Selective motor hyperreinnervation using motor rootlet transfer: an experimental study in rat brachial plexus

JAYME AUGUSTO BERTELLI, M.D., PH.D., JEAN CLAUDE MIRA, D.S.C., MONIQUE PECOT-DECHAUVASSE, D.S.C., AND ALAIN SEBILLE, M.D.

Department of Pharmacology, Universidade Federal de Santa Catarina, Florianopolis, Brazil; Laboratoire de Microchirurgie, Ecole de Chirurgie des Hopitaux de Paris, Paris, France; Laboratoire de Neurobiologie, Groupe “Regeneration des Nerfs Peripheriques et des Muscles Squeletiques,” Université René Descartes, Paris, France; Institut des Neurosciences-CNRS, Departement de Cytologie, Université Paris VI, Paris, France; and Laboratoire d’Electrophysiologie Fonctionnelle Neuromusculaire, Faculté de Medicine Saint-Antoine, Paris, France

When nerve roots of the brachial plexus are avulsed from the spinal cord, they do not spontaneously regenerate. Instead, the muscles innervated by these roots become permanently paralyzed. In some patients, nerve transfer using intercostal nerves, accessory nerve,1 cervical plexus,11 or C3–4 anterior rami11 to the musculocutaneous nerve or suprascapular nerves has been shown to restore some useful functions. Nevertheless, the results are still limited. Sensory fibers, which reach a muscle fiber by misdirection following peripheral nerve repair, are not able to generate motor functional synapses. These axons not only fail to establish functional contacts, but may also exclude appropriate motor axons from the pathways they occupy.

In the present experiment, an anterior motor rootlet of C-4 was directly attached to the musculocutaneous nerve using a peripheral nerve graft procedure, which was performed to increase the number of regenerating motor fibers and, thus, avoid growth of sensory fibers into the nerve grafts. In this manner, we induced motor hyperreinnervation of the biceps muscle, that is, muscle hyperreinnervation in which more motor neurons participate than is normal in rats that have not been surgically treated.

Materials and Methods

Animal Preparation

Experiments were performed on the brachial plexus in 16 young female Sprague–Dawley rats, each weighing approximately 230 g. Before surgery, anesthesia was induced in the rats by intraperitoneal administration of a 7% solution of chloral hydrate (0.6 ml/100 g body weight). The rats were handled in accordance with French laws governing animal use for experimental purposes.

Surgical Procedure

In the first group of animals (Group 1, 12 rats), dorsal laminectomy was performed and the C-5 anterior and posterior roots were avulsed from the spinal cord using traction. The C-4 dorsal root was dissected to expose the inferior C-4 anterior rootlet, which was then sectioned and connected to the proximal end of a 40-mm-long peripheral nerve graft (rootlet transfer) harvested from the left sural nerve. Via an anterior approach, the left musculocutaneous nerve was dissected in the axilar region and was sectioned. The proximal stump of the musculocutaneous nerve was ligated twice and turned proximally, whereas the distal stump was anastomosed to the distal end of the sural nerve graft (Fig. 1). Fibrin glue (Tissucol–Immuno France) was used for the entire nerve anastomosis. The dura mater was also repaired with an application of fibrin glue.

In a second group of animals (Group 2, four rats), the left musculocutaneous nerve was exposed, ligated on both ends, and cut between the ligatures.

Electrophysiological Measurements

Study of Biceps Brachii Motor Reinnervation. Selected animals in both groups (six from Group 1 and all four from Group 2) underwent electrophysiological studies 18 months after surgery. The peripheral nerve graft muscle preparation was removed from three of the animals in Group 1, transferred to a chamber, and bathed in an oxygenated physiological solution. The nerve graft was stimulated by a glass suction electrode with current pulses of varying
In three months after surgery. The cervical spinal cord was removed and placed in 30% sucrose for 24 hours. Longitudinally sliced serial frozen sections, 40-μ thick, were cut, mounted on slides, protected by coverslip, and examined using epifluorescence (excitation wavelength 395–425 nm, barrier filter 450 nm). Only labeled neurons with visible nuclei were counted. No corrections for split cell counts were made.

Histological Evaluation of Axonal Regeneration and Muscle Reinnervation

In animals from Group 1 (rootlet transfer) and Group 2 (control group), the biceps brachii muscle was removed bilaterally and weighed 18 months after surgery. In three animals from Group 1 reinnervated biceps muscles and their contralateral normal counterparts were used to study the morphology of the neuromuscular junctions by using a modified Koelle–Friedenwald method. In the remaining animals from Group 1 (nine rats) and in all animals from Group 2 (four rats), surgically treated and untreated normal contralateral biceps muscles were removed and frozen in isopentane that had been cooled in liquid nitrogen to ~160°C. Muscles were cut using a freezing microtome, stained according to hematoxylin and eosin and modified Gomori trichrome methods, dehydrated in serial concentrations of alcohol, and mounted on slides.

In six animals from Group 1, the musculocutaneous motor branch to the biceps muscle (Fig. 2) was harvested from the surgically treated side, fixed in 2.5% glutaraldehyde and postfixed in 1% osmium tetroxide for 1 hour, dehydrated in serial concentrations of alcohol, and embedded in epoxy resin. One-micron sections were obtained and were stained with toluidine blue for light microscopy.

Statistical Analysis

Morphological results of the retrograde labeling studies and fiber density (1–10 V) and duration (0.2–1 msec). Intracellular recordings of variations in muscle membrane potentials (muscle action potentials, endplate potentials, and miniature endplate potentials) were obtained using conventional electrophysiological techniques, with microelectrodes filled with a 3 M KCl solution. In some cases, endplate potentials were recorded after the addition of curare (2–8 × 10^{-6} M) to the bathing solution.

Study of Biceps Brachii Twitch and Tetanic Strength.

In three other animals from Group 1, twitches and tetanic tension of the biceps brachii were recorded bilaterally in situ. After general anesthesia had been induced, the animals were heated by warming lamps to ensure a local temperature of 25° to 27°C. The musculocutaneous nerve and the biceps brachii muscle were gently dissected and regularly soaked with saline solution (NaCl 0.9%, 27°C). The distal tendon was cut and the myotendinous junction was firmly tied with a silk suture. The forelimb was firmly attached to the recording table using two needles that passed through the shoulder and elbow joints. The silk suture was connected to an isometric force transducer. The sural nerve graft was proximally sectioned to avoid reflex responses and was gently suspended over two hooked Ag–AgCl stimulating electrodes. Supramaximal rectangular stimuli (2 msec in duration, 10 mA, 15 V) were delivered through a constant current isolator, either individually (for twitch), successively, or in trains (60–90 Hz, 450 msec for tetanus). There was a resting period of at least 5 minutes between two trains of stimulation to avoid fatigue. Amplified contraction signals (DC, bandwidth 0.3 Hz) were visualized on a storage oscilloscope (Hameg HN 205-2) connected to a thermal printer (Hameg HM 8148). The muscle length was adjusted to obtain the maximum isometric twitch tension, and this length was used for subsequent recordings. The peak amplitudes of the twitch and the tetanus were measured and calculated as a percentage of the recovery of normal muscle.

In all Group 1 animals the C-4 root was stimulated in situ with a surgical nerve stimulator (Concept, Inc.) and contraction of the shoulder muscles was observed. In Group 2 animals the surgically treated musculocutaneous nerve was stimulated in situ with the same surgical nerve stimulator and the contraction of elbow flexors was observed.

Retrograde Labeling Studies

Eighteen months after surgery, six animals from Group 1 were subjected to retrograde labeling studies. In three rats, the musculocutaneous nerve was sectioned and the proximal stump was exposed to an aqueous solution of 2.5% True Blue (Sigma); in the remaining animals, the motor branch to the biceps brachii muscle was exposed to the same solution (Fig. 2). Five days later, the rats were deeply anesthetized and perfused with 0.9% saline solution, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The cervical spinal cord was removed and placed in 30% sucrose for 24 hours. Longitudinally sliced serial frozen sections, 40-μ thick, were cut, mounted on slides, protected by coverslip, and examined using epifluorescence (excitation wavelength 395–425 nm, barrier filter 450 nm). Only labeled neurons with visible nuclei were counted. No corrections for split cell counts were made.

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Statistical Analysis

Morphological results of the retrograde labeling studies and fiber density.
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Counts from the grafted rats were compared to data obtained from normal untreated rats. The results of the electrophysiological studies, muscle strength and weight were compared with those obtained for the normal contralateral side. Means were compared using analysis of variance. A difference at the 1% level was considered significant.

Results

Electrophysiological Studies

In the animals that received grafts (Group 1), a strong contraction of the biceps brachii muscle was noted after in situ electrical stimulation of the musculocutaneous nerve or the sural graft. Contraction of the shoulder muscle was also observed after stimulation of the C-4 spinal root.

In isolated peripheral nerve graft–muscle preparations, action potentials evoked by electrical stimulation of the graft were recorded for all muscle fibers studied (Fig. 3). Typical, spontaneous, miniature endplate potentials were obtained at focal sites, in the region of the original endplates. At this site, endplate potentials, evoked by stimulating the grafted musculocutaneous nerve, were either detected as a step preceding the propagating active potential or were recorded alone after adding curare (2 × 10⁻⁷ M) to the medium. The cholineric nature of the transmission was further demonstrated by a gradual disappearance of the endplate potentials after adding higher concentrations of curare (up to 10⁻⁶ M). The recovery of twitch strength was 94% (standard deviation [SD] ± 5%) and that of tetanic strength was 96% (SD ± 8%) compared with the contralateral normal side (Fig. 4).

Morphology of the Biceps Brachii Muscle and Morphometrical Data

In Group 1, the average weight of the muscles from the surgically treated side was 312 mg (SD ± 44 mg). The average weight of the muscles from the contralateral normal side was 303 mg (SD ± 23 mg) and that of the muscle denervated by section of the musculocutaneous nerve was 49 mg (SD ± 13 mg).

When examined using light microscopy, the muscles reinnervated by the grafted nerve showed no signs of chronic denervation or adipose tissue deposits. However, muscles from Group 2 (denervation control) exhibited prominent signs of chronic denervation (Fig. 5).

In whole mounted preparations of the biceps brachii muscle processed for cholinesterase activity, the endplates had the typical appearance of normal endplates, displaying continuous and ramified pretzellike synaptic gutters and subneural lamellae, in both surgically treated and contralateral sides. No ectopic endplates were identified in the surgically treated rats (Fig. 6).

In Group 1 rats, an average of 570 (SD ± 65) myelinated fibers were counted in the motor branch to the musculocutaneous nerve (Fig. 7).

Retrograde Labeling Studies

Labeled neurons were found from C-4 to the superior portion of the C-5 level along 3 to 4 mm rostrocaudally in the animals that received grafts (Figs. 8 and 9). They were identified in Rexed laminae 7, 8, and primarily 9. No labeled neurons were found in the dorsal horn. Labeled motor neurons were 253 (SD ± 21) in number for the musculocutaneous nerve and 146 (SD ± 12) for the biceps motor branch.

The experimental results of grafted, denervated, and nontreated rats are shown in Table 1.

TABLE 1

Results of selective motor hyperreinnervation in rats*

<table>
<thead>
<tr>
<th>Characteristics of Muscle &amp; Nerve</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Nonop Contralat Limb</th>
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<tbody>
<tr>
<td>biceps weight in mg (12 rats)</td>
<td>312 ± 44</td>
<td>49 ± 13</td>
<td>303 ± 23</td>
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<tr>
<td>retrograde labeled motor neurons</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>musculocutaneous nerve (3 rats)</td>
<td>253 ± 21</td>
<td>—</td>
<td>192 ± 20</td>
</tr>
<tr>
<td>motor branch to biceps muscle (3 rats)</td>
<td>146 ± 12</td>
<td>—</td>
<td>112 ± 6</td>
</tr>
<tr>
<td>no. of myelinated fibers of the motor branch to biceps muscle</td>
<td>570 ± 65</td>
<td>—</td>
<td>288 ± 12</td>
</tr>
</tbody>
</table>

* Biceps weight in Group 2 was significantly lower (p < 0.001) than those in Group 1 and in the contralateral untreated limb. No significant difference was found between these latter two groups. The number of retrograde labeled motor neurons in the grafted side of Group 1 was significantly higher (p < 0.001) than in the contralateral side. This was also valid for the number of myelinated fibers inside the motor branch to the biceps muscle. Values are represented as mean ± SD. Abbreviation: — = not studied.
demonstrated. The number of myelinated fibers and motor neurons in the biceps muscular branch was significantly larger than that in normal nerves. Moreover, there were no functional consequences following rootlet section because full contraction of the shoulder muscle was observed after C-4 electrical stimulation. In fact, single motor units are able to grow to approximately five times their original size, resulting in the ability to compensate for up to 80% of motor neuron loss.24

According to Bell's and Magendie's law, motor fibers leave the spinal cord by the ventral root and sensory fibers enter the spinal cord by the dorsal root. It has also been proposed that sensory axons may enter the spinal cord via the ventral roots,14,27 placing Bell and Magendie's law in question. These sensory unmyelinated fibers in the ventral root were thought to have arisen from adjacent dorsal root ganglions.36 This hypothesis was further argued by Coggeshall, et al.,13 who found that most of the unmyelinated axons in the ventral roots of the cat disappear after removal of the corresponding dorsal root ganglion. However, the number of sensory fibers in the ventral root is only 10 to 15% of all fibers.28 In the experiments on motor rootlet transfer reported here, sensory fibers inside the motor rootlet would be expected to degenerate. Sensory fibers may reach motor roots by passing through the dorsal root and spinal cord or, alternatively, reentering the spinal cord by the ventral root. In the present model, all these possibilities have been eliminated because the dorsal and transferred ventral rootlet were sectioned. The reinnervated biceps muscle in this experiment was probably deficient in proprioceptive innervation. This could lead to poor functional recovery. However, it has been demonstrated that deafferent patients can compensate for functional deficits by using visual feedback.23,33 Moreover, in the current experimental model, skin and antagonist proprioceptive afferents were func-

**Fig. 5.** Photomicrographs showing sections of grafted (A) and denervated (B) muscles. There is no sign of chronic denervation in the surgically treated muscles and the muscle fibers have regained normal morphology. Note the fiber atrophy/necrosis and inflammatory cell infiltration in denervated muscle (B). Bars = 50 μm.

**Fig. 6.** Photomicrographs displaying a low-power view of endplates in the grafted group and (inset) a higher-power view of the motor endplates. The endplates show a typical appearance of continuous and ramified pretzellike synaptic gutters and subneural lamellae. No ectopic endplates are identified. Cholinesterase staining. A: Bar = 100 μm. B: Bar = 25 μm.

**Fig. 7.** Photomicrograph showing a transverse section of the biceps branch of the musculocutaneous nerve in an animal from Group A (grafted group) 18 months after surgery. Well-myelinated nerve fibers are noted at the lower portion of the figure but only a few thinly myelinated fibers are found at the upper portion. Toluidine blue, bar = 5 μm.
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Fig. 8. Electron micrograph showing motor neurons labeled in a longitudinal section after biceps motor branch exposure to True Blue aqueous solution. Bar = 50 μm.

Fig. 9. Camera lucida drawings of labeled neurons in longitudinal sections of the spinal cord in the normal rat (A) and in the grafted group (B) showing the common arrangement of labeled motoneurons. Only the left side of the spinal cord is represented. The upper part of the drawings corresponds to the upper root levels. CC = central canal.

The rôle of proprioception in motor control has been questioned. The delays in most sensorimotor loops are large, making feedback control too slow for rapid movements. The notion of an internal model, a system that mimics the behavior of a natural process, has now emerged as an important theoretical concept in motor control. The cerebellum is the proposed site for the model.40

An interesting finding of this study was motor hyperreinnervation of the musculocutaneous nerve and biceps brachii muscle after motor rootlet transfer. This was confirmed by the larger number (approximately 25%) of retrograde-labeled neurons in the musculocutaneous nerve and in the motor branch to the biceps muscle (approximately 35%) and by the larger number of myelinated fibers (approximately 50%) compared with normal animals.8

In the musculocutaneous nerve, it is probable that these supranumerary motor axons not only reinnervated the biceps brachii and brachialis anterior muscles but also reinnervated the cutaneous branch (that is, the lateral cutaneous nerve of the forearm) of the musculocutaneous nerve, thus transforming a sensory branch into a pure motor branch. This raises the possibility of using this branch to reinnervate other muscles in the forearm, such as wrist extensors, in brachial plexus injuries.

The supranumerary motor axons in the motor branch to the biceps muscle could lead to 1) formation of smaller motor units;29 2) reinnervation of muscle proprioceptors because sensory fibers were excluded from reinnervation; and 3) polyneural innervation. Polyneural innervation has been identified by other authors in various degrees after muscle hyperreinnervation.20,37 In our experiments, polyneuronal innervation was not observed. This finding could be related to the long period of assessment. It is well known that with time the level of polyneuronal innervation is reduced by the process of synapse elimination.29

The number of supranumerary motor neurons induced here (approximately 35%) is small in comparison to other reports (1200%).30 Finally, in the present model, sensory fibers were excluded from reinnervation. Sensory fibers in a motor nerve constitute at least half of the myelinated fibers.8,31 Therefore, competition for muscle fiber reinnervation was largely diminished. Not only did sensory fibers not compete for muscle fiber reinnervation, but their own sensory pathways were free to be reinnervated by superfluous motor fibers.

A few reports were found in the literature concerning muscle hyperreinnervation.20,29,32,35,37 To induce hyperreinnervation, investigators have crosstransferred nerves,20,37 implanted additional nerves in the innervated muscle,29,32 or partially removed the muscle to be reinnervated.29 However, to the best of our knowledge, this is the first report of selective motor hyperreinnervation using a motor rootlet. In recent reports we have demonstrated reinnervation of peripheral nerve grafts directly implanted into the spinal cord.56 Direct spinal cord implantation of a peripheral nerve graft connected to a single peripheral nerve also constitutes a model of hyperreinnervation.

After nerve transection, muscle often fails to regain its normal mass and strength.21 This is more clearly demonstrated when a nerve graft is interposed between the nerve stumps.7 The longer the graft, the more deficient the reinnervation.22 In the present report, in spite of a 40-mm-long nerve graft, there were minimal deficits in reinnervation. Therefore, selective motor hyperreinnervation seems to improve the quality of motor recovery. Better recovery in hyperreinnervated muscle has been demonstrated previously.28

After motor rootlet transfer motoneurons regenerated and reinnervated muscle endplates. Functional reinnervation was almost normal. This high degree of reinnervation in a long (40-mm) graft is attributed to the good chance of a muscle fiber being reinnervated by a motor fiber, first because the number of regenerating motor neurons is increased, and second, because the competitive sensory fibers are excluded from reinnervation.

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References


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Address reprint requests to: Jayme A. Bertelli, M.D., Ph.D., Praca Getulio Vargas, 322. Florianopolis-SC, Brazil 88 020 030.