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POLYSIALIC ACID EXPRESSION IS NOT NECESSARY FOR MOTOR NEURON TARGET SELECTIVITY

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Abstract

Introduction—Recovery after peripheral nerve lesions depends on guiding axons back to their targets. Polysialic acid upregulation by regrowing axons has been proposed recently as necessary for this target selectivity.

Methods—We reexamined this proposition using a cross-reinnervation model whereby axons from obturator motor neurons that do not upregulate polysialic acid regenerated into the distal femoral nerve. Our aim was to assess their target selectivity between pathways to muscle and skin.

Results—After simple cross-repair, obturator motor neurons showed no pathway preference, but the same repair with a shortened skin pathway resulted in selective targeting of these motor neurons to muscle by a polysialic acid-independent mechanism.

Conclusion—The intrinsic molecular differences between motor neuron pools can be overcome by manipulation of their access to different peripheral nerve pathways such that obturator motor neurons preferentially project to a terminal nerve branch to muscle despite not upregulating the expression of polysialic acid.

Keywords

femoral nerve; obturator nerve; pathway choice; PSA-NCAM; regeneration

When a peripheral nerve is repaired after injury, regenerating axons may or may not reestablish functional connections with their original end-organ targets. Although the capacity for regrowth is robust, functional recovery is often poor.¹ The key to obtaining functional recovery is the accurate regeneration of axons to their original end-organ targets. Any therapeutic intervention that enhances the accuracy of nerve regeneration and functional recovery after such nerve lesions would be a welcome change from the current, severely limited, recovery; permanent functional compromise occurs in up to 90% of adult nerve repairs.^{2–4} Because the finest sutures and needles available to peripheral nerve surgeon are still considerably larger than the size of axons to be repaired, the limits of microsurgical techniques have been reached. In order to develop intervention strategies that will increase regeneration accuracy, there will need to be a greater understanding of the basic cellular and molecular events that influence axonal growth choices within the complex *in vivo* environment of peripheral nerves.

The proximal femoral nerve contains a mixed population of motor and sensory axons, but as these axons course more distally they are progressively segregated into 2 anatomically distinct pathways of approximately equal size that project either to the quadriceps femoris muscle or to the skin via the saphenous nerve.^{2,5} The rodent femoral nerve has been a useful model for *in vivo* studies of nerve regeneration. It was originally introduced in 1945 by Weiss and Edds⁶ as a model system to study the fate of axons that originally innervated muscle or skin when they were forcibly misdirected into the inappropriate nerve branch. The original femoral nerve model was modified by the Brushart laboratory to examine the fate of regenerating motor neurons when they were given equal access to the terminal cutaneous and muscle branches rather than being forced into foreign nerve stumps.^{2,3} Subsequent work at several different laboratories demonstrated that, after a proximal lesion of the parent femoral nerve, regenerating motor neurons preferentially reinnervate the terminal nerve branch to muscle versus the terminal cutaneous branch.⁷⁻¹¹

In addition to the possibility of inherent pathway differences that might be responsible for motor neuron regeneration accuracy, it has been suggested recently that intrinsic differences that exist between motor neuron pools themselves might control selective targeting of regenerating motor neurons at a lesion site of a mixed peripheral nerve. Franz et al.^{5,12} suggested that polysialic acid (PSA) is a necessary requirement for motor neuron regeneration accuracy. Those motor neuron pools that are able to express PSA can accurately reinnervate a muscle pathway, and those motor neuron pools that do not express PSA do not.

We show here, however, that a simple surgical manipulation of a terminal nerve pathway dramatically influences the ability of motor neurons to correctly reinnervate a terminal nerve to muscle, regardless of neuronal PSA expression. This is further evidence that motor neuron regeneration accuracy is not determined solely by specific molecular signatures of either the motor neuron itself or the terminal nerve pathway. Rather, we suggest it is the relative levels of trophic support available in each terminal pathway that is the limiting factor that determines whether motor neurons will display regeneration accuracy, a general process that has been termed trophomorphism.^{13,14}

METHODS

All rodent procedures were approved by the animal use committee of the Veterans Affairs Medical Center. Rodents were housed on a standard 12-h light/dark cycle and fed *ad libitum*. Surgical procedures were carried out under deep anesthesia by intraperitoneal administration of ketamine, xylazine, and acepromazine (as a combined solution containing 100, 6, and 1 mg/kg, respectively) in normal (0.9%) saline. The fibrin sealant used for nerve repair was prepared according to the manufacturer's instructions (Tisseel; Baxter Healthcare Products, Glendale, California).

Rat Surgical Manipulations

For the "obturator-to-femoral-nerve" preparation, the inguinal area skin was clipped and cleaned with 70% ethyl alcohol and 5% povidone-iodine (Fig. 1). The parent femoral nerve was exposed by blunt dissection and transected with microscissors above the level of the iliacus branch, a region where axons to the 2 terminal nerve branches are randomly distributed.² The proximal portion of the femoral nerve was then ligated with 7-0 silk suture (Ethicon, Somerville, New Jersey). The obturator nerve, just medial to the parent femoral nerve, was exposed by blunt dissection and also transected with microscissors. The distal portion of the obturator nerve was also ligated. The proximal portion of the obturator nerve was then connected to the distal portion of the parent femoral nerve. After a small amount of fibrin sealer was placed at the apposition site, activator was applied to complete the sealant

repair. The site was then closed in layers, the skin sutured with 4-0 Vicryl (Ethicon), and the animal was allowed to recover.

For the “obturator-to-femoral-nerve-with-shortened-cutaneous” preparation, the proximal obturator nerve was attached to the distal femoral nerve as noted earlier, but, in addition, the terminal cutaneous branch of the femoral nerve was transected at a point where its length was equal to that of the intact muscle branch to the quadriceps muscle. Both the proximal and distal ends of the cutaneous branch were then ligated with 7-0 silk suture. The site was then closed in layers, sutured, and the rat allowed to recover.

Immunocytochemistry

PSA expression was examined in normal nerves and 5 days after a ligation of the femoral and obturator nerves from both rats [$N = 3$, female Sprague-Dawley rats, 100–150 g (Charles River)] and mice [$N = 3$, female C57BL/6, 20–25 g (in-house colony)]. Nerve samples were harvested from deeply anesthetized animals, fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4) for 30 min, washed extensively in PBS, and sucrose protected. Cryostat sections (12 μm) were mounted onto slides and stored at -20°C . Nerves from both species were processed for PSA immunocytochemistry using the same procedures [mouse on mouse (MOM); BMK-2202; Vector Laboratories, Burlingame, California]. Briefly, the sections were rehydrated in PBS for 5 min, covered in MOM block for 1 h, incubated in PSA antibody [1:200; #MAB5324; Millipore, Temecula, California (in MOM diluent)], washed extensively in PBS, incubated for 30 min at room temperature in an Alexa Fluor 555–conjugated goat anti-mouse IgM secondary antibody (1:1000; A21426; Invitrogen) in MOM diluent, washed, air dried, and coverslipped with Prolong according to the manufacturer’s instructions (P-7481; Molecular Probes). Incubations without primary or secondary antibodies confirmed specificity, and uninjured nerves served as normal controls.

Determination of Terminal Nerve Pathway Preference

The tendency for obturator motor neurons to project into either the terminal muscle or cutaneous branch of the cross-reinnervated femoral nerve was determined 8 weeks after nerve repair in the 2 surgery groups [$N = 8$ adult female Sprague-Dawley rats in each group, 100–150 g (Charles River)]. The terminal branches of the femoral nerve were reexposed and separated from each other by food-grade silicone grease dams. They were then trimmed to ~3 mm distal to the normal femoral nerve bifurcation, and randomly assigned to receive crystals of either Alexa Fluor 488 dextran (D-22910; Molecular Probes, Eugene, Oregon) or Alexa Fluor 594 dextran (D-22913; Molecular Probes). After crystal application, each branch was blotted and sealed with silicone grease. The surgical site was closed, sutured, and the rat allowed to recover. Three days later, each rat received an overdose of anesthetic and was perfused through the heart with PBS, followed by 4% paraformaldehyde in PBS. The lumbar spinal cord was removed, postfixed for several hours, and sucrose protected overnight. The spinal cord was frozen on dry ice and sectioned with a cryostat. Serial 25- μm frozen sections were thawed in PBS, mounted onto glass slides, air dried, and coverslipped using Prolong. Retrogradely labeled obturator motor neurons containing a nucleus were identified using a composite filter set that allowed simultaneous visualization of both labels (#51006; Chroma Technology, Brattleboro, Vermont) in a fluorescence-equipped Zeiss Axiophot microscope, at 250 $\times$ magnification.

Motor neuron counts were carried out by blinded independent observers and scored as either single-labeled (488 or 594 dextran only) or double-labeled (both 488 and 594 dextrans). Motor neurons labeled only from the muscle branch, only from the cutaneous branch or from both branches were tabulated and their counts corrected for split cells.¹⁵ All data are expressed as mean $\pm$ SD unless otherwise noted. In 5 control rats, the obturator nerve was

exposed as described earlier, then transected. After creating a small well around the cut proximal nerve using silicone grease, crystals of either Alexa Fluor 488 dextran or Alexa Fluor 594 dextran were applied as described previously. The surgical site was closed, sutured, and the rat allowed to recover. Three days later the rats were perfused as indicated, and the spinal cords were processed for visualization and counting of the labeled obturator motor neurons. Because our data involve quantifying retrogradely labeled motor neurons we consistently refer to regeneration choices made by the motor neurons themselves rather than their axons, which we did not attempt to quantify.

A regeneration pathway preference index for each animal was determined by dividing the number of retrogradely labeled motor neurons reinnervating only 1 pathway by the total number of labeled motor neurons and expressing the quotient as a percentage. Thus, “percent muscle pathway preference” was determined by dividing the number of motor neurons labeled from only the muscle pathway by the total number of labeled motor neurons; “percent cutaneous pathway preference” was determined in an identical fashion using the number of motor neurons labeled from only the cutaneous pathway as the numerator. Combining these 2 percentages for each animal is always less than 100%, because the number of double-labeled neurons was included in the denominator for each pathway preference (i.e., as part of the total number of labeled cells), but not in the numerator.

Statistics

Student *t*-tests for paired data were used within groups to compare the number of motor neurons labeled from the muscle and cutaneous branches. Differences were considered statistically significant when $P < 0.05$. Analysis of variance (using Student–Newman–Keuls *post hoc* comparisons) was used to determine differences among groups. Differences were considered statistically significant when $F < 0.05$ and $P < 0.05$.

RESULTS

PSA Immunocytochemistry

It has previously been shown in mice that PSA expression is low in normal femoral and obturator nerves and that a nerve lesion leads to significant increases in the femoral but not in the obturator nerve.^{5,12} We confirmed these observations in the mouse and extended them to the rat with the same results. In agreement with the previous mouse work, we found that PSA expression in the mouse femoral nerve was normally low, but expression noticeably increased 5 days after a nerve lesion (Fig. 2). A similar pattern and intensity of staining was seen in the rat femoral nerve. Also, similar to previous work with mice,^{5,12} we confirmed barely detectable PSA staining for the normal mouse obturator nerve, with no noticeable increase in PSA expression after a nerve lesion (data not shown); the same results were found for the rat obturator nerve (data not shown).

Obturator Motor Neuron Projections

Eight weeks after the surgical cross-reinnervation manipulations in the rat, shown in Figure 1, terminal nerve pathway preferences were determined by retrogradely labeling the regenerated obturator neurons that projected into either the terminal nerve branch to muscle or the terminal nerve branch to skin. In the obturator to femoral nerve group (Fig. 3, left panel), retrograde labeling from the 2 terminal pathways showed no pathway preference (147 ± 34 for muscle vs. 106 ± 57 for cutaneous). However, in the group with obturator to femoral nerve with shortened cutaneous (Fig. 3, right panel) the obturator motor neurons showed a significant preference for the terminal pathway to muscle as compared with skin (228 ± 60 vs. 77 ± 31 , $P < 0.01$). Comparisons between the 2 groups also showed

significantly more obturator motor neurons projecting to the terminal pathway to muscle in the group with obturator to femoral nerve with shortened cutaneous compared with the group with obturator to femoral nerve (228 ± 60 vs. 147 ± 34). In both groups, the total number of regenerated motor neurons did not differ from one another or from the normal rat obturator nerve (297 ± 39 , $N = 5$).

Terminal Nerve Pathway Preferences

We further examined regenerated motor neurons by analyzing the normalized pathway preference index, as described in Methods (Fig. 4). For comparison purposes, this figure includes data from standard femoral nerve preparations that have been described previously,¹¹ in which only the femoral nerve is transected and repaired with ($N = 8$) or without ($N = 14$) a shortened terminal cutaneous nerve branch. In looking at within-group differences, once again there was no difference between the muscle or cutaneous pathway preference for the group with obturator to femoral nerve, whereas all 3 of the other surgical groups showed a significant preference for the muscle pathway ($P < 0.01$, paired t -test; Fig. 4). Comparisons among surgical groups indicated that the muscle pathway preference for the group with obturator to femoral nerve (Fig. 4, upper far left) was significantly less than the muscle pathway preference for all 3 of the other surgical groups, whereas the muscle pathway preference for the group with femoral to femoral nerve with shortened cutaneous (Fig. 4, upper far right) was significantly greater compared with all 3 of the other surgical groups [analysis of variance (ANOVA), $F = 23.77$, $P < 0.0001$, followed by Student–Neuman–Keuls *post hoc* comparisons, $P < 0.05$]. The opposite pattern was found when comparing the cutaneous pathway preference among all 4 groups. The cutaneous pathway preference for the group with obturator to femoral nerve (Fig. 4, bottom far left) was significantly greater than the cutaneous pathway preference for all 3 of the other surgical groups (ANOVA, $F = 7.69$, $P < 0.0005$, followed by Student–Neuman–Keuls *post hoc* comparisons, $P < 0.05$). Similarly, the proportion of obturator motor neurons that maintained projections into both of the terminal nerve pathways was significantly greater in the group with obturator to femoral nerve (data not shown in Fig. 4) compared with all 3 of the other surgical groups (18.9 ± 13 vs. 7.7 ± 5 , 9.7 ± 5 , and 3.1 ± 3 , respectively; ANOVA, $F = 6.57$, $P < 0.001$, followed by Student–Neuman–Keuls *post hoc* comparisons, $P < 0.05$).

DISCUSSION

Extensive peripheral nerve injuries can result in effective paralysis of the entire limb or distal portions of the limb. Complete functional recovery after such nerve injuries requires accurate regeneration of axons to their original target end-organs. The experiments reported here address fundamental questions of regeneration accuracy of motor neurons at the level of a mixed peripheral nerve. Previous studies from several laboratories have demonstrated that regenerating femoral motor neurons preferentially reinnervate the appropriate terminal muscle branch rather than the inappropriate branch to skin. However, the underlying mechanisms that give rise to this ability of motor neurons to differentiate between terminal nerve branches are not completely understood. Some studies have suggested that there are inherent molecular differences between the nerve branches that are responsible, such as L2/HNK-1,^{16–18} enhanced BDNF/TrkB signaling by the muscle pathway,¹⁰ or specific motor and sensory Schwann cell identities that are recognized by regenerating motor axons.^{3,19} Other studies have suggested that there are inherent molecular properties of regenerating motor neurons that determine whether or not they are able to successfully distinguish between terminal nerve pathways.^{5,12} A third alternative, supported by our results, is that regenerating motor neurons assess the relative levels of trophic support in each pathway and preferentially retain projections to the one that provides the greater amount of such support; this is a general process that has previously been termed trophomorphism.^{13,14} Thus,

trophomorphism posits a more dynamic decision making process by regenerating neurons and their axons as compared with possible inherent properties of either the pathway or the motor neuron itself.

The differences among these 3 alternatives are not just of theoretical interest but would also direct possible clinical interventions. If motor neuron regeneration accuracy is dependent only upon inherent properties of either the motor neuron pools themselves or the terminal nerve pathways, then it follows that such accuracy would be limited to those motor neuron pools and pathways that express those specific properties. Possible therapeutic interventions would necessarily center around manipulations to influence the expression of specific molecules. Conversely, if motor neuron regeneration accuracy is dependent upon trophomorphism, then therapeutic intervention would include deciding which terminal pathway is the most critical for functional recovery and attempting to endow that pathway with the highest relative level of trophic support. We can consider each of these possibilities based on the available data.

Inherent Properties of the Terminal Nerve Pathway or Schwann Cells

As just detailed, there are several well-characterized molecular differences between the 2 terminal femoral nerve pathways. Although these may modify preferential pathway projections, it seems unlikely that they are essential causative factors. For instance, we have previously shown through surgical and transgenic manipulations that regenerating femoral nerve motor neurons can be preferentially directed to either the muscle or cutaneous terminal nerve branches,^{20,21} and regeneration comparisons using motor or cutaneous nerves as nerve grafts have identified parity between them.²² Finally, we have also used molecular targeting to remove the original population of Schwann cells in the two terminal nerve pathways and demonstrated that regenerating motor neurons still display a strong preference to reinnervate the terminal pathway connected to muscle.²³ All of these data suggest that specific molecular identities of Schwann cells or inherent properties of the terminal nerve pathways are not rigid determinants of motor neuron regeneration accuracy.

Inherent Properties of the Motor Neuron

It is a well-established general concept that motor neuron pools during development have distinct molecular identities that help shape nerve–muscle connectivity. Especially pertinent to this study is the demonstration that the motor neuron pools that project into the obturator and femoral nerves possess distinct early transcriptional identities that control axonal projections to their respective target muscles.²⁴ Within the femoral nerve regeneration model there is also a body of work suggesting that electrical stimulation modifies the molecular expression of femoral nerve motor neurons and results in acceleration of preferential projections to the terminal muscle branch (for review, see Ref. 25).

In keeping with such concepts, a recent series of evocative studies suggested that PSA expression is a *necessary* requirement for *regenerating* motor neurons to distinguish between the 2 femoral nerve terminal pathways and to be able to preferentially reinnervate the appropriate pathway to muscle versus skin.^{5,12} PSA is a large glycan with restricted expression and is well known to influence neuronal development, especially in view of its association with the neural cell adhesion molecule (NCAM). Two polysialyltransferases are involved in the synthesis of PSA, and these polysialyltransferases are only associated with 4 protein carriers: NCAM (the most common carrier); the alpha subunit of the voltage-gated sodium channel; CD36; and neuropilin.^{26–29} It has long been known that the level of PSA expression differs between motor neuron pools in the chick³⁰ and that it is required for accurate motor axon growth during development^{31,32} via its ability to decrease axon–axon adhesion³⁰ and thus allow axons to respond to various pathway guidance cues.

Franz et al.^{5,12} observed that PSA expression was upregulated in the mouse femoral nerve but not the obturator nerve during axon regeneration. To test the hypothesis of the necessity of PSA expression for such selective regeneration, they cross-sutured the proximal obturator nerve to the distal femoral nerve and demonstrated that, unlike femoral motor neurons that upregulate PSA expression, there was no preference for the obturator motor neurons (PSA negative) to project to the terminal muscle nerve branch. Likewise, if PSA in the femoral nerve of wild-type mice was removed via an injection of endoneuraminidase-N, or if NCAM was removed via transgenic technology (NCAM^{-/-}), femoral motor neurons failed to display accurate regeneration to the distal terminal muscle branch.

In this study we have revisited the possible role of PSA on selective motor neuron targeting. We first confirmed that PSA expression is upregulated in the mouse femoral nerve during axon regeneration, but not in the obturator nerve, and showed that the same expression pattern was found in the rat (Fig. 2). We also used the same cross-reinnervation model in the rat and confirmed that obturator motor neurons normally show no pathway preference, as they innervate the distal terminal pathways of the femoral nerve, the exact same result observed by Franz et al.¹² in the mouse. However, when we shortened the terminal cutaneous branch of the femoral nerve to be the same length as the intact muscle branch there was now a strong preference for regenerating obturator motor neurons to project to the muscle branch (Fig. 3). We also examined such terminal nerve pathway preferences for femoral motor neurons using the same surgical model of a shortened cutaneous pathway and found a very similar result (Fig. 4). Because we wanted to quantify the final motor neuron projection choices, we chose an 8-week survival time, because previous work has shown that at earlier time-points the motor neuron projections are still undergoing pruning and have not yet obtained their stable projection patterns.^{11,21,33} There is a strikingly similar increase in the preference for both motor neuron pools to reinnervate the terminal muscle branch due to this surgical manipulation. There was a 21% increase for obturator motor neurons (negative PSA expression) and an 18% increase for femoral motor neurons (positive PSA expression). It is unlikely that PSA is involved in this selective targeting of the muscle branch, because neither we nor Franz et al.¹² found increased expression of PSA in the obturator nerve.

Thus, we have confirmed many of the results of Franz et al.,^{5,12} but by adding a simple surgical manipulation to the distal femoral nerve we have come to a very different conclusion regarding the necessity of PSA expression as a mechanism for obturator motor neurons to accurately project to a terminal muscle branch pathway. There are several intriguing possibilities for these differences. First, we suspect that manipulations of PSA expression may have indirectly influenced several important related aspects of axonal regeneration. For instance, it has been known for some time that cellular responses to brain-derived neurotrophic factor (BDNF)^{34,35} or chemotaxis induced by platelet-derived growth factor³⁶ are modulated by PSA expression. It has also recently been suggested that PSA may modulate the availability and local concentrations of numerous cytokines and chemokines in a fashion very similar to that seen in glycosaminoglycans, and thus PSA may modulate various aspects of the immune response to injury.²⁹ The possible modulation of the humoral immune response is especially pertinent, because it has recently been shown in an elegant study that endogenous antibodies in peripheral nerve are instrumental in promoting both rapid myelin clearance and axonal regeneration.³⁷ All of these related consequences of PSA expression must be kept in mind when interpreting the observations by Franz et al.^{5,12} who found that disruption of PSA expression due to endoneuraminidase-N injections or NCAM removal via transgenic technology significantly impairs the ability of regenerating motor neurons to accurately reinnervate a terminal nerve branch to muscle.

Trophomorphism

We suggest a third alternative to explain the behavior of motor neurons as they “choose” to reinnervate terminal nerve pathways: “trophomorphism.” Trophomorphism is a general theory initially introduced by Crutcher and Saffran.^{13,14} It was suggested that regenerating neurons assess the relative levels of trophic support in each terminal pathway or target and, due to an intraneuronal competition among sibling axonal branches,³⁸ the neurons preferentially project to the pathway with the relatively greater amount of trophic support. Trophic support in this context is interpreted very broadly to include anything that impacts neuronal survival, such as growth factors, structural/metabolic precursors, and blood supply. We consider muscle to be the most potent primary source of such support, followed by the influence of Schwann cells.^{21,23} The interaction between these 2 sources may also be crucial.

Within the context of this study we suggest that all motor neurons are subject to trophomorphism and sibling neurite bias and that this competition occurs despite any differences in PSA expression. Although inherent molecular differences between motor neuron pools may play a modulating role in terms of the ability to distinguish terminal nerve branches, we suggest that trophomorphism is a more powerful underlying mechanism that shapes motor neuron regeneration. Such graded influences may occur during motor neuron regeneration into a distal femoral nerve stump when the terminal branches remain intact to muscle or skin. Under those circumstances, regenerating femoral nerve motor neurons, but not obturator nerve motor neurons, preferentially project to the terminal nerve branch to muscle, as shown in both studies by Franz et al.^{5,12} and in our experiments. Thus, the original femoral motor neurons may somehow be more responsive to cues from the terminal muscle branch compared with the “foreign” obturator motor neurons.

A corollary of the influence of trophomorphism using the femoral nerve model is that manipulations that increase the disparity between competing pathways will result in more robust selectivity. This would explain the preference of obturator motor neurons to project to the terminal muscle branch (under the potent influence of muscle and Schwann cells) when the competing cutaneous nerve branch is shortened, because the shortened cutaneous nerve would contain significantly fewer Schwann cells and no skin influence compared with an intact cutaneous nerve. Figure 4 demonstrates this idea that increasing the trophic disparity between pathways for both groups of motor neurons increases their target selectivities, regardless of whether or not their baseline preferences are already significant.

In conclusion, we suggest that manipulating the relative availability of sources of trophic support (primarily muscle and the number of Schwann cells) can account for selective motor neuron targeting. This mechanism is distinctly different from the suggestion that selectivity is based on inherent molecular differences between Schwann cells residing in the muscle and cutaneous branches themselves,^{3,19,39} or on inherent molecular differences between motor neuron pools themselves.^{5,12} We further suggest that if future therapeutic interventions to increase the accuracy of motor neuron regeneration are to be successful they will have to take into account the idea of trophomorphism and the intraneuronal competition for limited amounts of trophic support.

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Abbreviations

ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factor
MOM	mouse on mouse
NCAM	neural cell adhesion molecule
PSA	polysialic acid
PBS	phosphate-buffered saline

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Rat Surgical Models

FIGURE 1.

Surgical overview of the experiments in rats. In the *obturator to femoral nerve* preparation (top model), obturator motor neuron axons were given a choice to regrow into the muscle or cutaneous pathway of the femoral nerve for 2 months before retrograde labeling was used to determine pathway preference. Examples of such single (green or red) and double-labeled (green and red appear yellow) motor neurons are shown (far right panel, scale bar = 50 μm). In the *obturator-to-femoral-nerve-with-shortenedcutaneous* preparation (bottom panel), the same pathway choice was given to obturator motor neuron axons, but the possible trophic influences of the longer cutaneous pathway connected to skin were removed. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 2.

Mouse and rat polysialic acid (PSA) immunocytochemistry in injured and uninjured femoral nerve. Five days after ligation (see diagram) the proximal portion of the muscle branch to the quadriceps femoris muscle was processed for PSA immunocytochemistry and compared with uninjured (normal) nerve. In the mouse (top panel), normal nerve showed little staining. Five days after axotomy, PSA staining was intense. In the rat (bottom panel), the same axotomy-induced increase was seen in staining compared with normal. Scale bar = 100 μm for mouse and rat. P = proximal and D = distal to ligation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 3.

Counts of retrogradely labeled obturator motor neurons that projected into the 2 terminal femoral nerve branches 8 weeks after cross-reinnervation. The *obturator-to-femoral-nerve* group motor neurons showed no difference in the number of motor neurons labeled from the muscle or cutaneous branches of the femoral nerve (left panel). In contrast, significantly more neurons projected to the muscle branch compared with the cutaneous branch (asterisk) when the influence of the cutaneous pathway was reduced in the obturator-to-femoral-nerve-with-shortened-cutaneous group (right panel). Dotted lines represent the mean $\pm$ SD for total control motor neurons. Total counts of regenerating neurons were similar in both groups. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 4.

Pathway preference indices for obturator motor neurons with regenerating axons in the femoral nerve 8 weeks after nerve repair. The *obturator-to-femoral-nerve* group showed no pathway preference (first pair on left). In contrast, an increased preference for the muscle branch, and a decreased preference for the cutaneous pathway was observed after the influence of the cutaneous pathway was reduced in the obturator-to-femoral-nerve-with-shortened-cutaneous group (second pair on left). This significant increase in target specificity (21%) is similar to that seen for femoral motor neurons when the influence of the cutaneous branch is similarly reduced (an 18% increase, compare third and fourth pairs). The boxed “1” (red) represents the significantly lowest muscle pathway preference. The boxed “2” (red) represents the significantly highest muscle pathway preference. The boxed “3” (green) represents the significantly highest cutaneous pathway preference. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]