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Regeneration of an Adult Peripheral Nerve Preparation in Culture

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Contents

Abstract

The methods used to maintain the vagus nerve from the adult rat in culture and how regeneration is studied in this preparation are described. A hypothesis is presented on the triggering of the cell body reaction. It is suggested that this reaction is initiated by proteins synthesized in nonneuronal cells at the site of a nerve lesion. These proteins, referred to as regenerins, reach the nerve cell body by retrograde axonal transport, where they initiate the regeneration process.

Index Entry Phrases: Peripheral nerve regeneration; regeneration of the vagus nerve in vitro; triggering of the nerve cell body reaction; *ortho*-grade and retrograde axonal transport; vagus nerve.

Index Entries: Peripheral nerves; nerve regeneration; vagus nerve; growth factors; insulin-like growth factor I; axonal transport; axoplasmic transport; protein synthesis inhibitors.

Introduction

When the axons of a peripheral nerve are damaged, a series of events is initiated along the nerve. In the nerve cell body, RNA and protein synthesis are redirected. These changes are referred to as the cell body reaction and are aimed at the production of components required for regeneration of the severed neurite. Although we know several of the manifestations of the cell body reaction (Hoffman and Cleveland, 1988; Lieberman, 1971; Miller et al., 1987; Skene and Willard, 1981; Skene, 1984; Tetzlaff and Kreutzberg, 1985; Wong and Oblinger, 1987; Woolf et al., 1990), we know virtually nothing about how it is triggered. However, several trigger mechanisms have been proposed (Bisby, 1986; Cragg 1970).

We are nearly equally ignorant of the processes that are responsible for changes of macromolecular synthesis in cells at the site of the lesion and in the distal nerve segment. However, here interleukin-1 released from macrophages is important and triggers synthesis of nerve growth factor in Schwann cells (Heumann et al., 1987; Lindholm et al., 1987). Cells in the distal nerve segment also synthesize and release apolipoproteins (Boyles et al., 1990; Skene and Shooter, 1983) and several other so far unidentified proteins (Rotshenker et al., 1990). These local alterations appear to serve several main functions: One is to create a local environment that promotes growth of the regenerating fibers, and includes the production and release of basal laminae components and various growth factors, as well as removal of debris. A second function is to prevent degeneration of the target-deprived neurons by the production of neurotrophic factors. A third and early function could be to produce the factors that initiate the regeneration process.

During the last years, we have been interested in the mechanism that triggers the cell body reaction. I have suggested that this reaction is initiated by proteins made at the site of the lesion and then retrogradely transported to the soma, where they in an unknown manner turn on the "regeneration programme" (Kanje, 1991). The essentials of this hypothesis are shown in Fig. 1. Although the hypothesis might appear compelling, it has been difficult to test by in vivo experiments, particularly when a pharmacological approach is made. Thus, it is difficult, although possible, to treat only the crush area or the ganglia of a regenerating nerve with drugs (Kanje et al., 1988,1989). Still, the environment in which regeneration occurs is difficult to control and manipulate. Many of these problems could be overcome in an in vitro system. Two such systems are now available: the sciatic nerve of the adult frog, which survives and regenerates in vitro (Edström and Kanje, 1988) and the vagus nerve of the adult rat (Kanje, 1991). Here I will describe the latter system and the techniques employed for culturing the preparation. Some experimental results (Kanje, 1991; Kanje et al., 1991) that have bearings on the triggering of the cell body reaction will also be reviewed.

Experimental Techniques

Culture Conditions

Both vagus nerves together with the attached nodose ganglion are removed from female adult Sprague Dawley rats by dissection. The nerves are then mounted on a rectangular piece of ethanol-sterilized nitrocellulose paper, and the preparation immersed in a tissue culture dish containing serum-free RPMI medium. Neither the choice of medium, its buffer system (HEPES or carbonate) nor the lack of serum appears critical for survival. The preparations are kept at 37°C in a tissue culture incubator.

Survival

Survival can be monitored by measurements of the compound action potential or by measurements of the preparation's ability to maintain axonal transport.

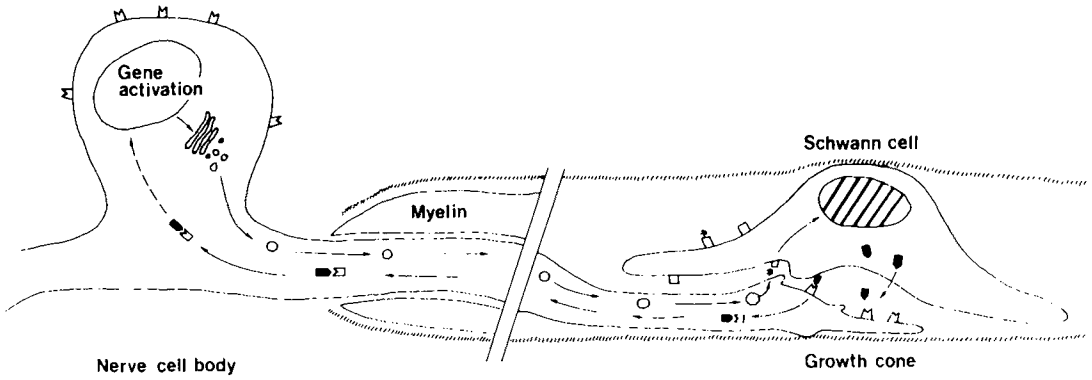

Fig. 1. Trigger hypothesis. According to the depicted hypothesis, a nerve injury induces synthesis of trigger proteins in cells surrounding the axon, in this case a Schwann cell. These proteins—regenerins—(■) are then excreted, picked up by receptors (□) on the damaged axons, and retrogradely transported (arrows) to the soma, where they activate a gene program required for the regeneration process. In an analogous manner, the neuron can send signals (*) to the nonneuronal cells via orthograde axonal transport (arrows). (O) transport vesicle.

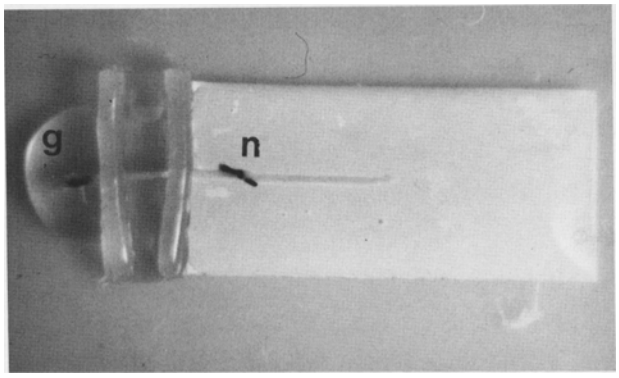

Fig. 2. Ligated vagus nerve mounted for an axonal transport experiment. Note silicone grease barriers that separate the nodose ganglion (g) from the vagus nerve proper (n).

Axonal Transport

Axonal transport is measured by incubating the nerve in a multicompartment chamber constructed on a piece of Parafilm using silicone grease barriers to separate the nodose ganglion from the vagus nerve proper (Figs. 2 and 3). A radiolabeled amino acid, usually ^{35}[S]-methionine, is then added to the ganglionic compartment. To suppress leakage of isotope and concomitant local incorporation along the nerve, extra silicone grease barriers can be used and unlabeled methionine can be added to the nerve compartment. After overnight incubation at 37°C, a flat whole mount preparation is made of the nerve. To this end, the preparation is fixed in trichloro acetic acid (TCA) between two objective slides, briefly rinsed in water, and then dried. The nerve is then exposed to an autoradiographic film. Retrograde axonal transport can be studied in the same manner, but then the isotope is added to a distal compartment.

The vagus preparation offers several experimental advantages for axonal transport experiments. It is sufficient to add 25 µL medium to cover the nodose ganglion or a distal nerve segment. Thus, small amounts of isotopes at high concentrations can be used for labeling. It is also possible to study axonal transport of ^{3}[H]-labeled materials. Because of the lower energy of this isotope, the whole mount is immersed in a scintillator before drying and film exposure.

Regeneration

Regeneration is induced by the infliction of a crush injury on the nerve by a pair of watch makers forceps. The crush site is labeled by application of a small amount of carbon particles. The

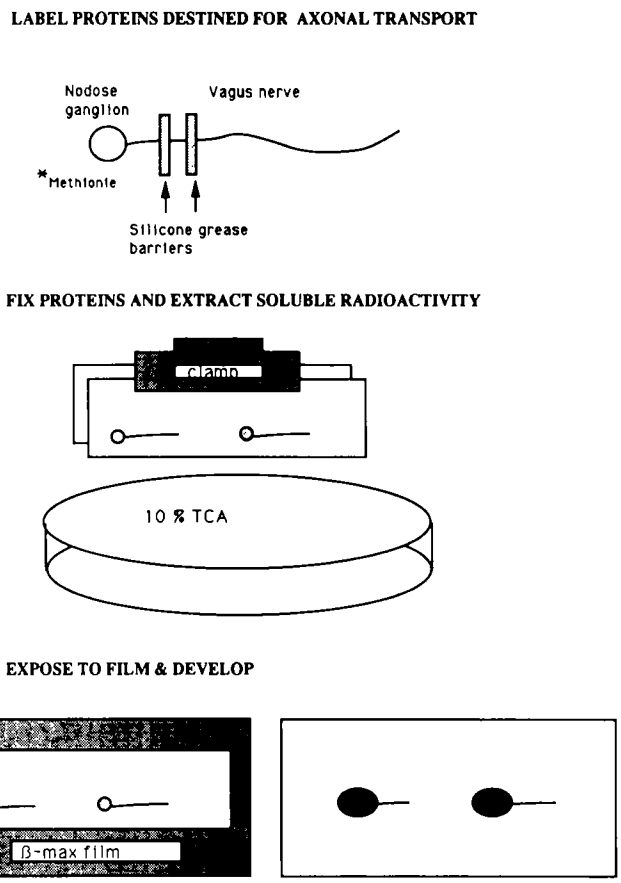

Fig. 3. Schematic representation of the methods used to study axonal transport and regeneration in the vagus nerve. For details, *see* Experimental Techniques.

preparation is then cultured on the nitrocellulose paper. Regeneration distances are obtained by measuring how far distal to the crush lesion axonally transported radioactive proteins can be detected.

Results and Discussion

Survival

An initial assumption (that the vagus nerve should be thin enough to survive on oxygen dissolved in the tissue culture medium) proved correct. Cultured preparations retained their excitability (Fig. 4) during at least 7 d in culture. The compound action potential decreased in amplitude during culturing partly because of degeneration of motor fibers that are severed from their cell bodies at the time of dissection. The remaining activity could be ascribed to sensory fibers, since it vanished within a few days if the nodose ganglion was severed from the nerve (Kanje, 1991). Ortho- and retrograde transport of ^{35}[S]-methionine-labeled proteins could also be demonstrated in preparations cultured for a week.

Regeneration

At this point, the possibility that regeneration could be induced in the cultured preparation was tested. To this end, a crush lesion was made on the nerve. Several days later, the nodose ganglion was labeled with ^{35}[S]-methionine. Recovery of labeled axonally transported proteins distal

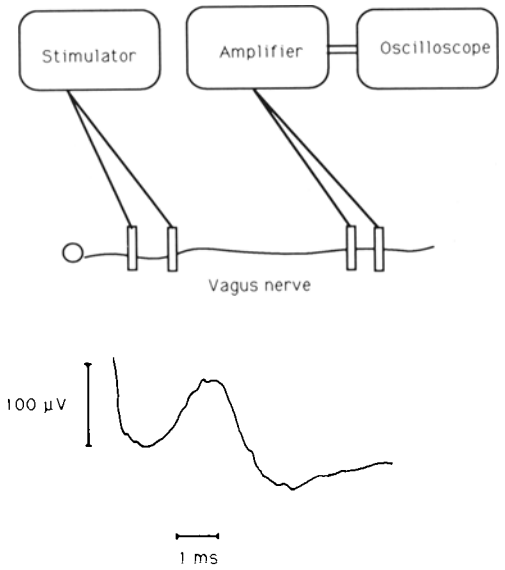

Fig. 4. Compound action potential. Experimental setup and record from a vagus nerve kept for 4 d in culture.

to the crush lesion should signify regeneration. Indeed, axonally transported ^{35}[S]proteins could be detected distal to the crush lesion, and regeneration proceeded at a rate of 1.4 mm/d.

It was also possible to condition the nerve, i.e., increase its regenerative potential, by a conditioning lesion. If a crush lesion was made on nerves cultured for 2 d (conditioning interval), the regeneration distance, measured after an additional 2 d, was nearly doubled as compared to nerves subjected to a single crush lesion at the time of dissection.

Next, the possibility that the nerve could withstand prolonged culturing under conditions where the nodose ganglion was separated from the nerve proper by a silicone grease barrier was tested. If so, it should be possible to expose separately the ganglion or the nerve to drugs. Fortunately, such barriers did not affect the survival of the preparation, as revealed by measurements of the compound action potential or axonal transport.

The Trigger Hypothesis

The preparation was subsequently used to test some aspects of the trigger hypothesis (Fig. 1). The first question asked was if locally synthesized

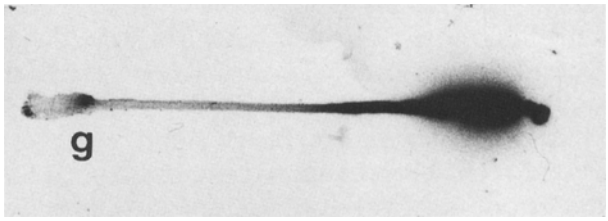

Fig. 5. Autoradiogram of a vagus nerve containing ^{35}[S]-labeled retrogradely transported proteins. The label was administered to a distal nerve segment. Note accumulation of label in nodose ganglion (g).

proteins in a distal nerve segment could be picked up by the sensory axons and transported to the cell body. The answer is yes. Proteins produced and labeled in a distal nerve segment were found to accumulate in the nodose ganglion (Fig 5). This accumulation was prevented if cycloheximide—an inhibitor of protein synthesis—was added distally, or if vinblastine was added or a crush lesion was made between the distal site of synthesis and the nodose ganglion. To the best of my knowledge, this is the first demonstration that proteins synthesized in nonneuronal cells are excreted, taken up by the nerve, and transferred to the cell bodies by retrograde axonal transport. It can be argued that even if proteins synthesized around a lesion can be recovered in the nerve cell body, this has no bearing on triggering of the cell body reaction. However, if such proteins are important, it should be possible to block regeneration by inhibiting protein synthesis around the lesion. Therefore, testing was performed to determine whether treatment of the lesioned area with cycloheximide could block regeneration. Indeed, this treatment severely impaired outgrowth of sensory fibers (Kanje, 1991), lending some support to the idea that initiation of the regeneration process depends on protein synthesis in the lesioned area. Further support for this idea stems from experiments with the cultured frog sciatic nerve in which regeneration also is impeded by local treatment with cycloheximide, but only when the drug is added at the time when the crush lesion is made, and not if it is added when regen-

eration has started (Edström et al., 1990). Interestingly, in vivo perfusion of the rat sciatic nerve with inhibitors of protein and RNA synthesis around a crush lesion also inhibits outgrowth of nerve fibers (Kanje et al., 1988).

Regeneration can also be triggered by the loss of target contact and a subsequent decrease in the amount of target-derived factors that reach the soma. Although this possibility is not supported by the cycloheximide experiments, it has been suggested that a regeneration-related protein—GAP-43—could be upregulated by such a mechanism (Woolf et al., 1990). This appears, however, not to be the case for ornithine decarboxylase, which increases in frog dorsal root ganglia following sciatic nerve injury (Kanje et al., 1981).

Preliminary attempts to characterize a putative trigger by separating the retrogradely transported proteins on SDS polyacrylamide electrophoresis show that there are several candidates, including those with a mol wt of NGF and apolipoprotein E. It still remains to be elucidated if any of these proteins can initiate a "regenerative response" in the soma similar to that observed after administration of the suggested trigger candidate, insulin-like growth factor 1 (IGF-1) (Kanje et al., 1991).

If time aspects are considered in the vagus experiments, it follows from the hypothesis that a trigger protein first must be synthesized in the nonneuronal cells and then transferred to the cell body before it can exert its action. Based on current estimates of the retrograde transport rate, it would take about an hour plus the time required for synthesis until the signal reaches the nodose ganglion. Consequently, newly synthesized and rapidly transported regeneration-related proteins cannot reach the growth cones until at least 2 h have elapsed since the crush lesion. Interestingly, GAP-43 synthesis increases in excised dorsal root ganglia within this time frame, whereas induction in vivo after a crush lesion is much slower (Woolf et al., 1990).

The question arises as to the cellular origin of the trigger proteins, or as they are tentatively called, the *regenerins*. It is tempting to suggest that they are produced in Schwann cells because of the close association of these cells with the axons. Other candidates are various sheath cells or macrophages. The latter cell type is particularly interesting, since it releases interleukin-1, which stimulates synthesis of NGF in Schwann cells (Heumann et al.,1987; Lindholm et al., 1987). However, macrophages, at least those derived from circulating monocytes, are probably not required for initiation of the regeneration process in the cultured frog sciatic or rat vagus nerve, since both preparations should be deficient in cells derived from the circulation.

Summary

In this article, the techniques used to culture the adult vagus nerve from rats are described. Regeneration can be induced in this preparation, which lends itself to selective pharmacological manipulations of the nodose ganglion or an isolated segment of the vagus nerve proper. Some experimental findings in this preparation are reviewed and a hypothesis in which it is suggested that proteins synthesized at a lesion triggers the cell body reaction is presented.

Acknowledgments

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