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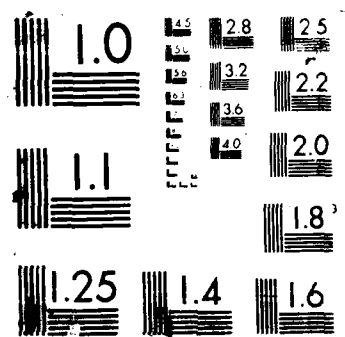
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Medical Report No. 271

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Observations of Ocular Problems From the
Regimental Cavalry (D. and S.) Ocular Nerve
with Neurological Lesions

Michael A. Dwyer, PhD, CPT, MS
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and
John A. Pugh, MS, PhD

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DIVISION OF OCULAR HAZARDS
and
DEPARTMENT OF CELL BIOLOGY
VANDERBILT UNIVERSITY MEDICAL SCHOOL
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ABSTRACT: Three proteins, with apparent molecular weights of 16, 30, and 42 kilodaltons, made by glial cells of the goldfish optic nerve undergo enhanced synthesis during regeneration of the retinotectal axons. If, as has been postulated, these enhanced glial proteins (or EGPs) assist in axonal regeneration, they may interact with the retinotectal neurons. Two possible modes of interaction, 1) endocytosis/retrograde transport and 2) binding to optic nerve membranes, were examined using gel electrophoresis of radiolabelled glial proteins. The results suggest 1) that two glial proteins, which may be EGP-16 and EGP-42, are retrogradely transported to the retina during axonal regeneration and 2) that a third glial protein, perhaps EGP-30, binds in a specific and saturable manner to the membranous fraction of the optic nerve.



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INTRODUCTION: Teleost fishes, such as the common goldfish (*C. auratus*), and anurans possess the ability to regenerate central nervous system (CNS) axons following trauma to the nerve. Unidentified factors made by glial cells have been shown to potentiate regeneration of axons following axotomy (1-4). The effects tend to be greater if the factors are gleaned from glial cells of nerves that are themselves in the process of regenerating (5-8). Recent work suggests that these unknown factors may enable injured mammalian CNS neurons, which normally do not regenerate, to extend neurites well beyond the site of trauma (9, 10). Three proteins made by glial cells of the goldfish optic nerve undergo at least five-fold increases in synthesis following injury to the retinotectal axons (11). Thus these three proteins are likely candidates for the unknown factor(s) which potentiates regeneration. These proteins have been tentatively named *enhanced glial proteins*, or EGPs, and each is identified by its apparent molecular weight in kilodaltons (KD), as shown in Figure 1 (12-16). If one or more of the EGPs is the unknown factor(s), it (they) might well interact with regenerating axons. Two possibilities for such interaction were tested in this study: retrograde transport of EGPs to the retina and binding of EGPs to the optic nerve.

METHODS: FIRST PARADIGM (retrograde transport) - Each fish (n=3) received a unilateral intraorbital left optic nerve crush. Ten days later, 500 uCi of [³⁵S]methionine in a total volume of 5 μ L was introduced intracranially to the right optic tract¹. The cranium was then resealed, and 16 hours were allowed for [³⁵S]met incorporation into glial proteins and subsequent retrograde transport to the retina. The left retina was then excised and prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (19) and gel fluorography (20).

¹The goldfish optic axons decussate completely at the optic chiasm. Between the eye and the chiasm the fibers are referred to as the optic nerve; between the chiasm and the optic tectum they are referred to as the optic tract. Glial cells (oligodendrocytes, astrocytes, and microglia) reside around and in between the axons (17, 18).

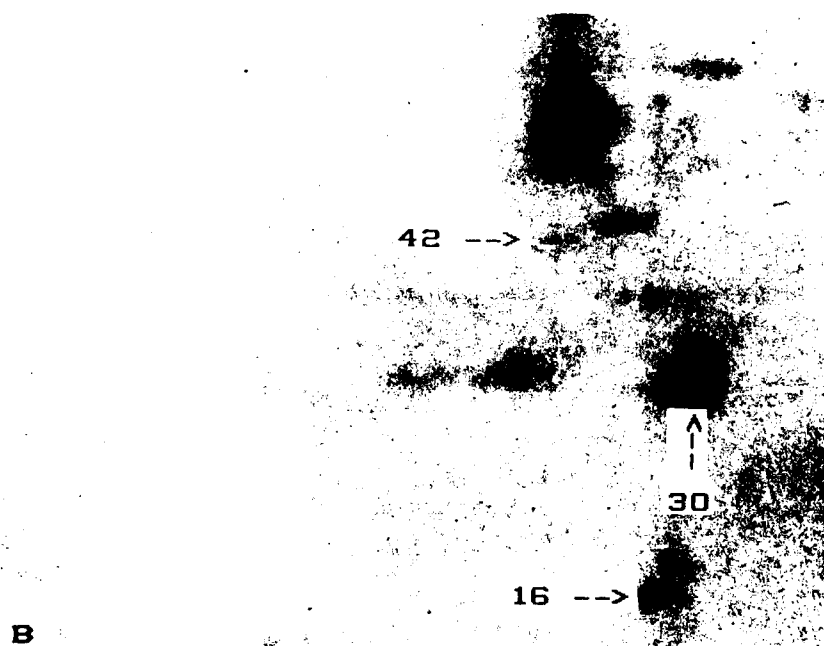
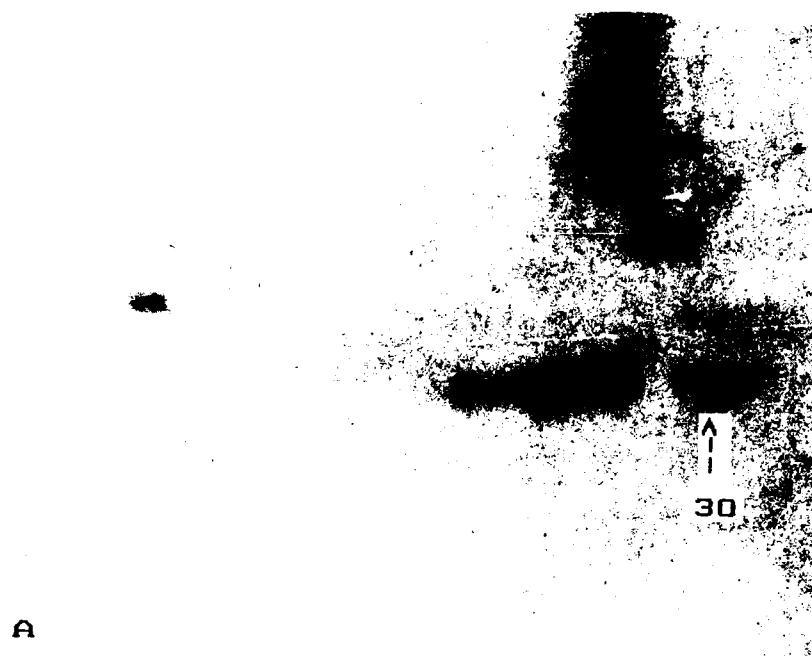


Figure 1. Enhanced glial proteins of the goldfish optic nerve. Optic nerves were incubated for two hours in 100 uL of Ringer's solution that also contained 60 uCi [35S]met. Radiolabelled proteins that had been released into the medium were then subjected to 2-D PAGE and fluorography. A, normal control. B, 10 days after intraorbital optic nerve crush. The nerve crush caused three glial proteins to undergo enhanced synthesis, hence the term enhanced glial proteins, or EGPs. Similar results were obtained when enucleation was substituted for optic nerve crush.

RESULTS AND CONCLUSIONS: FIRST PARADIGM - The retinae from the fish used in the first paradigm (retrograde transport, Figure 2A) contained two radiolabelled proteins which migrated electrophoretically to positions corresponding to those of EGP-16 and EGP-42 (Figure 2B). The most likely source of these two proteins is the glia surrounding the optic nerve. This assertion is based on three factors. First, the only structures in the optic tract that are capable of synthesizing significant amounts of protein are the glial cells (17). Second, the minimum amount of time required for radiolabelled proteins present in the optic tract to be retrogradely transported to the retina is approximately 16 hours (21, 22). Thus, even if the radiolabel had made its way into neuronal cells surrounding the injection site, the proteins made by those cells would not have had time to be secreted into the interstices, cross into the optic axons, and be retrogradely transported to the retina. Third, it is unlikely that the radiolabelled proteins found in the retina at the end of 16 hours were made by retinal cells from free [35S]met which was carried to the retina by the bloodstream. The reasoning for this third assertion is as follows: our calculations indicate that only $2 \times 10^{-2}\%$ of the radiolabel injected into the goldfish cranium makes its way into the circulatory system over the course of 16 hours. If one assumes that all the radiolabel in the bloodstream is unbound and that one retina occupies as much as 1% of the total blood volume (a generous assumption), then only $2 \times 10^{-4}\%$ of the total injected radiolabel would be available for protein synthesis in the retina. Thus of the 500 uCi introduced into the cranium, only 2000 cpm would be available to the retina. We recovered precipitable radioactivity from the retina which was on the order of 15,000 cpm (12). We postulate, therefore, that the 16 and 42 KD proteins were made by the glia, and appeared in the retina as a result of

endocytosis and retrograde transport.

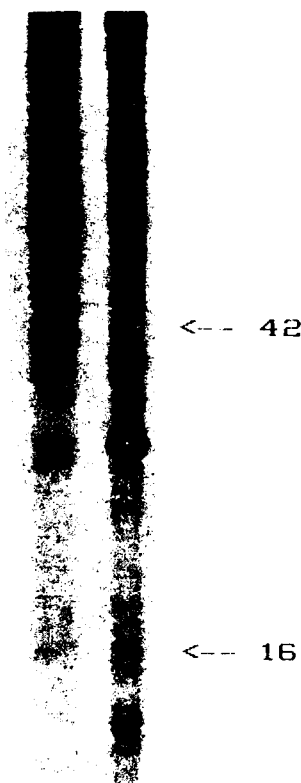


Figure 2. Two proteins, which may be EGP-16 and EGP-42, appear in the retina 10 days after optic tract crush. A, each fish (n=3) received an intraorbital optic nerve crush. Ten days later [³⁵S]met was applied intracranially directly to the affected optic tract. Sixteen hours later, the corresponding retina was excised and subjected to SDS-PAGE and fluorography. Two proteins with apparent molecular weights of 16 and 42 KD appeared in the retinæ of these animals. B, SDS-PAGE preparation of the conditioned medium described in Figure 1B is shown here for comparison. The most likely source of the labelled proteins shown in A is the glial cells of the optic nerve. Thus, these proteins probably appear in the retina as a result of endocytosis and retrograde transport.

METHODS: SECOND PARADIGM (specific binding of EGPs to axons) - Conditioned medium containing [³⁵S]met-labelled glial proteins (to include the EGPs) was prepared as previously described by incubating 10-day post-crush goldfish optic nerves for 2 hours in 100 uL of goldfish Ringer's solution that also contained 60 uCi [³⁵S]met (12-16). Concomitantly, optic nerves (back of the eye to optic chiasm) from three sets of fish (n=3 for each set) were excised and homogenized into Ringer's solution (23). Set 1 was prepared from normal optic nerves. Set 2 was made from regenerating optic nerves, 10 days after nerve crush. Set 3 was prepared from degenerating optic nerves, 6 weeks

after enucleation. The amount of each homogenate was adjusted such that each contained approximately 210 ug total protein.

An aliquot of the conditioned medium containing 85 ug total protein and an aliquot of 4500 ug of bovine serum albumin (BSA, used to inhibit nonspecific binding) were added to each of the nine homogenates. Each preparation was then brought to equal weight, incubated at 20°C for 30 minutes, and then centrifuged at 45,000 x g for two hours; each resulting cytosolic supernatant and membranous pellet was prepared for SDS-PAGE and fluorography.

As a control, one group of these preparations (n=1 from sets 1, 2, and 3) was made in which no BSA was added. Thus nonspecific binding of radiolabelled glial proteins to the axonal membranes was not inhibited. In this group, virtually every radiolabelled protein made by the glial cells bound to and co-sedimented with the membranous fraction of the optic nerve homogenates (data not shown).

In another control, the optic nerve homogenates were incubated for 2 hours with 60 uCi each of [35S]met. The homogenates were shown by SDS-PAGE/fluorography to be incapable of incorporating [35S]met into protein. All lanes on the resulting fluorograms were blank.

RESULTS AND CONCLUSIONS: SECOND PARADIGM - A 30 KD radiolabelled glial protein from the conditioned medium bound to and co-sedimented with the membranous fractions from normal, regenerating, and degenerating optic nerves (Figure 3, lanes D-F) when BSA was added in a 50-fold excess to inhibit nonspecific binding. A detectable amount of this 30 KD protein also remained in the cytosolic fraction (Figure 3, lanes A-C). Thus, the protein appears to bind specifically and saturably to the membranous component of the optic nerve. It is interesting to note that, at least qualitatively, not as much of the 30 KD protein binds to the degenerating optic nerve membranes as binds to either those from the normal or regenerating optic nerves (compare lane F to D and E, Figure 3). Thus if a receptor for the 30 KD protein exists, it appears to decrease in quantity as the optic nerve fibers degenerate. This suggests that if a receptor is present, it is located on the axonal membrane.

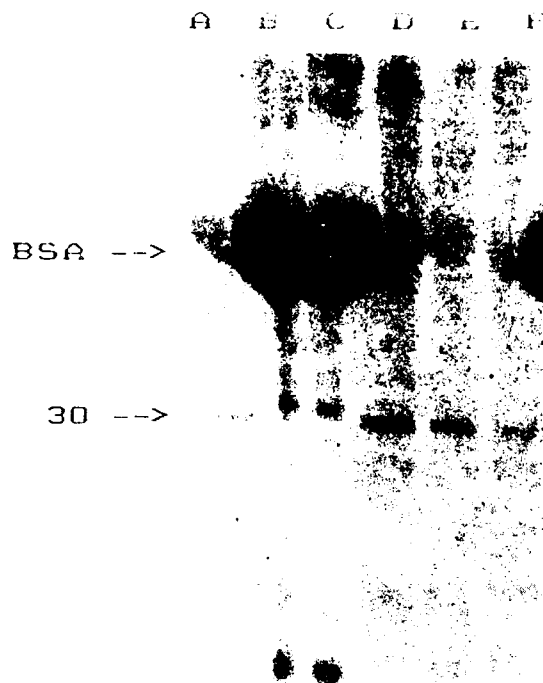


Figure 3. A 30 KD glial protein co-sediments with the membranous fraction of the optic nerve. Conditioned medium containing radiolabelled glial proteins (to include the EGPs) was prepared as for Figures 1B and 2B. An aliquot of this medium was added to whole homogenates of optic nerve. Also, an aliquot of crude BSA was added to each mixture. Each such mixture was incubated for 30 minutes and then centrifuged at 45,000 x g for two hours. The resulting pellets and supernatants were separated and prepared for SDS-PAGE and fluorography. A 30 KD glial protein from the conditioned medium bound to and co-sedimented with the membranous fraction of

the optic nerves, lanes D-F. A readily detectable amount also remained in the supernatant, lanes A-C. Normal optic nerves were used to prepare the homogenate used for lanes A and D. Regenerating optic nerves, 10 days after intraorbital nerve crush, were used for lanes B and E. Degenerating optic nerves, six weeks after enucleation, were used for lanes C and F.

DISCUSSION: Several controls remain to be performed before it can be said whether any of the three proteins described here are the same as EGP-16, EGP-30, or EGP-42. The results of this preliminary study, however, make the hypothesis that EGPs interact with regenerating neurons less speculative.

Using these results, one can propose a mechanism whereby glial cells potentiate the process of regeneration of axons. For instance, it is likely that EGP-30 is an

apolipoprotein (24-28). Thus, when nerve trauma occurs, a population of the glia begins to synthesize this apolipoprotein, perhaps as a way to supply the regenerating axons with the lipids they require to accomplish neurite/axon extension. This scenario would explain why we see binding of the 30 KD protein to the axonal membranes without subsequent retrograde transport; the 30 KD protein is probably being used at the axon tips.

In the cases of the 16 and 42 KD proteins, it is possible (but still highly speculative) that one or both is a growth factor made by the glia following nerve trauma. It (they) is then endocytosed by the injured axons and retrogradely transported to the retina where it potentiates/triggers reactions in the retinal ganglion cell bodies that lead to successful axonal regeneration. Many of the known growth factors (fibroblast growth factor, to name but one) have molecular weights in the range of that of EGP-16.

Given that spinal and other CNS injuries occur in the military environment in times of both peace and war, this area of research warrants further investigation by the military medical research community. Discovering the difference between teleosts/anurans and mammals that enables the former to successfully regenerate CNS axons may someday result in regimens that allow us to effectively treat CNS axonal injuries.

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