Peripheral Pathways Regulate Motoneuron Collateral Dynamics

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Motor axons regenerating after repair of mixed nerve reinnervate pathways leading to muscle more often than those leading to skin [preferential motor reinnervation (PMR)]. Motoneurons that initially project collaterals to both muscle and skin prune incorrect projections to generate specificity. The number of motor axon collaterals maintained entirely within cutaneous or muscle pathways, however, is unknown. To overcome this shortcoming, dorsal root ganglion excision has been used to allow only motor axons to regenerate after a peripheral lesion. Motor axon number in reinnervated cutaneous and muscle pathways can then be correlated with the number of parent motoneurons determined by retrograde labeling. The number of collaterals per neuron can be calculated for each environment and the relative roles of pathway and end organ assessed by blocking the distal pathways to prevent target reinnervation.

Without sensory competition, PMR develops in two stages: a limited response to muscle nerve and then a robust response to muscle that may involve retrograde signaling to the proximal pathway. Motoneurons maintain more collaterals in cutaneous nerve than in muscle nerve, even without muscle contact. This difference could result either from increased collateral formation in cutaneous nerve or from increased collateral pruning in muscle nerve. In either instance, these findings confirm that muscle and cutaneous pathways have functionally significant identities that can be recognized by motor axons and can regulate their arborization. Decreased arborization in muscle pathways could promote regeneration by focusing neuronal resources on high-yield projections; increased arborization in cutaneous pathways, conversely, would enhance pathfinding abilities.

Key words: axon guidance; retrograde labeling; regeneration; dorsal root ganglion; deafferentation; peripheral nerve

Introduction

Preferential motor reinnervation (PMR) is the tendency for motor axons regenerating in mixed nerve to reinnervate muscle nerve (M) and/or muscle rather than skin (Brushart, 1988). During early regeneration, individual motoneurons often project collaterals to both cutaneous (C) and muscle pathways. Specificity is then generated by pruning collaterals from cutaneous nerve while maintaining those in muscle nerve [the pruning hypothesis (Brushart, 1990)]. In young animals, PMR occurs in response to muscle nerve alone, suggesting that this difference does not result from increased collateral formation in cutaneous nerve or from increased collateral pruning in muscle nerve. In either instance, these findings confirm that muscle and cutaneous pathways have functionally significant identities that can be recognized by motor axons and can regulate their arborization. Decreased arborization in muscle pathways could promote regeneration by focusing neuronal resources on high-yield projections; increased arborization in cutaneous pathways, conversely, would enhance pathfinding abilities.
Femoral nerve repair. Each tributary nerve can then be characterized by the number of myelinated motor axons it contains and by the number of parent motoneurons that produced these axons. The mean number of myelinated collaterals per neuron in that environment can then be calculated. By blocking the distal pathway, the relative contributions of pathway and end organ to collateralization can be determined. Applying these techniques, motoneurons were found to maintain more collaterals in cutaneous nerve than in muscle nerve, regardless of whether muscle was reinnervated. Muscle and cutaneous nerve thus differ in ways that can be recognized by motor axons and that can regulate their arborization. Decreased arborization in muscle pathways could focus resources on axons with a demonstrated potential for success; increased arborization in cutaneous pathways, conversely, would focus continued efforts on pathfinding.

Materials and Methods

Surgical procedures. Experiments were performed on the femoral nerves of juvenile (3–4 weeks of age; 50–75 g) female Sprague Dawley rats (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) (Brushart, 1988). Juveniles were chosen for these experiments because of previous evidence of pathway recognition in this age group (Brushart, 1993). In the proximal femoral nerve, at the site of transection and repair, axons destined for skin and muscle intermingle. Regenerating axons that contact the distal stump will thus have access to Schwann cell tubes that lead either to muscle or to skin. Distally, the nerve bifurcates into a muscle branch to the quadriceps, containing both motor efferent and muscle afferent fibers and a purely afferent cutaneous branch. Myelinated axon counts are greater in the cutaneous branch (muscle, 1251; cutaneous, 1586), whereas total axoplasmic area is greater in the muscle branch (muscle, 12,263 μm²; cutaneous, 7505 μm²), ensuring a balance of factors that might contribute to preferential reinnervation (Brushart, 1988).

Animals were anesthetized by intramuscular injection of ketamine (87 mg/kg) and xylazine (13 mg/kg). Two types of experimental nerve repair were each assessed at three time periods, resulting in six experimental groups. In "pure motor-end organ" preparations (EO), dorsal root ganglionectomy ensured that only motor axons could reinnervate the distal nerve stump after femoral nerve transection and repair performed under the same anesthetic. In these animals, the distal femoral cutaneous and muscle branches remained connected to their respective end organs. Unilateral laminectomy was performed from L2–L5, and adjacent facet joints were excised with a 1 mm rongeur to expose the underlying DRGs. The peripheral nerve from all 8-week-old animals was processed for demonstration of HRP within neurons (Mesulam, 1982). Sections were viewed with fluorescent (405 nm) and transmitted light at 20–40×. The presence of split cells in adjacent sections was corrected as described by Abercrombie (1946). Each of the six experimental groups was then characterized by three means: the mean number of motoneurons projecting correctly to the cutaneous branch, the mean number projecting incorrectly to the cutaneous branch, and the mean number of double-labeled neurons, those projecting axon collaterals to both branches. Multiple regression analyses were used to compare means of correct, incorrect, and double-labeled neuron counts to one another both within and among each of the six groups.

Axon counting. Cross sections of cutaneous and muscle nerve from 8-week-old end organ and no-end organ groups were examined at 1370× by using a Nikon (Tokyo, Japan) Optiphot microscope interfaced with Bioquant software (R&M Biometrics, Nashville, TN). Myelinated axons were counted by standard stereologic methods (Mayhew, 1988). Each nerve was characterized by the total number of myelinated fibers regenerating and the mean diameter of these fibers. A Poisson regression analysis was used to compare the number of axons per motoneuron in the cutaneous and muscle branches at 8 weeks with and without end
organ. The 8 week time period was chosen for analysis because nearly all regenerating motor sprouts are myelinated by this time (T. M. Brushart, unpublished data) and can be quantified by light microscopy. Furthermore, limiting the study to myelinated axons includes all motor collaterals that have matured to the point of functional competence, regardless of their destination.

Control experiments. Control experiments were performed to validate the pure motor model. At issue are the damage caused to motor axons by DRG excision and the ability to correlate labeled motoneuron counts with the number of myelinated axons remaining in the muscle and cutaneous branches. The number of motoneurons projecting to the muscle and cutaneous branches of untransected femoral nerves was determined with HRP/FG double labeling in normal animals (n = 6), both 1 week (n = 5) and 8 weeks (n = 3) after generating pure motor nerve by excising the L2–L4 DRGs. These time periods were picked to assess the immediate effects of ganglion excision, as soon as Wallerian degeneration has cleared the denervated sensory Schwann cell tubes, and the later consequences, when sprouts from injured motor axons have had an opportunity to regenerate distally. Femoral cutaneous and muscle branches from the 1 week pure motor control group were embedded in plastic, sectioned at 1 μm, and evaluated to determine the number and size of remaining myelinated axons.

Results

Motoneuron counts

In the pure motor-end organ groups (EO), preferential motor reinnervation was already present at 2 weeks, with significantly more motoneurons projecting correctly to muscle than incorrectly to skin [M, 126 (SE 13); C, 57 (SE 8); p < 0.0001] (Fig. 1). The number of correct projections increased dramatically during each subsequent time interval [EO-M, 2 weeks, 126, EO-M, 3 weeks, 194 (SE 13), p < 0.001; EO-M, 3 weeks, 194, EO-M, 8 weeks, 279 (SE 16), p < 0.0001]. During this process, the number of motoneurons projecting incorrectly to skin increased between 2 and 3 weeks [EO-C, 2 weeks, 57 (SE 8); EO-C, 3 weeks, 91 (SE 9); p = 0.003] but then leveled off at a value not significantly different from the means at 2 or 3 weeks [EO-C, 8 weeks, 76 (SE 7); p = 0.100 (vs 2 weeks) and p = 0.165 (vs 3 weeks)]. By 8 weeks after nerve repair, more than three and one-half times as many motoneurons were double-labeled if end-organ access was denied at both 2 weeks [EO-D, 2 weeks, 31; NEO-D, 2 weeks, 50 (SE 10); p = 0.042] and 3 weeks [EO-D, 3 weeks, 33; NEO-D, 3 weeks, 74 (SE 12); p = 0.001], suggesting that collateral pruning is stimulated by contact with muscle. The final value at 8 weeks, however, was not end-organ dependent [EO-D, 8 weeks, 25; NEO-D, 8 weeks, 33; p = 0.177].

Axon counts

Myelinated motor axon counts were obtained from the muscle and cutaneous branches of EO and NEO animals 8 weeks after ganglion excision and nerve repair. When end-organ contact was permitted, a mean of 818 (SE 56) myelinated axons reinnervated the muscle branch, and a mean of 513 (SE 35) reinnervated the cutaneous branch (p < 0.0001). If end-organ contact was denied, a mean of 560 (SE 58) axons reinnervated the muscle branch, and a mean of 501 (SE 95) reinnervated the cutaneous branch (p = 0.6004). Comparing EO and NEO groups, end-organ contact significantly influenced axon counts in the muscle.

Figure 1. Motoneuron counts at 2 weeks (2 wks), 3 weeks (3 wks), and 8 weeks (8 wks) with and without end-organ contact. Motoneurons are scored as projecting correctly to the muscle branch (filled bar), incorrectly to the cutaneous branch (open bar), or simultaneously to both branches (striped bar). PMR develops by 2 weeks when competition from sensory axons is eliminated by ganglion excision. End-organ contact results in over three times as many motoneurons projecting to muscle as to skin by 8 weeks. No additional PMR develops if muscle contact is denied. PMR thus occurs in two stages: an early, limited response to muscle nerve, followed by a robust response to muscle itself. Double-labeled motoneurons, those with collaterals in both cutaneous and muscle nerves, are more numerous at both 2 and 3 weeks when contact with muscle is prevented, suggesting a role for muscle in collateral pruning.
branch ($p = 0.0071$) but not in the cutaneous branch ($p = 0.8829$).

The outcome of these experiments can be better appreciated by determining the number of myelinated axon collaterals per motoneuron in the cutaneous and muscle branches of EO and NEO animals. For these calculations, the total motoneuron count for each branch is taken to be the number of motoneurons projecting only to that branch plus the number of double-labeled motoneurons. Although double-labeled motoneurons may have fewer collateral projections than these calculations assume, their relative numbers are small (7% of labeled motoneurons), so that any resulting inaccuracy will have little effect on the results. The total number of myelinated axons in each branch is then divided by the total motoneuron count labeled from that branch to determine the number of collaterals per motoneuron.

When end-organ contact was permitted, motoneurons maintained a mean of 5.66 (SE 0.41) collaterals in the cutaneous branch and 2.84 (SE 0.16) collaterals in the muscle branch ($p < 0.0001$) (Fig. 2). If end-organ contact was denied, a mean of 6.15 (SE 0.66) collaterals was maintained in the cutaneous branch, whereas a mean of 3.06 (SE 0.36) persisted in the muscle branch ($p < 0.0001$). Comparing EO and NEO groups, end-organ contact significantly influenced the number of myelinated collaterals maintained by each motoneuron in the muscle branch ($p = 0.017$) but had no significant effect on the number of collaterals persisting in the cutaneous branch ($p = 0.156$). In aggregate, these results show that motoneurons maintain fewer collaterals in the muscle branch if end-organ contact is permitted but respond to the cutaneous branch regardless of end-organ availability. Additionally, motoneurons are clearly able to respond to pathway identity alone, because the muscle branch contained only half as many collaterals as the cutaneous branch even if these collaterals were unable to interact with muscle.

The mean diameter of myelinated motor axons did not differ significantly between cutaneous and muscle nerve in NEO animals but increased significantly in the muscle nerve when it led to muscle (Fig. 2).

### Control experiments
In normal animals ($n = 6$), a mean of 347 (SE 14) motoneurons projected to the muscle branch, and none projected to the cutaneous branch. One week after DRG excision without nerve repair ($n = 10$), a mean of 369 (SE 19) motoneurons projected to the muscle branch, and none projected to the cutaneous branch. DRG excision thus had no significant effect on baseline motoneuron projections ($p = 0.60$). Eight weeks after DRG excision without nerve repair ($n = 5$), however, a mean of 402 (SE 24) motoneurons projected to the muscle branch, a mean of 74 (SE 7) projected to the cutaneous branch, and a mean of 27 (SE 9) were double-labeled, significant increases in all categories ($p < 0.01$). Many of these additional motoneurons lay outside the anatomical confines of the normal femoral motoneuron pool (Fig. 3), suggesting innervation of the femoral nerve by nonfemoral axons as a result of DRG excision. Evaluation of peripheral nerve from pure motor animals 1 week after surgery ($n = 5$) revealed a mean of 356 (SE 21) myelinated axons in the muscle branch and none within the cutaneous branch (Fig. 4). This number compares favorably with the mean of 369 motoneurons labeled from these same branches (see above), indicating a nearly one-to-one correspondence between motoneuron labeling and myelinated axon counts within the same nerve. Ventral root remaining at the site of DRG excision contained no labeled sensory neurons, consistent with effective deafferentation.

### Discussion

#### The modified femoral nerve model

DRG excision to remove afferent axons followed by HRP labeling of motoneurons was introduced by Peyronnard et al. (1986) to evaluate labeling efficiency. When DRG excision is added to the femoral nerve model, only motor axons will regenerate after nerve repair. We are thus able to correlate, for the first time, the number of motor axon collaterals in a peripheral pathway with the number of motoneurons that generated these collaterals. Although deafferentation is known to compromise motoneuron development (Kalb and Hockfield, 1992) and function (Mendell...
et al., 1999), it may also have a positive impact on regeneration through upregulation of BDNF in the neuron (Johnson et al., 2000).

Control experiments were necessary to confirm successful removal of afferent axons and to evaluate possible damage to remaining motor axons. One week after deafferentation, myelinated axons were no longer present in the femoral cutaneous branch, whereas areas of degeneration resulted from loss of smaller myelinated afferents in the muscle branch (Fig. 4). The mean number of motoneurons labeled from the deafferented muscle branch at this time (369) was similar to the number labeled in normal animals (347), confirming that DRG excision can be performed without significant loss of motor axons. Ventral root remaining at the site of DRG excision contained no labeled sensory neurons, consistent with effective deafferentation.

The mean number of motoneurons projecting to the muscle branch after deafferentation increased from 369 at 1 week to 402 at 8 weeks. Ventral roots may be stimulated to sprout by the injury of DRG excision, a process no doubt influenced by adjacent degenerating pathways. These suspicions are confirmed by the location of labeled motoneurons beyond the confines of the normal femoral pool, indicating that they originally projected to other peripheral nerves (Fig. 3). Although this sprouting would defeat attempts to quantify normal anatomy, it does not detract from the current use of the femoral model, as only motor axons are permitted to regenerate. The addition of these neurons explains the increased total regeneration compared with previous experiments in which DRGs were not excised (Brushart, 1993). An increase, rather than a decrease, of the number of labeled motoneurons also indicates that deafferentation has not interfered with motoneuron labeling, a possibility suggested by Peyronnard and Charron (1983).

Preferential motor reinnervation

These experiments demonstrate that PMR develops in two distinct phases (Fig. 1). Two weeks after nerve repair in EO animals, twice as many motoneurons project to the muscle branch as to the cutaneous, and few are double labeled. A nearly identical pattern is seen in NEO animals, confirming that the initial phase of PMR results from axon/pathway interaction without reference to muscle. The course of subsequent regeneration is determined by end-organ availability. If muscle contact is permitted, the number of correct projections increases dramatically. If muscle contact is denied, however, there is no significant change in correct projections between 2 and 3 weeks, or between 3 and 8 weeks. Muscle contact is therefore a prerequisite for the second phase of PMR. In previous experiments in which cutaneous and motor axons regenerated together (Brushart, 1993), the more prolonged development of PMR masked this now clear distinction between pathway- and muscle-dependent phases.

The pattern of change in the pool of double-labeled motoneurons, those with simultaneous projections to distal cutaneous and muscle nerve at the time of labeling, was influenced by the pres-
Collateral generation and pruning

In the EO group, motoneurons that project correctly to muscle maintain significantly fewer myelinated collaterals than do those that project incorrectly to skin (2.84 \( \pm \) 5.66; \( p < 0.0001 \)), and these collaterals are of larger caliber (1.92 vs 1.27 \( \mu \)m; \( p < 0.0001 \)). This apparent pruning and maturation in response to muscle contact is consistent with the results of early regeneration experiments (Sanders and Young, 1946; Aitken et al., 1947). Axon counts distal to transection and repair of predominantly motor nerve have been found to be elevated by a factor of three to five, depending on the model and postoperative time (Shawe, 1955; Evans and Murray, 1956; Jenq and Coggeshall, 1984; Mackinnon et al., 1992). One would thus expect to find progressively fewer collaterals in the EO muscle branch after longer postoperative intervals.

The number of collaterals in a given environment must reflect the combined effects of collateral generation and pruning. In NEO animals, the muscle pathway may be conducive to collateral pruning and/or the cutaneous pathway conducive to collateral generation. Pruning in muscle pathways could result from the selective elaboration of factors that partially substitute for muscle-derived support such as the HNK-1 carbohydrate. Normally found in muscle nerve but not in cutaneous nerve, HNK-1 is re-expressed only when motor axons reinnervate previously motor Schwann cell tubes (Martini et al., 1992, 1994). Alternatively, factors limited to cutaneous nerve may selectively promote collateral formation. The expression of both NGF and BDNF is upregulated by a factor of 30 in denervated cutaneous nerve but barely at all in denervated ventral root (Hoke et al., 2004). These factors not only promote sprouting from sensory (Diamond et al., 1992; Gallo and Letourneau, 1998) and retinal ganglion (Cohen-Corey and Fraser, 1995; Lom and Cohen-Corey, 1999) neurons, but their antibodies reduce sprouting from motoneurons (Streppel et al., 2002). The substantial increase in collateralization within cutaneous nerve may thus result from increased sprouting rather than decreased pruning, an adaptation that could promote eventual pathfinding success.

References


