Protein Modification by Amino Acid Addition Is Increased in Crushed Sciatic But Not Optic Nerves

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Rat optic and sciatic nerves were crushed, and 10 minutes to 3 days later nerve segments between the crushed site and the cell body were removed and assayed for posttranslational protein modification by amino acid addition. Protein modification was comparable in intact optic and sciatic nerves, but in sciatic nerves increased to 1.6 times control levels 10 minutes after crushing and reached a maximum of ten times control levels by 2 hours. In optic nerves activity was decreased throughout the time course studied. The results indicate that, in a nerve which is capable of regeneration (sciatic), protein modification by the addition of amino acids increases immediately after injury, but a nerve incapable of regeneration (optic) is incapable of activating the modification reaction. These findings may be important in understanding the reasons for the lack of a regenerative response after injury to central mammalian nerves.

AXONS OF NEURONS WITH CELL bodies in the mammalian central nervous system (CNS) fail to regenerate after injury, while axons that extend from the CNS into the peripheral nervous system do regenerate (*1*). There has been much speculation as to the reasons for this phenomenon, but recent results (*2*) have supported the view, first enunciated in 1928 (*3*), that the lack of regeneration in the mammalian CNS is not due to an intrinsic inability of a central neuron to regrow but is dictated by environmental events at the site of the injury. Thus, the response of Schwann cells to the injury of a peripheral nerve appears to be supportive or permissive to growth, whereas central glia either do not support growth or actively inhibit it.

The biochemical events taking place immediately after injury by crushing are likely to be critical in determining whether subsequent nerve regeneration will be successful. Crushed optic nerves are capable of reactive sprouting within 1 week of injury, but the sprouts never extend more than a few millimeters into the damaged region (*3, 4*). This suggests that the sprouts may encounter an impenetrable barrier, such as an astrocytic scar (*5*). Since peripheral nerve sprouts are able to traverse the region of the injury, it appears that the early response of a central nerve to injury is different from that of a peripheral nerve.

For several years we have been investigating a group of reactions that occur in axons (*6*) (as well as in glia and Schwann cells) and that increase in activity 10 to 100 times in the rat sciatic nerve 6 days after injury (*7*). These reactions involve the posttranslational addition of a variety of amino acids to axonal and glial proteins; in the case of the neuron this process appears to be a way of modifying axonally transported proteins. The reactions are likely to utilize transfer RNA (tRNA) as the amino acid donor and are regulated (or inhibited) in vivo by the presence of low molecular weight substances (*6, 7*). Thus far we have studied these reactions in vertebrate nerves capable of regeneration. In the experiments reported here, we examined the early response (from 10 minutes to 3 days after crushing) of the rat optic nerve (a central nerve, incapable of regeneration) and the rat sciatic nerve (a peripheral nerve, capable of regeneration) with respect to posttranslational protein modification by amino acid addition and protein synthesis.

Intact rat sciatic nerves are capable of a low level of protein modification by the addition of amino acids (*7*). To determine whether these reactions occur in intact optic nerves, male Sprague-Dawley rats (125 to 150 g) were decapitated and two sciatic or four optic nerves were rapidly removed, desheathed, pooled, homogenized in buffer, and assayed for the modification reaction (*8*). Samples obtained from intact optic nerves were able to incorporate [^{3}H]arginine and [^{3}H]lysine into protein to approximately the same extent as sciatic nerve segments. Leucine was also incorporated into protein in the optic nerve, but at somewhat higher levels than in sciatic nerves (Fig. 1). Thus intact optic nerves, like sciatic nerves, are capable of the posttranslational addition of amino acids to endogenous proteins.

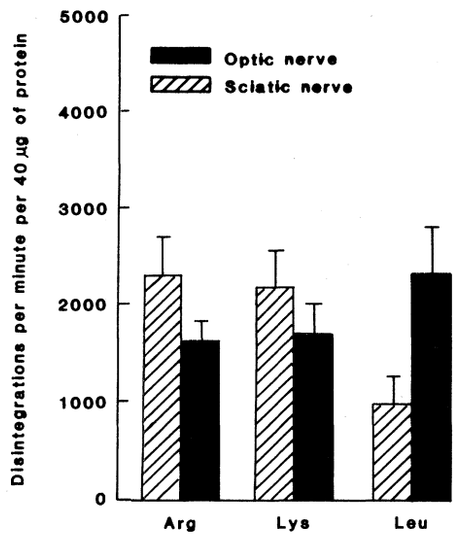

Fig. 1. Comparison of the posttranslational incorporation of [^{3}H]arginine, [^{3}H]lysine, and [^{3}H]leucine into proteins in intact rat sciatic and optic nerves. Values are based on 40 µg of protein (the average amount of protein per reaction), and controls for nonspecific binding (inactivated by the addition of 200 µl of 10 percent TCA to each sample) have been subtracted from the enzymatically active fractions. Assays for each amino acid were performed in triplicate; data are expressed as means ± standard errors for five (sciatic nerve) or ten (optic nerve) separate experiments.

In the next series of experiments we examined the posttranslational addition of [^{3}H]lysine to proteins after the nerves were crushed (lysine was chosen as the representative amino acid for the remainder of the experiments). Animals were anesthesized with ketamine (10.0 mg per 100 kg) and the right optic or sciatic nerve was crushed (*9*). The contralateral nerve remained intact and served as a control. Ten, 30, 60, or 120 minutes or 3 days later the rats were decapitated and nerve segments between the crushed site and the cell body (6 mm of sciatic nerve and 3 mm of optic nerve) were pooled and assayed.

Crushing the optic nerve also compresses the optic artery, so it is important that the blood supply to the optic nerve and retina remain intact after surgery. The following results indicate that the optic artery remained patent. Six rats were anesthesized and the left optic nerve was crushed as described above. The contralateral optic nerve was cut and both eyes were continually irrigated with normal saline. The retinal blood vessels in both eyes were examined 10 to 15 minutes after surgery with a Wild Heerbrugg dissecting microscope (10×) and compared with normal retinas. The retina associated with the crushed nerve was indistinguishable from retinas of unoperated controls, while blood vessels in the retina associated with the cut nerve were either not visible or were shrunken. In addition, lenses of rats with cut optic nerves became opaque within 20 minutes of surgery, while lenses associated with crushed nerves remained clear. In three of the six rats, the crushed nerve was cut and lens opacification developed within 15 to 20 minutes. Furthermore, histological examination of horizontal sections of the optic nerve 6 days after

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crushing showed zones of (i) degeneration at and central to the crushed site; (ii) dying back, with end bulb formation (1 to 2 mm retinal to the crushed site); and (iii) intact myelinated axons (2 to 3 mm retinal to the crushed site). There was no evidence of ischemic necrosis. These results indicate that the optic artery and hence the blood supply to the optic nerve and retinal ganglion cells remained patent.

Protein modification in injured optic nerves decreased approximately 40 percent 10 minutes after crushing and remained depressed by 40 to 60 percent for up to 3 days after the injury (Fig. 2). In contrast, the posttranslational incorporation of [^{3}H]lysine into protein in the sciatic nerve was 1.6 times higher than that in controls 10 minutes after injury and continued to increase to a maximum of ten times control values 2 hours after injury (Fig. 2). Thus, while elements in the sciatic nerve responded to injury by an immediate (10-minute) activation of the protein modification reaction, protein modification in the optic nerve decreased in response to injury.

We also examined the ability of the nerve to synthesize proteins under the same conditions. Five groups of four rats each were injected intravenously with 100 μCi of a mixture of [^{3}H]arginine, [^{3}H]lysine, and [^{3}H]leucine. One hour later, sciatic or optic nerves were crushed bilaterally in half of these rats and in the remainder (the controls) the nerves were left intact. The rats were killed 10, 30, 60, 120 minutes, or 3 days after nerve crushing. Nerve segments comparable to those used in the experiments described above were removed and assayed for incorporation of label into protein (*10*).

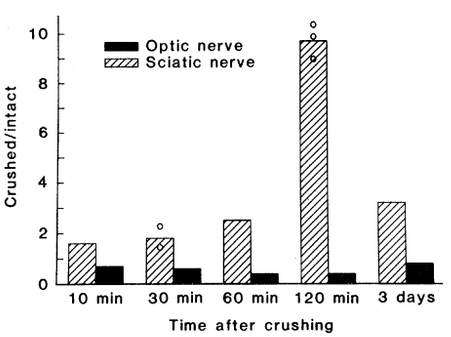

Fig. 2. Posttranslational incorporation of [^{3}H]lysine into proteins in rat sciatic and optic nerves 10 minutes to 3 days after injury. Contralateral noninjured nerves served as controls so that comparisons of protein modification activity could be made in each experiment. Values are for single experiments and are expressed as the ratio of crushed to intact nerves (values for TCA-inactivated samples have been subtracted from active samples). Circles represent individual data points at 30 and 120 minutes. Each data point represents two pooled sciatic nerves or four pooled optic nerves.

Protein synthesis in injured sciatic nerves decreased initially but by 2 hours had increased to approximately twice control levels, an increase that was still sustained 3 days later (Fig. 3, top). Protein synthesis in injured optic nerves decreased within 10 minutes and remained low (Fig. 3, bottom).

Since protein synthesis has not been demonstrated in axons, it is likely that the activity observed occurred in nonneuronal elements of the nerve. Thus the increased incorporation of tritiated amino acids into proteins in the sciatic but not the optic nerve suggests that cells in the sciatic nerve respond to nerve injury by activating their protein-synthesizing machinery while cells in the optic nerve do not.

The protein modification experiments show that both optic and sciatic nerves contain the elements necessary for modification of proteins by amino acid addition. However, after being crushed, the optic nerve is unable to activate these reactions for at least 3 days. In contrast, the sciatic nerve responds to such injury by an immediate and sustained activation of the reactions.

The increase in protein modification activity in the sciatic nerve could be due to increased synthesis of components of the reaction or to activation of elements already in the nerve. Since protein modification activity is elevated at 10, 30, and 60 minutes after crushing—time points when protein synthesis is decreased (Fig. 3)—this increase is probably not due to increased synthesis of any of the reaction components, but rather to activation of components already in the nerve. Another possibility is that endogenous modification reactions decrease in sciatic and increase in optic nerves in response to injury, resulting in more (sciatic) or fewer (optic) sites for in vitro modification by [^{3}H]lysine. These experiments do not discriminate between this possibility and the possibility that there is an activation of components in the sciatic nerve and a failure to activate these components in the optic nerve.

The molecules required for these reactions are present in nerves as high molecular weight complexes of enzymes (aminoacyl tRNA synthetases and protein transferases), tRNA, and a low molecular weight inhibitor (*7*). This inhibitor appears to be linked to a component of the reaction, preventing its expression in vivo and in unfractionated homogenates of nerve. Removal of the inhibitor by gel filtration chromatography results in activation of the reactions (*6*). We propose that the activation in the rat sciatic nerve results from removal of the inhibitor from the high molecular weight complex, thus releasing the protein modification reactions. If this is the case, then the failure of the activation in the optic nerve may be due to failure to "disinhibit" the protein modification reactions.

The modification reactions may be occurring in axons or in nonneuronal cells of the nerve. In nerves we examined previously [squid stellate (*6*), rat sciatic (*7*), and goldfish optic (*11*)], the reaction did occur in axons, but the present experiments do not yield any direct evidence that the changes are due to increases in these reactions in axons. However, the fact that increases in protein synthesis do not appear to be requisite for increases in the modification reactions is consistent with an axonal locus for at least some of the protein modification activity.

The role of these reactions in injured sciatic nerves is not known. A modified protein might be retrogradely transported to signal the cell body that axonal damage has taken place (*12*). This view is supported by the finding that mouse and rat retinal ganglion cells do not respond to nerve injury by increasing their synthesis of RNA and protein (suggesting that they may not receive a signal of axonal injury), as do cells capable of regeneration (*13*). Another possibility is that certain axonal or Schwann cell

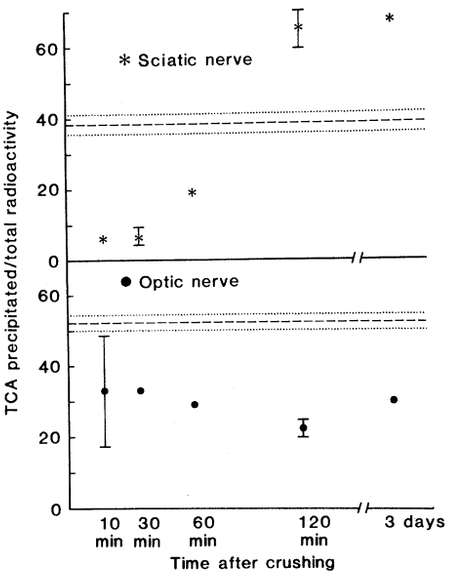

Fig. 3. Protein synthesis in sciatic and optic nerves at various times after crushing. Values give the ratio of radioactivity incorporated into hot and cold TCA-insoluble material to the total radioactivity in the nerve segment (TCA-soluble plus -insoluble). Each data point represents values for two sciatic or four optic nerves; error bars represent the standard error of the mean for three or four separate experiments at those time points. The mean value for all controls (nine sciatic and ten optic nerves) is shown by the dashed lines, with the standard error indicated by the dotted lines.

enzymes, which play a role in sealing off membranes of cut nerve endings (*14*) and in establishing a path for regeneration, may become activated subsequent to modification. A third possibility is that these reactions may be involved in proteolytic events. The nonlysosomal ubiquitin proteolytic pathway, a system for hydrolyzing damaged proteins, has an absolute requirement for tRNA (*15*). The function of tRNA in the ubiquitin reaction is not known; it may serve as a posttranslational amino acid donor to damaged proteins, thereby targeting those proteins for degradation. If that is the case, then the modification reactions could act to participate in the breakdown of damaged proteins, thus clearing the way for successful regeneration.

In conclusion, both sciatic and optic nerves contain the components necessary for, and are capable of, protein modification by the addition of amino acids. However, the finding that these reactions are dramatically increased in sciatic but not optic nerves after injury suggests a fundamental biochemical difference between them, a difference that may be related to the ability of one and the inability of the other to regenerate.

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9. The connective tissue on the inferior-lateral side of the eyeball was gently teased away with fine forceps to expose the optic nerve. The nerve was then separated from the surrounding connective tissue and crushed for 10 seconds with curved jeweler's forceps at the point where the optic nerve enters the orbital cavity (approximately 4 mm from the back of the eye). Nerves were crushed rather than cut to avoid severing the retinal artery. Sciatic nerves were crushed by the procedure of M. F. Zanakis *et al.* (*7*).
10. Nerves were excised, desheathed, homogenzied in 250 μl of buffer, and extracted with hot and cold 6.5 percent TCA until the wash contained negligible amounts of radioactivity. The pellet was dissolved in Protosol and counted in toluene:Liquifluor. Aliquots of the TCA-soluble fraction were counted in Hydrofluor (National Diagnostics). The calculation for protein synthesis was made by dividing the TCA-insoluble radioactivity by the total radioactivity (TCA-soluble plus -insoluble) in the nerve segment. Thus increases in protein synthesis are indicated by an increase in the percentage of incorporation. (This determination includes radioactive amino acids that may have been incorporated into proteins by modification reactions, but this is likely to be a minor proportion of the amino acids incorporated into proteins by protein synthesis.)
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Herbivores' Direct and Indirect Effects on Algal Populations

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The increase in algal reproductive rates caused by nitrogen regeneration from herbivorous zooplankton approximately equaled the zooplankton-caused mortality. This result demonstrates that nutrient regeneration by herbivores is at least sometimes a strong indirect effect in natural communities.

HERBIVORES AFFECT THE POPULATION dynamics of plants in at least two distinct ways: directly through consumption and indirectly through regeneration of limiting nutrients. Although there has been interest in this dual role of herbivory in both terrestrial (*1*) and aquatic (*2*) plant communities and in indirect effects in general (*3*), until now there have been no studies that have simultaneously assessed the relative importance of these two processes in a natural community. Such experiments were conducted on a natural algal community with a cooccurring crustacean herbivore, *Daphnia pulex*, to separate the direct effect of herbivory from the indirect effect of fertilization. The indirect effect caused by nitrogen regeneration had about as large an impact on the phytoplankton community as the direct grazing effect did.

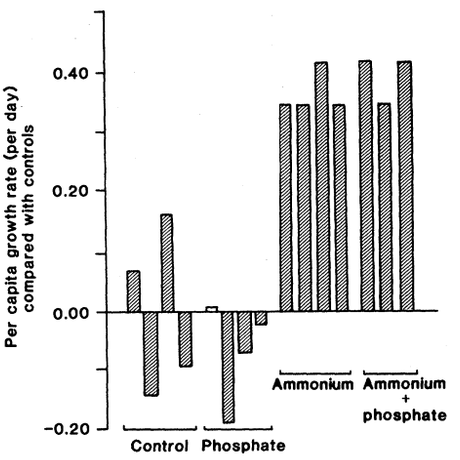

Fig. 1. The difference between growth rates of chlorophyll fluorescence in each vessel and the mean growth rate for controls—as determined from final densities by the equation

$$[\ln F - (\text{mean } \ln F \text{ for controls})]/6$$

where F is fluorescence on day 6—shows a significant nitrogen effect [$F(1, 11) = 86.90$, $P < 0.005$]. The mean for the nitrogen flasks was 0.348 (SE, 0.067), and the mean for the nonnitrogen flasks was −0.035 (SE, 0.068). Nitrogen regeneration could potentially stimulate phytoplankton growth.

The general experimental procedure consisted of establishing an herbivore gradient and monitoring phytoplankton growth rates when influenced by both grazing and nutrient regeneration and when influenced by only nutrient regeneration. In addition, I performed nutrient addition experiments to establish whether regenerated nitrogen or phosphorus or both were potentially limiting (*4*).

In the absence of grazers the algae were limited by nitrogen, but not by phosphorus, as shown by chlorophyll fluorescence (*5*) (Fig. 1) and population densities. Chlorophyll increased at 0.41 per day (SE, 0.09) faster in the nitrogen treatments than in the nonnitrogen ones, providing an estimate of the maximum potential effect of nitrogen regeneration on algal reproductive rates. Population densities of two of four domi-

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