal limb, whereas the dorsolateral portion projects to distal limb. Note that the distribution of labeled neurons innervating the shoulder muscles are complimentary to those innervating the distal forelimb muscles.

known as Rexed's laminae VIII and IX, respectively (Rexed, 1952). Following muscle injections, retrogradely labeled motoneurons formed longitudinal columns in the ventral horn of the spinal cord. In agreement with previous descriptions in macaques (Jenny and Inukai, 1983; Rouiller et al., 1996), we observed a somatotopic pattern of motoneuron innervation of the forelimb muscle groups in squirrel monkeys and galagos (Figures 1 and 2; also see Figure 6 for summary). As a general rule, motoneurons innervating axial body parts are located in the medial motoneuron pool, whereas motoneurons innervating the limb muscles are located in the lateral motoneuron pool. Furthermore, motoneurons involving muscles of the upper arm were located in the ventral horn at C4–C8 levels, whereas motoneurons involving muscles of distal forelimb were at C6–T2 levels. Also, the ventromedial portion of the lateral motoneuron pool innervated the proximal limb muscles, whereas the dorsolateral portion of the lateral motoneuron pool innervated the most distal limb. Injections involving only small hand muscles labeled the extreme dorsolateral portion of the lateral motoneuron column in C8–T2 levels (data not shown). Although the numbers of labeled neurons varied with the type and amount of tracer injected, the general location of labeled neurons after injections in the same muscle groups was consistent across tracers and cases.

Motoneurons innervating upper trunk and shoulder were also examined. Nerves controlling shoulder muscles originated in the medial and/or lateral groups through the C3–T3 levels of the spinal cord (Figures 1B and 6). Upper trunk muscle injections labeled medial motoneuron pools from the C8–T3 levels (individual case data not shown, but see Figure 6 for summary illustration). These observations are consistent with earlier reports that medial motoneuron groups terminate in the axial muscles, whereas lateral ones synapse mainly on the limb muscles (Kuypers, 1982).

Both shoulder and forelimb muscles were innervated by the lateral motoneuron pools at the C4–C8 levels. However, motoneurons controlling shoulder muscles were largely separated from those controlling forelimb muscles. Furthermore, the motoneuron pools serving distal forelimb muscles and shoulder muscles were completely separated. Until the rostral portion of C5, motoneurons innervating shoulder muscles remained in the ventromedial portion of the lateral motoneuron group, and gradually shifted ventrally and medially as distal limb motoneuron columns appeared. At the caudal portion of C8, motoneurons controlling the shoulder disappeared from the lateral motoneuron group. Motoneurons controlling the hand muscles constituted the remainder of the lateral motoneuron groups, whereas upper trunk and shoulder motoneurons constituted the medial motoneuron group. This topographical organization is illustrated in the summary reconstruction in Figure 6.

Reorganization of Motoneurons Innervating the Stump and Shoulder of the Forelimb-Amputated Animals

For comparison with the amputated side, motoneuron pools serving forelimb and/or shoulder muscles were localized by injection of tracers into these muscles on the intact side (see Table 1). The distributions of retrogradely labeled neurons following injections into shoulder, forearm, and hand muscles of the intact side of the forelimb amputees were similar to those found in the normal controls (Figures 1–3 and 6).

The distributions of the motoneurons innervating the arm stump were examined in three forelimb-amputated animals. Densely placed injections in the end of the stump labeled only a few motoneurons in the distal forelimb column, and these neurons were small and appeared to have fewer and shorter dendritic branches (Figure 2). Many more labeled neurons were found in the proximal forelimb or shoulder columns in the lateral motoneuron pools rostral to the C6 segment (individual case data not shown, but see Figure 6 for summary illustration).

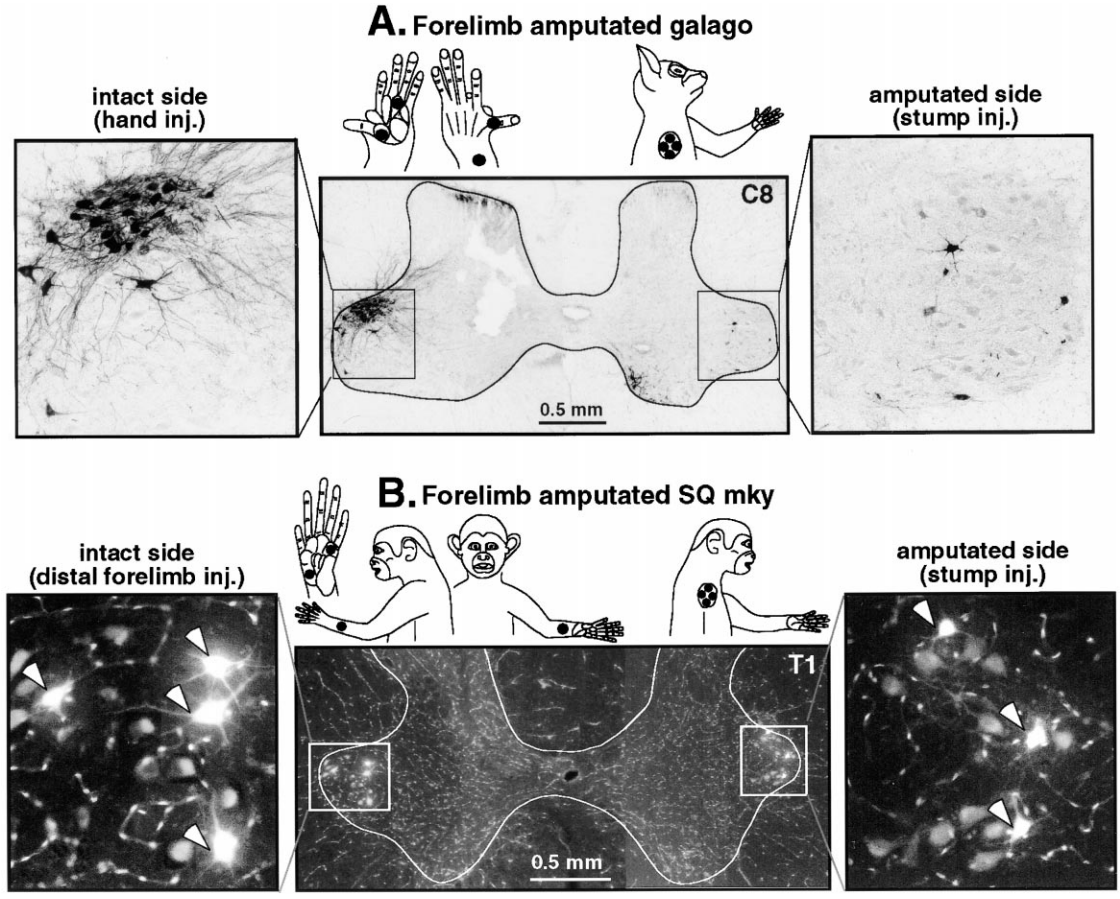

Figure 2. Photomicrographs of a Series of Spinal Cord Cross-Sections from Forelimb-Amputated Galago and Squirrel Monkey Showing the Distribution of Retrogradely Labeled Spinal Motoneurons

The lower magnification views of the sections are in the middle. The lateral motoneuron pools on both sides of the spinal cord are shown in the enlarged views.

(A) Sections from a forelimb-amputed galago (case 97-100) prepared for CTB exhibit the well-defined somata and dendritic arbors of individually labeled neurons. Following CTB injections into muscles of the intact hand, labeled neurons on the intact side of the spinal cord were found in the most dorsolateral extreme of the lateral motoneuron pools. Following CTB injections into the limb stump, labeled neurons on the amputated side were found in the dorsolateral portion of the lateral motoneuron pools controlling the distal forelimb. Note that labeled dorsolateral neurons on the amputated side are small and have simpler processes than those on the intact side. Also note the apparent reduction in volume of both ventral and dorsal horns on the amputated side of the spinal cord.

(B) Dark-field photomicrographs from a forelimb-amputated squirrel monkey (case 98-61) showing retrogradely labeled neurons (arrowhead) following FB injections into the distal forelimb and limb stump muscles. Injections into the limb stump labeled lateral motoneuron pools that would normally innervate the distal forelimb muscles, as identified by the location of labeled neurons after forelimb injections on the intact side. Similar results were obtained in case 98-64.

Since injections densely placed in the limb stump left most of the distal forelimb motoneurons unlabeled, we investigated the possible reinnervation of muscle targets adjacent to the stump by severed forelimb nerves. Tracers were placed in the shoulder muscles of the amputated limb. In both control animals, and on the intact side of animals with amputation, motoneurons innervating the shoulder muscles in the lateral motoneuron pool are restricted to the ventromedial portion within C5– C8 segments. In contrast, following tracer injection in the shoulder muscles of the amputated side, labeled neurons were found not only in the motoneuron pool innervating the shoulder muscles but also in the dorsolateral portion of the lateral motoneuron pool caudal to the C5 segment, as well as in the entire lateral motoneuron pools caudal to the C8 level (Figure 3). These latter two motoneuron pools normally innervate the proximal forelimb muscles and distal forelimb muscles, respectively (see Figure 6). This observation was repeated in the other forelimb amputee in which nerves controlling the shoulder muscles of the amputated limb originated from both the shoulder and former forelimb motoneuron pools. Therefore, we conclude that the former forelimb motoneurons changed their targets to innervate the shoulder muscles.

Organization of Motoneurons Innervating the Hindlimb and Tail in the Normal Animals

As a control for the hindlimb amputees, the origins of motoneurons innervating proximal hindlimb (i.e., upper leg) and distal hindlimb (i.e., foot) muscles in normal galagos were examined. Similar to the organization of the distal forelimb motoneurons, motoneurons innervat-

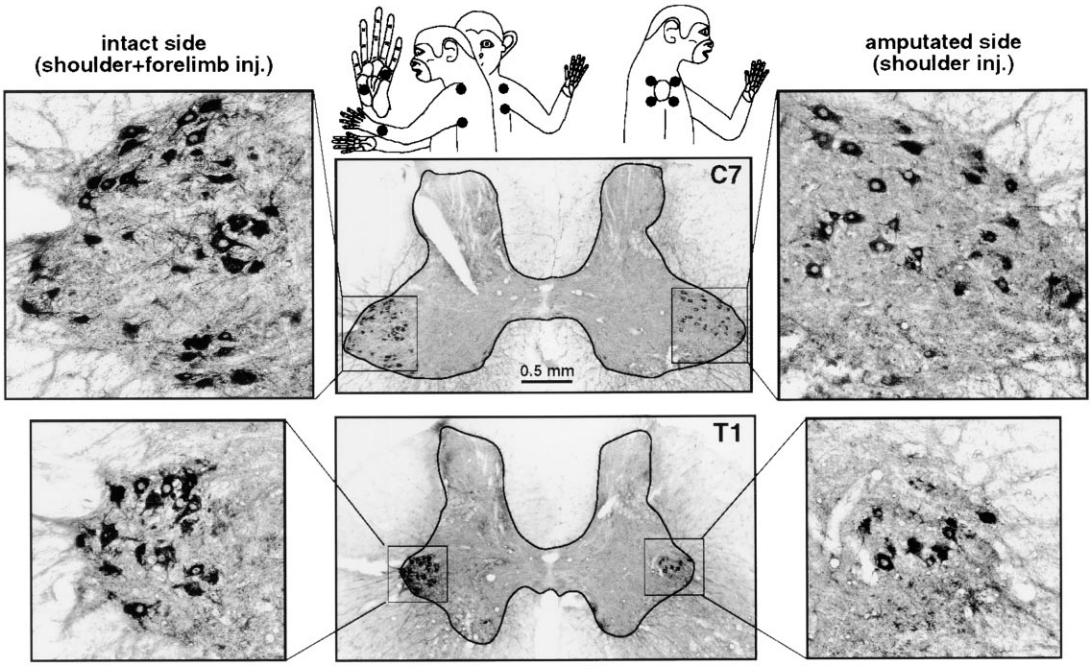

Figure 3. Photomicrographs of a Series of Spinal Cord Cross-Sections from a Forelimb-Amputated Squirrel Monkey

Photomicrographs from case 98-64 show the distribution of retrogradely labeled spinal motoneurons in sections processed for CTB. Following CTB injections into muscles of the forelimb and the shoulder on the intact side, labeled neurons were found in both lateral and medial motoneuron pools throughout C5–T2 segments (left). Injections of CTB into the shoulder muscles on the amputated side resulted in a similar extent of label as on the intact side (right). This indicates that some motoneurons formerly controlling the amputated limb have changed their muscle targets to innervate the shoulder muscles.

ing the distal hindlimb muscles were located in the most dorsolateral extreme of the lateral motoneuron pools at L6–S2 levels, whereas motoneurons innervating the proximal hindlimb were located ventromedial to the motoneurons controlling movement of the foot. The highest density of labeled motoneurons following distal hindlimb injections was found at L6–S2 levels, caudal to the highest density of labeled motoneurons following proximal hindlimb injection (Figure 4A).

Reorganization of Motoneurons Innervating the Hip Joint in the Hindlimb-Amputated Animal

Figure 4B illustrates the locations of labeled motoneurons following bilateral and symmetrical injections of tracers into hip muscles in a hindlimb-amputated galago. Consistent with the results from forelimb amputees, labeled neurons on the amputated side covered a larger extent of the spinal cord than on the intact side. Most importantly, labeled neurons were found in the dorsolateral extreme of the lateral motoneuron pools that normally only project to the distal hindlimb. This indicates that after long-term hindlimb loss, the distal motoneurons have shifted their targets to the muscles proximal to the site of amputation.

Changes in the Morphology of Motoneurons Formerly Supplying the Missing Limb

In agreement with earlier observations (Ranson, 1906; Donoghue and Sanes, 1988; Jones and Pons, 1998), the sizes of the dorsal horn, cuneate fasciculus, and ventral horn ipsilateral to the amputation or denervation were evidently reduced. Measures of surface area from coronal sections indicate that the lateral motor columns were also reduced by ~20%–35% in the lower cervical enlargement for forelimb amputees, and in the lumbosacral segments for the hindlimb amputee. To understand the possible causes for the shrunken motor nuclei, the numbers and sizes of the motoneurons on the amputated side were analyzed and compared to those on the intact side.

Number of Distal Limb Motoneurons in the Amputated Animals

Neurons in the dorsolateral extreme of the lateral nuclei at C7–T2 of the forelimb amputees or at L6–S2 of the hindlimb amputees were counted in the same sampled sections for both intact and amputated sides. Nearly equal numbers of distal limb motoneurons were sampled in the two sides of the amputees with an amputation at either young or adult ages (Table 1). This result suggests that the reduced size of the ventral horn on the amputated side was not due to a significant loss in the total number of distal limb motoneurons.

Size of Motoneurons Innervating the Missing Limb of the Amputated Animals

As previously described (Bryan et al., 1972; Strick et al., 1976; Hashizume et al., 1988), motoneurons of different sizes were intermingled in the longitudinal motor nuclei. On the intact side of the amputees, a bimodal size distribution was observed in the distal limb motoneuron group (i.e., the dorsolateral extreme of the lateral motor nuclei in C7–T2 or L6–S2; see Figure 5). Since it has been shown that γ motoneurons are systematically smaller in average soma diameter than α motoneurons (Bryan et

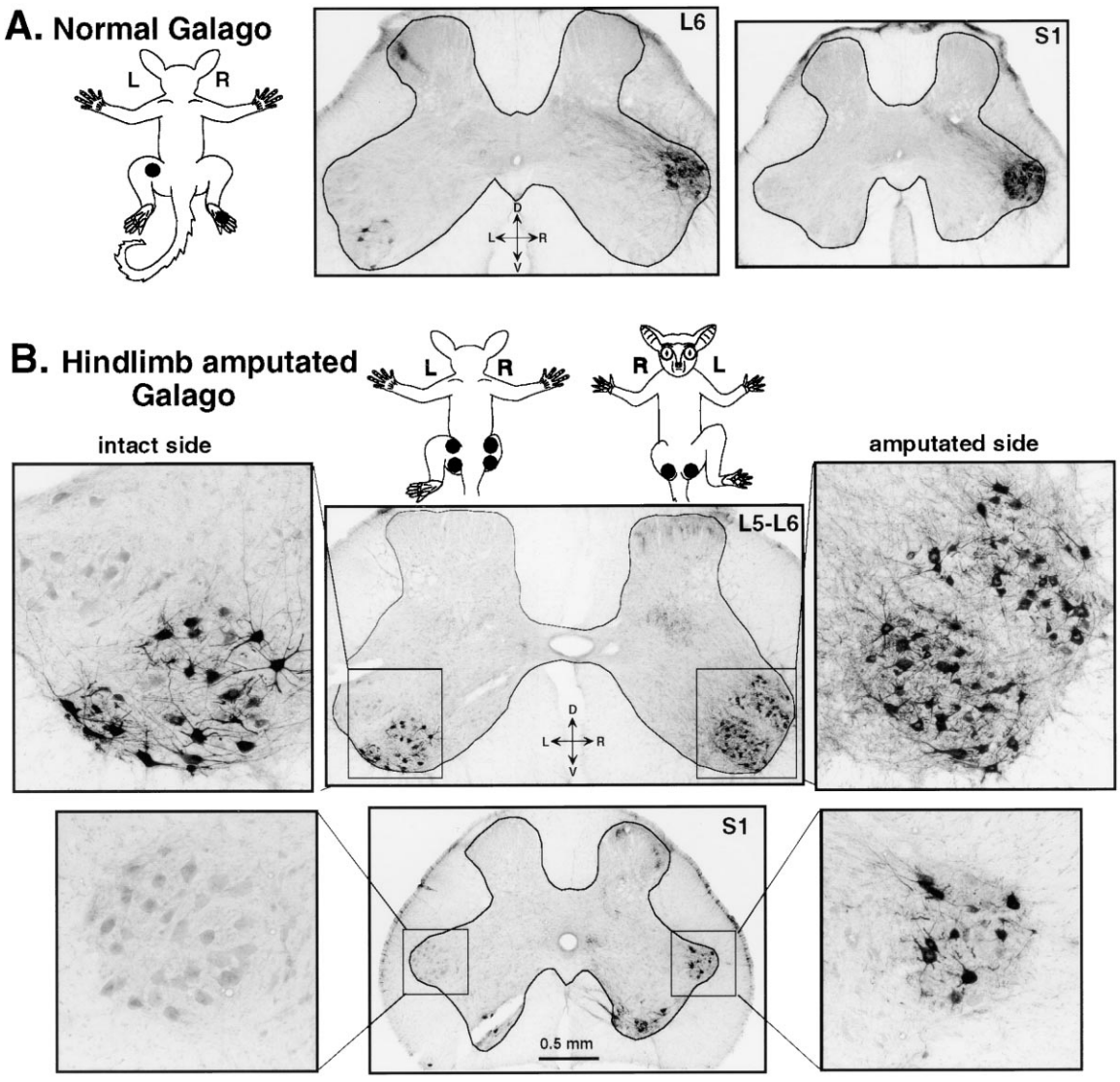

Figure 4. Reorganization of Motoneurons in Hindlimb-Amputated Animals

(A) Photomicrographs of a series of spinal cord cross-sections showing the distribution of retrogradely labeled motoneurons in the ventral horn of normal galagos after CTB injections into the muscles of the left upper leg and right foot. Motoneurons innervating the muscles of the feet were located in the lateral extreme of the lateral motoneuron pools at L6–S2 levels, whereas motoneurons innervating upper leg muscles were located in the ventromedial portion of the lateral motoneuron pool at L4–S1 levels.

(B) Photomicrographs of a series of spinal cord cross-sections from a leg-amputated galago (case 97-134) showing the distribution of retrogradely labeled spinal motoneurons in sections processed for CTB. Following bilateral and symmetrical injections of CTB into muscles of the hip, labeled neurons on the intact and amputated sides exhibited similar distributions, but more extensive labeling was found on the amputated side at lower levels, including dorsolateral portions of the lateral motoneuron pools that normally innervate distal hindlimb muscles.

al., 1972; Cullheim and Ulfhake, 1979; Westbury, 1982), we assume that cells with the smaller average diameter were γ motoneurons and that those with the larger average diameter were α motoneurons. Depending on the animal species and spinal segments, the total range of cell sizes and transition points between α and γ motoneurons varied. The distal forelimb motoneuron diameters were between 300–800 μm^2 and 900–1600 μm^2 in the galago and between 300–900 μm^2 and 1000–1800 μm^2 in squirrel and owl monkeys, for motoneurons in γ and α size ranges, respectively. The distal hindlimb motoneuron diameters were between 300–700 μm^2 and 800–1400 μm^2 in the galago and between 300–1500 μm^2 and 1600–1900 μm^2 in the squirrel monkey, for motoneurons in γ and α size ranges, respectively. In each case, a valley of lower numbers separated the peaks of the two subpopulations.

In contrast, a bimodal distribution of cell sizes was not apparent for the distal limb motoneurons on the amputated side. In addition, the distribution of the neurons on the amputated side was concentrated within the smaller sizes, with a significant reduction in the number of larger neurons in the presumed α motoneuron size range, accompanied by a significant increase in the number of smaller neurons in the presumed γ motoneuron size range as compared with the intact side (χ^2 test,

Table 1. Amputated Animals Used in This and the Parallel ICMS Studies[a]

Amputee Cases	Age at Time of Amputation	Level of Amputation	Survival Duration (Years)	Injection Sites (Amputated Side)	Injection Sites (Intact Side)
Squirrel monkeys					
Case 98–61	2 months old	near shoulder joint	8	CTB in shoulder FB in stump	CTB in shoulder FB in distal forelimb
Case 98–64	4 months old	shoulder joint	5	CTB in shoulder FB in stump FR in stump	CTB in the forelimb and shoulder FB in distal forelimb FR in proximal forelimb
Case 97–127	6 years old	hip joint	12		
Galagos					
Case 97–100	1.5 months old	shoulder joint	4	CTB in stump	CTB in distal forelimb
Case 97–134	1.5 months old	hip joint	7	CTB in hip	CTB in hip
Owl monkey					
Case 98–60	adult	above elbow	≥2		

[a] Wu and Kaas, 1999.

smallest difference among cases: $\chi^2 = 36.5$, $p < 0.001$). Since the total cell count on the amputated side was not significantly different from that on the intact side, the increased number of neurons in the γ motoneuron size range is likely due to a decrease in the size of neurons in the α motoneuron size range. Although γ motoneurons may have also undergone some atrophy, the effect of limb loss appears to be much greater on the larger α motoneurons. This suggests that the atrophy of the larger motoneurons and their associated neuropil is a major contributor to the size reduction of the ventral horn following long-standing limb loss. The atrophy and size reduction were apparent in various tissue preparations, including Nissl, neurofilament SMI-32, and acetylcholinesterase (AchE) stains (Figure 7). Furthermore, the packing density of the cells appears to be greater on the amputated side, with decreased spacing between motoneurons (Figure 7).

Appearance of Distal Limb Motoneurons Innervating the Stump and Shoulder or Hip of the Amputated Animals

Since neurons with smaller sizes were significantly greater in number on the amputated side, we determined whether these atrophied neurons maintained functional connections. One motoneuron function that is probably important for initiating and supporting successful muscle reinnervation is the ability to acquire and retrogradely transport material from the most distal component of the regenerated axon (Vanden Noven et al., 1993). Following injections of tracers into the muscles of the limb stump and shoulder or hip, the labeled distal limb motoneurons were smaller and simpler than the normal distal limb motoneurons labeled by distal limb injections on the intact side. Micrographs taken at high magnification revealed that the distal limb motoneurons were either lightly labeled or had very small and faint dendrites on the amputated side (Figures 2, 3, and 4B). Processing for AchE- and neurofilament-positive neurons also demonstrated a great reduction in the sizes of the soma and primary dendrites for motoneurons in locations normally projecting to the amputated limb (Figure 7). However, motoneurons of the former proximal limb muscles were less affected then those of the distal limb muscles. Some proximal limb motoneurons retained their normal sizes and processes.

Discussion

Motoneurons Affected by Limb Loss

The present results indicate that long-standing deprived motoneurons can successfully survive after long-term limb loss, as reflected in the unchanged total number of neurons and their capability of retrograde transport. The survival of neurons following injuries is related to the species of animals and the age of the animals at the time of injury (Oppenheim, 1991). In adult rats or cats, motoneurons can remain viable weeks to months after denervation (Carlson et al., 1979; Swett et al., 1991; Vanden Noven et al., 1993). In contrast, peripheral nerve injury in newborn or very young rats or cats results in massive motoneuron death (Romanes, 1964; Schmalbruch, 1984; Aldskogius and Thomander, 1986; Figure 10 of Donoghue and Sanes, 1988). The results of our study provide evidence for the survival of most of the motoneuron population supplying the missing limb in primates years after amputation at young or adult ages. Furthermore, we found that motoneurons of different sizes and in different locations were affected differentially by the injuries. Within motoneuron pools controlling the former distal limb, limb loss predominantly reduced cell size in the larger α component, whereas it had no apparent effect on cell size among the small, γ component of motoneurons. Such changes were less severe for the motoneurons supplying missing proximal muscles. While a reduction in the volume of the ventral horn does occur following long-standing limb loss, this is probably due to the atrophy of large motoneurons and associated neuropil controlling the former limb, rather than the loss of motoneurons. The preferential atrophy of large α motoneurons after limb loss may be similar to the atrophy that normally occurs during the aging process or after nerve injury (Mittal and Longmani, 1987; Hashizume et al., 1988; Vanden Noven et al., 1993).

The Reinnervation of New Targets by Deprived Motoneurons

The present study provides evidence that motoneurons supplying the former limb not only survive amputation injury but also reinnervate new muscle targets proximal to the amputation, as demonstrated by transported anatomical tracers. Since tracers were not picked up by the

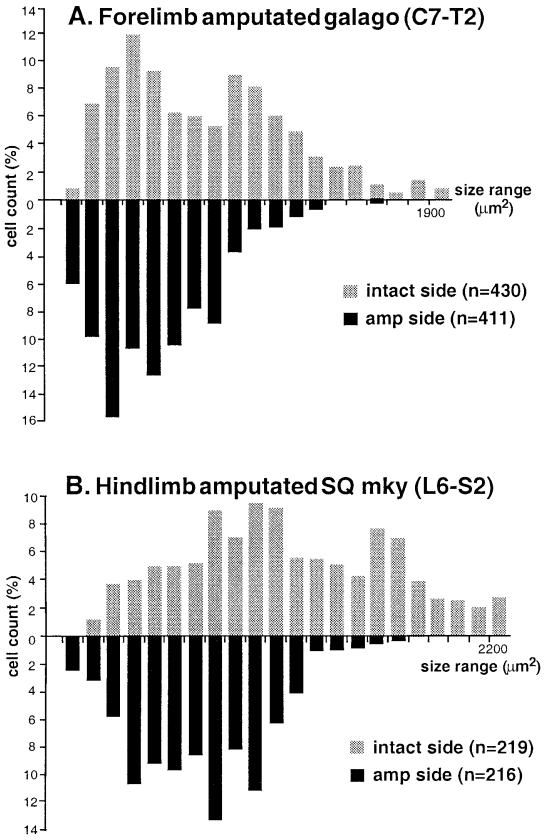

Figure 5. Frequency Distribution of Cross-Sectional Areas for the Population of Dorsolateral Motoneurons on the Amputated Side and Intact Side in Animals with an Amputated Limb

Black bars indicate the amputated side and gray bars indicate the intact side. Motoneurons were sampled from the C7–T2 levels in the forelimb-amputated galago (left) as well as L6–S2 levels of the hindlimb amputated squirrel monkey (right). Note the bimodal distribution of motoneurons on the intact side, with a reduction in numbers between the presumably smaller γ motoneurons and the larger α motoneurons. In contrast, on the amputated side, there is an obvious decrease in the number of motoneurons within the α motoneuron size range. Note that the total numbers of motoneurons sampled from the two sides are not significantly different within the same animals. Bin size is 100 μm^2. The last bin represents values equal to or greater than the value indicated for that bin.

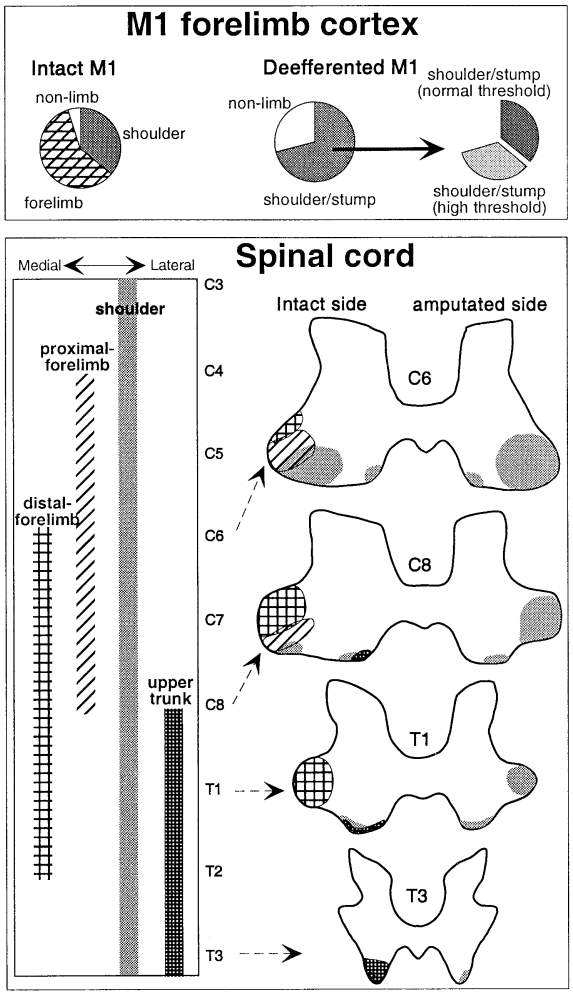

Figure 6. Summary Diagram of Reorganization at Different Levels of the Motor System after Long-Term Limb Loss

(Top) Pie charts showing the percentage of intracortical microstimulation sites for different body movements in the forelimb cortex of M1 in a forelimb-amputated squirrel monkey (case 98-61; Wu and Kaas, 1999). In M1 ipsilateral to the missing limb (i.e., intact M1), evoked movements predominantly involve forelimb and shoulder muscles, with a small percentage of sites devoted to nonlimb movements involving the face and trunk muscles. By contrast, in M1 contralateral to the missing limb (i.e., deefferented M1), there is a significant increase in the percentage of sites devoted to shoulder or stump and nonlimb movements.

(Bottom) Schematic drawings of the somatotopic organization of various motoneuron pools of the spinal cord innervating different muscle groups. The segmental distribution of motoneurons controlling distal forelimb, proximal forelimb, shoulder, or upper trunk muscles on the intact side are illustrated on the left. On the right, the mediolateral and dorsoventral distributions at different segmental levels of the motoneurons on the amputated side are compared to those on the intact side.

en passant nerve trunks following matched injections on the intact side of amputees or normal controls, we believe that labeled distal motoneurons on the amputated side following shoulder and stump injections are due to the reinnervation by the severed nerves of muscles proximal to the amputation. Although it has been reported that motoneurons can survive nearly half a year without forming synaptic contacts (Pinter and Vanden Noven, 1989; Vanden Noven et al.,1993), it seems unlikely that completely deprived motoneurons would survive and transport macromolecules 4–8 years after injury, especially if injury occurred at young ages during which motoneurons are especially vulnerable (Kuno, 1990; Oppenheim, 1991). Moreover, because motoneurons originally supplying missing limb and proximal body parts were labeled following injection of tracers in muscles proximal to the amputated stump, we believe that muscles proximal to the amputation were hyperinnervated by intact and deprived motoneurons (also see Manger et al., 1996). This finding was somewhat unexpected. Whereas synaptic contacts can be formed between a regenerated nerve and foreign denervated or paralyzed muscles, the innervation of host muscles is typically prevented by the presence of their normal in-

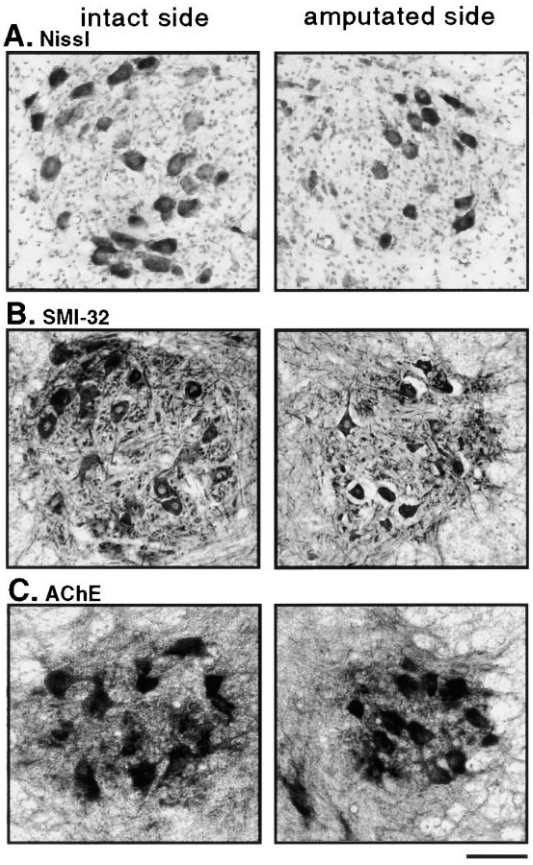

Figure 7. Photomicrographs of Cross-Sections from the S1 Segment of the Spinal Cord in Hindlimb Amputees Showing Changes in Motoneuron Morphology

Photomicrographs of cross-sections reacted for Nissl are shown in (A), for SMI-32 in (B), and for AChE in (C). Pairs of images are enlarged from intact (left) and amputated (right) sides of the same spinal sections. The general decrease in the size of dorsolateral nuclei on the amputated side can be clearly seen in these sections. Also note that the somata and primary dendrites of motoneurons on the amputated side are smaller than those on the intact side. Scale bar, 100 μm.

nervation (Brown et al., 1981; Dennis, 1981; Jansen and Fladby, 1990; Boss and Wigston, 1992; Vanden Noven et al., 1993; Chen et al., 1995). However, we cannot rule out the possibility that proximal muscles were also damaged and partially denervated during therapeutic amputation, and therefore allowed end-plate competition for the foreign nerves.

Motor nerve innervation is coordinated by signals between motoneurons and muscle fibers. Material released from degenerating axons and myelin of injured nerve, as well as growth factors and neurotrophic factors produced by the inactivation of muscles, can trigger the motoneurons to regenerate and reinnervate (Brown, 1984; Oppenheim, 1989; Diaz and Pecot-Dechavassine, 1990; Chiu et al., 1993; Chen et al., 1995; Bisby et al., 1996; Kishino et al., 1997; Novikov et al., 1997). The synaptic contacts of reinnervation can be further stabilized by the retrogradely transported, activity-dependent molecular signals produced by muscle fibers (Dennis, 1981; reviewed by Connor and Smith, 1994). Since axons can regenerate from the neuromas of the nerve stump (Sunderland, 1978), the tissue of the limb stump, including injured portions of the muscles controlling shoulder and/or hip movements, might have triggered the deefferented motoneurons to innervate it.

Target-derived molecules are crucial in the regulation of neuronal survival (Koliatsos et al., 1993; Nishi, 1994; reviewed by Oppenheim, 1989). Results from the present study suggest that motoneurons formerly supplying the missing limbs likely recover from their axotomized state by successfully reinnervating new targets (see also Foehring et al., 1986; Einsiedel et al., 1992). Although nearly all motoneurons survived amputation injury in the present cases, the full extent of the reinnervation remains uncertain, since tracers were not placed at all locations on the shoulder or hip stump. Factors such as the position and origin of motoneurons and muscles can determine the extent of reinnervation (Wigston and Sanes, 1985; Hardman and Brown, 1987; Wigston and Kennedy, 1987; Laskowski and Sanes, 1988; Boss and Wigston, 1992; Greensmith et al., 1997). This may explain our observation that proximal limb motoneurons were more resistant to atrophy than the distal limb motoneurons after limb loss.

However, successful innervations of foreign muscles do not guarantee regain of normal synaptic efficacy in new connections. Atrophy in motoneurons supplying the missing limb likely results from reduced synaptic efficacy for motoneurons with foreign compared to original targets. Such weaker synaptic efficacy may be due to competition with motoneurons supplying the original innervation or the lack of function and usage of the new target muscles proximal to the limb stump. Primary dendrites and other processes are also likely affected by the synaptic strength (Carlson et al., 1979).

Spinal Cord Contribution to the Reorganization in the Motor Cortex

In an earlier study, we reported a significant increase of cortical sites devoted to the shoulder or hip and stump representations in the deefferented M1 cortex after long-standing limb loss (Wu and Kaas, 1999). Expansions of the representation proximal to the amputated stump have been demonstrated in rats (Donoghue and Sanes, 1988; Sanes et al., 1990), galagos, and monkeys (Schieber and Deuel, 1997; Wu and Kaas, 1999; Qi et al., 2000), and humans (Woolsey et al., 1979; Hall et al., 1990; Cohen et al., 1991; Brasil-Neto et al., 1993; Kew et al., 1994; Pascual-Leone et al., 1996; Chen et al., 1998). Although modification of cortical circuits may be largely responsible for motor cortex reorganization, as suggested in recent studies (Jacobs and Donoghue, 1991; Chen et al., 1998; Ziemann et al., 1998a, 1999b), changes in the connections of spinal motoneurons with the muscle targets, such as those demonstrated in the present study, suggest that rewired spinal cord motoneurons may contribute to M1 reorganization.

Such spinal cord reorganization may account for some of the behavioral observations of amputated patients. It has been consistently reported that cortical stimulation in the deefferented M1 can evoke the sensation of the phantom limb, including vivid sensation of movement in the missing limb, accompanied by actual

stump movements or contraction in the muscles proximal to the stump (Woolsey et al., 1979; Cohen et al., 1991; Pascual-Leone et al., 1996). Moreover, vigorous involuntary contractions of the proximal muscles were observed when amputees were asked to imagine phantom limb movements (Ramanchandran, 1993). Since the corticospinal connections are preserved after limb loss (Kew et al., 1994; Chen et al., 1998; Wu and Kaas, 1999), activation of the deefferented cortex could activate the spinal motoneurons that have lost distal limb targets. These motoneurons would have reinnervated and activated proximal limb targets.

As muscles remaining after amputation receive a much larger number of spinal connections than those on the intact side, the total influence upon the hyperinnervated muscles may be stronger, even after a reduction in synaptic efficacy is taken into account. When relatively large portions of M1 cortex were activated by noninvasive transcranial magnetic stimulation, larger motor-evoked potentials (MEPs) were found in muscles proximal to the site of amputation (Cohen et al., 1991; Topka et al., 1991). This greater response could reflect involvement of a larger percentage of the spinal motoneurons in stump movements. Recent studies using intracortical microstimulation in deefferented M1 of monkeys with long-standing amputations found that an elevated average threshold is needed to evoke movements in remaining forelimb muscles, with some sites requiring higher thresholds and others remaining normal (Schieber and Deuel, 1997; Wu and Kaas, 1999). Perhaps cortical sites originally representing distal limb movements, due to weaker synaptic efficacy (as revealed by atrophy of distal limb motoneurons), now require higher currents to activate newly innervated proximal muscles through the same descending influence upon spinal motoneurons formerly supplying the missing limb. Therefore, simultaneously stimulating both low-threshold and high-threshold pathways by transcranial magnetic stimulation in M1 of human amputees could produce larger than normal MEPs.

Experimental Procedures

Animal Cases

Experiments were carried out in three squirrel monkeys (*Saimiri sciureus*), two prosimian galagos (*Galago garnetti*), and one owl monkey (*Aotus trivirgatus*), each with a long-standing therapeutic amputation. These amputated animals were obtained from other facilities where they had received serious injury to a limb and treatment in the form of surgical amputation was necessary. Each of these animals had lived 2 or more years after the amputation and had undergone intracortical microstimulation mapping as described previously (Wu and Kaas, 1999; Wu et al., 2000). Some of the present results have been briefly described before (Wu and Kaas, 1999, Soc. Neurosci., abstract). Details about the cases, including the extent and age of amputation, as well as the muscles in which tracers were injected, are summarized in Table 1. Since unilateral limb loss affects only motoneurons on the side of the missing limb, the contralateral side was used as an undamaged control for direct comparison. Results were also compared to those from normal animals. Animals were cared for in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and the guidelines of the Vanderbilt University Animal Care and Use Committee.

Tracer Injections

Animals were anesthetized with isofluorane gas for the muscle injections. Tracers were injected with a Hamilton syringe that was used to penetrate the skin and enter the muscles after the skin was cleaned with disinfectant. Ten minutes after each injection, the syringe was slowly withdrawn from the muscle. The distribution of motoneurons innervating the various muscle groups was examined by placing the same tracers into different muscle groups across individuals, or injecting different tracers into different muscle groups within individuals. Tracers used in this study included cholera-toxin B subunit, (CTB; 1% in distilled water, 20–25 μl/site), wheat germ agglutinin conjugated to horseradish peroxidase (WGA-HRP; 4% in saline, 20–25 μl/site), choleragenoid-HRP (B-HRP; 1% in saline, 20–25 μl/site), fast blue (FB; 5% in distilled water, 15–20 μl/site), and fluoro-ruby (FR; 10% in saline, 15–20 μl/site). Although the tracers for each injection may differ in spread within the tissue or in uptake efficacy, the overall locations of the labeled motoneuron pools in the spinal cord after injections made in the same muscle group were consistent across individuals or tracers.

For comparison with amputated cases, tracers were injected at several sites in muscles mediating distal limb ($n = 8$), proximal limb ($n = 5$), or axial body ($n = 9$) movements in normal animals. In the amputated animals, we were interested in determining whether the motoneurons controlling missing limbs lose their function and undergo degeneration, or whether they survive amputation and are still capable of reinnervating muscles. Therefore, tracers (20 μl of CTB, FB, or FR at each site) were injected into both sides of the body. In the forelimb amputees, the locations of injection sites were chosen to allow direct comparison of motoneurons innervating the shoulder on both sides or innervating the amputated stump and normal forelimb muscles in the same individuals (see Table 1). Therefore, tracers were placed in the normal forelimb and amputated stump or bilaterally and symmetrically in the shoulder muscles (see Table 1). In addition, tracers were placed bilaterally in the hips of a hindlimb amputee.

Perfusion and Histology

Typically, following a session of stimulation of motor cortex with microelectrodes (see Wu and Kaas, 1999, for detail) and a survival of 3–10 days after muscle injections (10 days for FB and FR, or 3–8 days for CTB, WGA-HRP, and B-HRP), the animals were deeply anesthetized with a lethal dose of sodium pentobarbital and when areflexive were perfused through the heart with cold saline followed by fixative (4% paraformaldehyde in 0.1 M phosphate buffer [pH 7.4]). After the perfusion, the spinal cord was exposed, and the borders between segments were judged as lying between the most caudal and rostral rootlets of adjacent segments. Borders between segmental levels C3–T3 or L5–S2 were marked on the ventral surface of the cord with a fine pin. Tissue blocks containing these segments were placed in 30% sucrose buffer at 4°C overnight. Serial sections in the coronal plane were cut at 50–60 μm thickness on a freezing microtome.

Sets of every sixth section were mounted unstained for fluorescent tracers, after immunocytochemical processing for CTB (goat anti-CTB, Vector Laboratories) following standard procedure (Angelucci et al., 1996), or after tetramethylbenzidine (TMB) treatment to reveal WGA-HRP or B-HRP (Gibson et al., 1984). Additional sections were reacted for AChE (Geneser-Jensen and Blackstad, 1971), cytochrome oxidase (Wong-Riley, 1979), Nissl substance, or neurofilament antibody SMI-32 (Sternberger Monoclonals; see Preuss et al., 1997, for details) to reveal the architectonic features and cell morphology.

Data Analysis

The spinal cord sections were examined serially under bright-field microscopic illumination for neurons containing the CTB reaction product. The locations of cells labeled by WGA-HRP or B-HRP injections were determined under dark-field illumination. Labeled neurons were drawn at the final magnification of 100× along the segmental levels. The locations of fluorescence-labeled cells were charted with a Leitz microscope connected to an x–y plotter, with 360 nm (for FB) and 530–560 nm (for FR) wavelength excitation filters. Since no attempt was made to prevent the tracers from being uptaken by the cutaneous receptors of the skin or by muscle spindle receptors during the muscle injections, in addition to the motoneuron groups label was also observed in the fibers distributed in the

superficial laminae of the dorsal horn of the spinal cord. Although the diffusion of tracers throughout the muscles was likely uneven, so that some sectors of the muscle probably contained heavier deposits of tracers than others, this potential problem was reduced by placing tracers at multiple sites within given muscle groups. We found that the motoneuron groups were consistently labeled in the same spinal segments and in the same locations for injections made in the same muscle groups.

A digital image (Leaf Lumina digital camera) was captured at 100× magnification of the distal limb motoneuron pools of the limb-amputated animals. Photomontages of tissue sections were composed using Macintosh versions of Photoshop (Adobe System) and Canvas (Deneba System) softwares. Cell counts and cell size measurements were made from sections stained for Nissl substance. A cell was counted when its nucleus was included in the section or when a nucleolus could be visualized. To reduce sampling errors in cell counts (see Swett et al., 1991), a large number of cells (205–430 in each side) were counted from 12–14 serial sections, all at least 4 sections apart, over several spinal segments (e.g., C7–T2 for forelimb amputees or L6–S2 for hindlimb amputees).

The surface areas of cross-sections of the spinal ventral horn and the sizes of motoneurons were measured from a series of coronal spinal cord sections. Digital images were captured and the ventral horn was outlined in each section. The volume of the spinal lateral motoneuron column in the ventral horn from Rexed's laminae IX was calculated from series of histological sections from C7–T2 in the forelimb amputees and from L6–S2 in the hindlimb amputees. Motoneuron sizes were determined by outlining the contours of somas and their primary dendrites at high magnification. The areas within the contours were automatically integrated by closure of the profile's perimeter. Since we compared the two sides of the same sections for each case, no correction for tissue shrinkage was used. Cell counts and size measures were conducted in all animals with amputation of a limb except for two forelimb-amputated squirrel monkeys that received spinal cord injections. Results from the amputated side were directly compared to those from the normal side.

Acknowledgments

We thank Drs. C. Collins, N. Jain, and S. L. Florence for helpful comments on the manuscript as well as M. Feurtado for assistance with tracer injections. We are also grateful to J. Ives and L. Trice for histological assistance. This research was supported by National Institutes of Health grant NS 16446 to J. H. K.

Received July 28, 2000; revised September 27, 2000.

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