Incorporation of Axonally Transported Glycoproteins into Axolemma during Nerve Regeneration

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ABSTRACT The insertion of axonally transported fucosyl glycoproteins into the axolemma of regenerating nerve sprouts was examined in rat sciatic motor axons at intervals after nerve crush. [3H]Fucose was injected into the lumbar ventral horns and the nerves were removed at intervals between 1 and 14 d after labeling. To follow the fate of the "pulse-labeled" glycoproteins, we examined the nerves by correlative radiometric and EM radioautographic approaches.

The results showed, first, that rapidly transported [3H]fucosyl glycoproteins were inserted into the axolemma of regenerating sprouts as well as parent axons. At 1 d after delivery, in addition to the substantial mobile fraction of radioactivity still undergoing bidirectional transport within the axon, a fraction of label was already associated with the axolemma. Insertion of labeled glycoproteins into the sprout axolemma appeared to occur along the length of the regenerating sprouts, not just in sprout terminals. Once inserted, labeled glycoproteins did not undergo extensive redistribution, nor did they appear in sprout regions that formed (as a result of continued outgrowth) after their insertion. The amount of radioactivity in the regenerating nerves decreased with time, in part as a result of removal of transported label by retrograde transport. By 7-14 d after labeling, radioautography showed that almost all the remaining radioactivity was associated with axolemma. The regenerating sprouts retained increased amounts of labeled glycoproteins; 7 or 14 d after labeling, the regenerating sprouts had over twice as much of radioactivity as comparable lengths of control nerves or parent axons.

One role of fast axonal transport in nerve regeneration is the contribution to the regenerating sprout of glycoproteins inserted into the axolemma; these membrane elements are added both during longitudinal outgrowth and during lateral growth and maturation of the sprout.

Regeneration of a transected axon requires both the restoration of a substantial volume of axoplasm and the addition of new axolemma to the regenerating sprouts. Because the axon itself is unable to synthesize significant amounts of protein, transfer of new protein from the cell body to both axoplasm and axolemma must be mediated by the axonal transport systems. Slow axonal transport provides the bulk of the axoplasmic and cytoskeletal proteins (15, 18, 24, 25), whereas several lines of evidence suggest that fast transport contributes elements to the axolemma (4, 12, 13, 19). During regeneration, fast axonal transport continues at the normal rates (2, 13, 19, 30, 32), passing unimpeded through the site of axotomy into regenerating sprouts (2, 17, 19, 31). Rapidly transported materials accumulate in the distal regions of the regenerating sprouts soon after delivery (17, 19, 28, 31, 33, 38). The subsequent disposition of rapidly transported materials, however, is poorly understood.

The present study was designed to examine the fate of rapidly transported glycoproteins in regenerating rat sciatic nerves. We were interested in several related problems: first, whether rapidly transported glycoproteins were inserted into sprout axolemma; second, at what stages of sprout outgrowth and maturation insertion occurred; third, how the proportion of transported glycoproteins associated with axolemma (rather than intraaxonal constituents) changed with time after delivery; and fourth, how insertion into sprout axolemma compared in
To correct for intrinsic radioactivity in the segment distal to the ligature, the dpm in the second segment from the ligature was subtracted from that in the first. The resulting value is a better measure of the radioactivity arriving by retrograde transport.

**Materials and Methods**

**Nerve Crush**

All studies were performed on 250-g Sprague-Dawley female rats. In rats under chloral hydrate anesthesia, the right sciatic nerve was crushed for 10 s with watchmaker’s forceps just distal to the branching of the nerve to the biceps femoris or, in some groups, at the merging of lumbar roots 4 and 5. In all animals, the contralateral side was used as a control.

**Radio metric Studies**

These studies were done to analyze the distribution and relative amount of transported radioactivity along regenerating and control nerves at different times after labeling (i.e., after delivery of a “pulse” of transported glycoproteins to the axon). Groups of animals were subjected to unilateral crush at various times from 7 to 90 d before labeling. [3H]Fucose (sp act, 40 or 60 Ci/mM; New England Nuclear, Boston, Mass.) was injected into the lumbar ventral horns as previously described (19). In the present studies, three injections—each of 0.8 µl and separated by 0.75 mm—were made symmetrically in each side. Animals were returned to their cages after labeling. Groups of four animals each were killed 1, 3, 7, or 14 d after labeling. Table I summarizes the timing of the various experimental groups.

The nerves were dissected, solubilized, and counted as previously described (19). In preliminary studies, the amount of nontransported radioactivity present along the sciatic nerves was assessed by measuring disintegration per minute per 3-mm segment in regions distal to nerve ligations. The highest values, found 24 h after labeling, were 70 dpm in control nerves and 240 dpm in regenerating nerves. The values (for each interval after labeling) were subtracted from the measured values to obtain values for transported radioactivity. Results were plotted as disintegrations per minute per 3-mm nerve segment against position of each segment along the nerve.

An additional experiment was designed to determine the extent to which inserted glycoproteins might be carried distally as the sprouts continue to elongate.

We compared the distribution of glycoproteins in the regenerating nerves 7 d after labeling with the position of the growing tips of the regenerating nerves at the time of nerve removal. Two animals were subjected to a crush at the merging of L-4 and L-5 lumbar roots in the pelvis. [3H]Fucose was injected into the lumbar ventral horns 7 d later, and on day 13 after crush, [35S]methionine (sp act, 40 Ci/mM) was injected into the lumbar ventral horns. On day 14 after crush, the nerves were removed and counted by double-label techniques.

**Ligation Studies**

These experiments, done in parallel with the radiometric studies, were designed to estimate the proportion of radiolabel that continued to be transported at various times after labeling. Animals were labeled 7 d after crush; on the day on which the animals had been killed in the studies described above, the animals in these studies were anesthetized and the regenerating nerves were ligated at a level 15 mm proximal to the crush (see Table I). These animals were killed 24 h after ligation, and the nerves were removed, divided into segments, and counted.

We related the distribution of radioactivity in these ligated nerves to that of the corresponding unligated nerves (Table II). The amount of radioactivity accumulating distal to the ligature was used to calculate a ”mobile fraction” and the radioactivity remaining in the regenerating sprouts of the ligated nerves was used to calculate a ”stationary fraction.”

**Radioautographic Studies**

Regenerating nerves from animals 1 and 7 d after labeling were examined by radioautography (8 d and 14 d after crush). The animals were perfused with 50 ml of 4% paraformaldehyde followed by 1 liter of 5% glutaraldehyde in sodium phosphate buffer, pH 7.3, and samples were taken from the regenerating nerve proximal to the crush, as well as from the first eight 3-mm segments distal to the
Each curve is the mean of four nerves (± SD). (a) Control nerves (contralateral to the regenerating nerves shown in b). The slightly lower levels of radioactivity in the distal segments of the nerve are the result of nerve branching (particularly the nerve to the biceps femoris). △, 1 d postinjection; ○, 3 d postinjection; ●, 14 d postinjection. (b) Regenerating nerves, labeled 7 d after crush and removed at times shown. Note the general conformation of the curves is maintained with time although the relative prominence of the peak in region D decreases. Note also that relatively little transported radioactivity is found distal to region D at late times after labeling, even though the regenerating sprouts are growing out >4 mm/d (cf. Fig. 7). △, 8 d postcrush, 1 d postinjection; ○, 10 d postcrush, 3 d postinjection; ●, 21 d postcrush, 14 d postinjection. (c) Mean differences in disintegrations per minute between regenerating and contralateral control nerve at intervals after labeling. Note that 1 and 3 d after labeling, more radioactivity was present in region A on the regenerating side. However, by 14 d after labeling, the amount of radioactivity was essentially identical on the two sides. Note also that the amount of radioactivity in the regenerating sprouts (regions B, C, and D) was greater than comparable segments of the control nerves at all times after labeling (see also Table III). △, 8 d postcrush, 1 d postinjection; ○, 10 d postcrush, 3 d postinjection; ●, 21 d postcrush, 14 d postinjection. (d) Ligature studies. Plots of the distribution of radioactivity in regenerating nerves labeled 7 d after crush and subjected to nerve ligation at intervals after labeling. Note that the amount of radioactivity accumulating distal to the ligature decreases dramatically with time. Note also that, 1 d after labeling in the group ligated, the amount of radioactivity in region D is much less than in unligated nerves (b). In contrast, nerves ligated 14 d after labeling have a mean curve similar to that of the 14-d group in b. As described in the text and Table II, these changes reflect the decrease in the mobile fraction and relative increase in the stationary fraction with time after labeling. △, 9 d postcrush, 2 d postinjection, 1 d postligation; ○, 11 d postcrush, 4 d postinjection, 1 d postligation; ●, 15 d postcrush, 8 d postinjection, 1 d postligation; ●, 22 d postcrush, 15 d postinjection, 1 d postligation.

FIGURE 1 Plots of the distribution of transported radioactivity along rat sciatic nerves at intervals after injection of [3H]fucose into the lumbar ventral horns, showing retention of [3H]fucosyl glycoproteins in (a) control nerves and (b-d) regenerating sprouts. Each curve is the mean of four nerves (± SD). (a) Control nerves (contralateral to the regenerating nerves shown in b). The slightly lower levels of radioactivity in the distal segments of the nerve are the result of nerve branching (particularly the nerve to the biceps femoris). △, 1 d postinjection; ○, 3 d postinjection; ●, 14 d postinjection. (b) Regenerating nerves, labeled 7 d after crush and removed at times shown. Regions A-D are as defined in the text. Note that the general conformation of the curves is maintained with time although the relative prominence of the peak in region D decreases. Note also that relatively little transported radioactivity is found distal to region D at late times after labeling, even though the regenerating sprouts are growing out >4 mm/d (cf. Fig. 7). △, 8 d postcrush, 1 d postinjection; ○, 10 d postcrush, 3 d postinjection; ●, 14 d postcrush, 7 d postinjection; ●, 21 d postcrush, 14 d postinjection. (c) Mean differences in disintegrations per minute between regenerating and contralateral control nerve at intervals after labeling. These curves represent the differences between the curves in b and those in a, with the mean values of the control nerves in each segment plotted as 0 on the y-axis. Note that 1 and 3 d after labeling, more radioactivity was present in region A on the regenerating side. However, by 14 d after labeling, the amount of radioactivity was essentially identical on the two sides. Note also that the amount of radioactivity in the regenerating sprouts (regions B, C, and D) was greater than comparable segments of the control nerves at all times after labeling (see also Table III). △, 8 d postcrush, 1 d postinjection; ○, 10 d postcrush, 3 d postinjection; ●, 21 d postcrush, 14 d postinjection. (d) Ligature studies. Plots of the distribution of radioactivity in regenerating nerves labeled 7 d after crush and subjected to nerve ligation at intervals after labeling. The animals in each group were sacrificed 1 d after ligation. Note that the amount of radioactivity accumulating distal to the ligature decreases dramatically with time. Note also that, 1 d after labeling in the group ligated, the amount of radioactivity in region D is much less than in unligated nerves (b). In contrast, nerves ligated 14 d after labeling have a mean curve similar to that of the 14-d group in b. As described in the text and Table II, these changes reflect the decrease in the mobile fraction and relative increase in the stationary fraction with time after labeling. △, 9 d postcrush, 2 d postinjection, 1 d postligation; ○, 11 d postcrush, 4 d postinjection, 1 d postligation; ●, 15 d postcrush, 8 d postinjection, 1 d postligation; ●, 22 d postcrush, 15 d postinjection, 1 d postligation.
loosely packed membranous organelles including branched growth cones (Fig. 3b and c). The growth cones, containing myelinating axons, were seen, ranging from clusters of fine sprouts within single axonal swellings, which were mostly smaller and had not begun to myelinate (Fig. 6b), to myelinating axons as well as in axons that had not yet begun to grow out effectively (8, 20). They explain the small peak of radioactivity in region B.

In region C, sprouts in varying degrees of maturity were seen, ranging from clusters of fine sprouts within single Schwann cells to larger sprouts already beginning myelination (Fig. 3a). Growth cones were rare. The grain distribution (Fig. 4b) showed that a fraction of transported label was already associated with axolemma; the distribution of grains in both regions was nearly identical, and closely approximated those predicted for a hollow circle (Fig. 4a, C, and E). In the sprouts, the same association of grains with axolemma was found in myelinating axons as well as in axons that had not yet begun to myelinate.

These grain distributions were assessed from normalized grain densities. For example, 7 d after labeling, there was a greater density in the small sprouts than in the more proximal regions. For example, 7 d after labeling, the density was 2.6-fold greater in region D (the former growth cone region) than in region C (just distal to the crush).

Extent of Redistribution of Transported Glycoproteins

We next asked to what extent labeled glycoproteins might be redistributed after delivery. For example, we wondered whether labeled constituents in the growing tips would be carried more distally as the sprouts continued to grow out, and whether substantial intramembranous migration might occur. At 7 and 14 d after labeling, the amount of radioactivity in the regenerating sprouts decreased by >70% (Fig. 1b and Table II). In addition, the relative distribution of radioactivity between regions C and D changed. Region D became progressively less prominent (Figs. 1b and 2), although a small peak corresponding to region D could still be distinguished 14 d after labeling (Fig. 5a).

The ligature studies showed a progressive increase in the proportion of radioactivity in the stationary fraction. 36% of the total radioactivity in the regenerating sprouts was in the stationary fraction on day 1; by day 14 the stationary fraction was 90% of the total (i.e., the amount of radioactivity within the regenerating sprouts was nearly the same in ligated and unligated nerves) (Table II). There was a corresponding reduction in the mobile fraction from 25 to 6% between days 1 and 14 (Table II).

Quantitative radioautography was used to assess the association of label with axolemma, with the intraxonal compartment, and with extraxonal structures, and also to compare the patterns in sprouts of different degrees of maturity. Sections from the crush site showed a small proportion of axons that were markedly enlarged and contained dense collections of membranous organelles. These axonal swellings, which were heavily labeled, presumably represented axons that had not begun to grow out effectively (8, 20). They explain the small peak of radioactivity in region B.

In region C, sprouts in varying degrees of maturity were seen, ranging from clusters of fine sprouts within single Schwann cells to larger sprouts already beginning myelination (Fig. 3a). Growth cones were rare. The grain distribution (Fig. 4b) showed that a fraction of transported label was already associated with axolemma; the distribution of grains in both regions was nearly identical, and closely approximated those predicted for a hollow circle (Fig. 4a, C, and E). In the sprouts, the same association of grains with axolemma was found in myelinating axons as well as in axons that had not yet begun to myelinate.

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FIGURE 3  Electron microscope radioautograms from regenerating sprouts 1 d after injection of $[\text{H}]$fucose into the lumbar ventral horns. (a) region C, early myelinating axon (A), with silver grains present both near the axolemma and within the axon. SC, Schwann cell. Bar, 1 $\mu$m. $\times$ 17,000. (b) Region D, an early myelinating axon (A) and two growth cones (GC) containing particulate organelles. Bar, 1 $\mu$m. $\times$ 20,100. (c) Region D, several densely labeled growth cones (GC) containing tubulovesicular profiles. Bar, 1 $\mu$m. $\times$ 27,700.
The double-labeling experiment allowed direct comparison within individual nerves of the distribution of radioactivity 7 d after labeling (14 d after crush) with the position of the sprout terminals at that time. In this experiment (Fig. 7), the sciatic nerve was crushed; on day 7 after crush, \(^{3}H\)fucose was injected; on day 13 after crush, \(^{35}S\)methionine was injected; and on day 14 after crush, the animal was killed. The results showed that proteins labeled by \(^{35}S\)methionine extended 20 mm beyond the segments labeled by \(^{3}H\)fucose. Thus, these results indicated that inserted glycoproteins were not carried distally with the growing tip, nor was there any substantial redistribution of transported glycoproteins to regions distal to region D in spite of continued outgrowth.

**Amount of Transported Radiolabel in Regenerating Nerves**

The regenerating sprouts retained considerably more radioactivity than either parent axons or contralateral control nerves; even 14 d after labeling, there was more than twice the amount of radioactivity in the regenerating sprouts as in the control nerve (Fig. 5 A and Table III). Increased retention was not restricted to region D, although a small peak persisted in this region (Fig. 5 A). Region C also showed more radioactivity than control nerves, as is best seen in animals labeled 21 d after crush and sacrificed 7 d later (Fig. 5 B and Table III). In these animals, the growing tips had grown beyond the available length of nerve. The regenerating sprouts contained 2.22 times the amount of radioactivity in comparable control nerves (Table III). However, in animals labeled 90 d after crush and killed 2 wk later, the same amounts of radioactivity were found all along the regenerating and control nerves (not shown).

It is of interest that the amount of radioactivity in the parent axons (region A) was increased at early times after labeling, presumably reflecting an increased amount of fucosyl glycoproteins undergoing fast transport (13). However, by 14 d after labeling, the number of counts in this region was identical on the two sides (Fig. 1 c).
DISCUSSION

This study was designed to construct a spatial and temporal picture of aspects of the insertion of glycoproteins into the axolemma of regenerating sprouts. Interpretation of these correlational radiometric and radioautographic studies is based on the fact that labeled fucosyl glycoproteins are synthesized in the cell body and delivered to regenerating axon in relatively "pulsed" fashion, with fast axonal transport providing the means of delivery (1, 11, 12, 16, 26, 39, 40). Because, in young rats, a large proportion of sciatric motor axons grow out in a coherent fashion (17, 19, 31, 33), it was possible to examine regions of the regenerating nerve rich in growth cones, in mature sprouts, or in maturing sprouts, and to follow the fate of a "pulse" of transported glycoprotein over time.

The autoradiographic results showed that some axonally transported [3H]fucosyl glycoproteins were incorporated into the axolemma of the regenerating sprouts within the first day after synthesis. 1 d after labeling, in addition to the silver grains located within the axon, another fraction was clearly associated with axolemma (Fig. 4 B and D). It is likely that these labeled glycoproteins within the axolemma correlate in large part with the stationary fraction as determined by ligature studies. 1 d after labeling, a definite stationary fraction was present within the sprout (Fig. 1 d and Table II). In addition, there was a relatively large mobile fraction, presumably corresponding to the intraxonal glycoproteins still undergoing bidirectional transport (Fig. 1 d and Table II). In contrast, by 7 d after labeling, the grains remaining in the regenerating sprouts were associated almost exclusively with axolemma, and the grain distribution closely approximated that expected for a hollow circular source (Fig. 4 C and E). In the ligature studies, most of the radioactivity was stationary, with a much reduced mobile phase (Fig. 1 d and Table II). Thus, at late times after labeling, the radioactivity remaining in the sprouts can be equated with the stationary axolemmal radioactivity.

We next asked at what stage or stages of sprout development axolemmal insertion of transported glycoprotein occurred. In particular, we tested whether insertion of transported glycoproteins might occur only at the growing tip (4), or whether a significant proportion might be inserted locally all along the regenerating sprout (lateral insertion). Our data suggests that both terminal and lateral insertion contribute transported glycoproteins to the axolemma. Indirect evidence suggesting that terminal insertion occurred was found in the peak of stationary radioactivity in the growth-cone-rich region D in the ligature studies (e.g., in nerves ligated 1 and 3 d after labeling [Fig. 1 d]). A similar persistent peak in region D was found in the radiometric studies, even 14 d after labeling (Fig. 5 A). These patterns are consistent with the possibility of terminal insertion.

On the other hand, several related observations indicated that the growth cone was not the sole site of addition of transported glycoproteins. Radioautography showed that even large myelinating sprouts, whose growth cones must be many millimeters distal to the level of the histologic section, contained label within axolemma, even 1 d after labeling (Fig. 3 a). In addition, local insertion all along the regenerating sprouts was suggested by the relatively large amounts of radioactivity found in region C 7 d after labeling. This retained radioactivity is best seen in groups labeled 14 or 21 d after crush (Figs. 2 and 5 B, respectively), because of the greater length of region C available for examination. Taken together, these radioautographic and radiometric observations are best explained by local axolemmal insertion of glycoproteins all along the regenerating sprouts.

An alternative interpretation might be that terminal insertion was followed by retrograde intramembranous movement of glycoproteins resulting in general axolemmal labeling. Although such a process (27, 29) cannot be excluded by present data, intramembranous redistribution (27, 29) would have to be rapid (to account for widespread axolemmal labeling within 1 d), extensive, selective (the abrupt increase in the amount of radioactivity at the level of the crush never ascended into the parent axon [Figs. 1 c and 5]), and unidirectional (see below). Local insertion seems a more likely mechanism for addition of the bulk of axolemmal glycoproteins.

We next asked whether labeled glycoproteins, once delivered to the sprouts, might subsequently be carried along as the sprouts continued outgrowth. The radiometric studies showed no evidence of a major redistribution of transported glycoproteins into sprout regions that develop after delivery. In particular, the double-labeling experiment demonstrated that most of the new sprout growth that developed after delivery of the pulse of fucosyl glycoprotein remained unlabeled (Fig. 7). There is a small difference in the most distal segment containing transported radioactivity 14 d after labeling (compared with 1 d) (Figs. 1 b, 2, 5 A, and 7); this difference is best accounted for by the continued delivery of small amounts of material still moving distally 3 or 7 d after intraspinal injection of isotope (i.e., the radioactivity proximal to the ligation in Fig. 1 d). Thus, we conclude that the sprout axolemma with the greatest proportion of recently synthesized glycoproteins is always the most distal (the most heterogeneous sprout axolemma with regard to the day of synthesis of axolemmal constituents would be the segment just distal to the crush).

There was a progressive reduction of the prominence of region D relative to region C with time after labeling. This change in conformation of the radiometric curves suggests that
FIGURE 6 Electron microscope radioautograms from regenerating nerves removed 7 d after intraspinal injection of [³H]fucose, showing association of silver grains with axolemma. (a) Region C, sprout in advanced stage of myelination (cf. Fig. 4 C). Bar, 1 μm. x 17,000. (b) Region D, large axon that has not yet begun to myelinate (cf. Fig. 4 E) Bar, 1 μm. x 19,500.

A portion of the radioactivity that accumulates in the growth cones at early times turns over more quickly after delivery than that in region C (Figs. 1 a, 2, and 7). As demonstrated by the ligation studies, this turnover occurs in part by retrograde transport back towards the cell body (Fig. 1 d) and also, presumably, by local degradation. It is possible that some of the transported glycoproteins reaching the growth cone undergo a cycle of insertion, endocytosis, and subsequent retrograde transport (7), similar to that suggested at the neuromuscular junction (23).

Retention of [³H]fucosyl glycoproteins within regenerating sprouts occurred in amounts considerably greater than either parent axons or control nerves (in regions comparable to the regenerating sprouts). For example, 2 wk after labeling, the 24 mm distal to the crush contained 2.3 times the amount of radioactivity present in the same region of the contralateral nerve (Figs. 1 c and 5 A; Table III). That this increase was not simply the result of the increased fast transport of fucosyl glycoprotein that occurs during regeneration (13) was shown by the fact that no greater retention was seen in parent axons than in the control nerves 7 or 14 d after labeling (Figs. 1 c and 5). Increased retention continued in sprout axolemma at least 3 wk old (the region just distal to the crush in Fig. 5 b), but, by 3 mo after crush, counts in control and regenerating nerves were comparable.

The following model relates previous models of membrane
the lateral growth or enlargement of the axolemma. Calculations indicate that the total area of axolemma added must be considerably greater during lateral growth (e.g., from 1 to 10 μm) than during longitudinal outgrowth of a 1-μm sprout terminal. In addition, the added glycoproteins could contribute to an increase in particle density within the sprout. Freeze-fracture studies (34) have suggested a low particle density within growth cone axolemma and intraaxonal vesicles, with more proximal regions of the sprouts showing a progressive increase in particle density toward that of mature axolemma. Finally, a speculative possibility might include a role for inserted glycoproteins in "signaling" Schwann cells by axon-Schwann cell contact. There is evidence that axonal membrane-associated elements can influence Schwann cell division (22, 37) and they possibly could be involved in signaling the initiation of myelination.

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