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6. AUTHOR(S)
Damien Kuffler

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Institute of Neurobiology
Univ. of Puerto Rico
201 Blvd. del Valle
San Juan, PR 00901

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9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
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13. ABSTRACT (Maximum 200 words)

ABSTRACT

The broad aim of the work carried out under the support of this grant was to examine the factors involved in promoting and directing axon regeneration. How do injured nerves find and recognize their targets and reestablish the correct types of synaptic contacts with them? Cultures have been established of adult sensory and motor neurons, as well as intact muscle fibers. These cells survive for weeks *in vitro* and the neurons extend processes. In electrophysiological experiments we have characterized several membrane properties of the neurons. The peripheral nerve tube, as well as isolated intact muscle fibers, have been shown to release trophic factors that promote and modify process outgrowth from the sensory neurons, and one of the trophic factors is being characterized. An extracellular matrix molecule that independently also promotes process outgrowth has been isolated and characterized. A novel role of macrophages, as directors of process growth, has been shown. Preliminary experiments demonstrate that factors released from peripheral nerve can direct process outgrowth by means of diffusible concentration gradients.

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Forward

The principal aims of the experiments supported by this grant are to determine what factors are responsible for promoting and directing the outgrowth of regenerating axons towards their targets and how, once the axons have reached their targets they can be recognized. The answers to these questions are important to understanding how neurons recognize and interact with their targets and associated cells by providing synaptic input and by giving and receiving nurture or support during to periods of physiological activity, as well as information on the status of their normal or patho- physiological states.

INTRODUCTION

In vivo it has been demonstrated that muscle fibers and pieces of peripheral nerve release diffusible factors that direct process outgrowth. As a result of these factors the axons then grow towards their targets and upon contacting them establish characteristic interactions. The Schwann cells of the nerve tube myelinate the regenerating axons and the tropic factor is no longer released from the nerve tube. The axons reaching the muscle fibers recognized them and establish functional synapses, which results in the muscle fibers stopping their production of the tropic factor. The experiments carried out during the present grant period were principally aimed at understanding the molecular mechanisms that underlie this directed growth and attempting to isolate and characterize the responsible factors.

To examine these questions it was necessary to establish the phenomena of promoted and directed process outgrowth *in vitro.* This first required the establishment of cultures of adult sensory and motor neurons, cells of the peripheral nerve tube and muscle fiber. Only then could the neurons and target cells be co-cultured to look for trophic and tropic interactions.

To know whether the cultured neurons were in a physiologically healthy state, experiments were carried out to examine their membrane properties, to determine what receptors and types of channels they possess. The results of these experiments could then be compared with those on acutely removed intact ganglia and spinal cord sections.

One difficulty in looking for tropic interactions is that to get a tropic influence a concentration gradient of the diffusible molecules must be established. This necessitated the development of a system in which such gradients could not only be established but the steepness of the gradients could be controlled. This has required the testing a variety of approaches.

Our present experiments have focused on *in vitro* approaches to address these questions. However results from some of these experiments will now allow us to return to the *in vivo* preparation where we can attempt to determine whether the results obtained *in vitro* have physiological implications *in vivo.*

SUMMARY OF MAJOR RESULTS

(1) Establishment of cultures of adult dorsal root ganglion neurons.

Adult dorsal root ganglion neurons can now be reliably isolated, free of associated cells and debris. These neurons are plated individually in low density cultures using a mouth sucker pipette. This allows the neurons to be separated from one another and their outgrowing processes and their branches be observed without overlap with those from other neurons. These neurons survive for more than 4 week and extend processes (Kuffler and Megwinoff, 1994).

(2) Improvement of conditions for maintaining adult motoneurons in culture.

Although we have been successful in culturing adult motoncurons, methods have been improved that now allow us to isolate and maintain *in vitro* a population of large motor neurons for which this was not previously possible. These neurons survive well in a defined medium and rapidly extend long processes. These neurons do not require exogenous neurotrophic factors for their survival or to promote process outgrowth, however, they respond to neurotrophins by increasing process outgrowth (Broesamle, 1994).

(3) Study of the membrane properties of cultured dorsal root ganglion and motor neurons.

Initial experiments have been carried out to examine the properties of the dorsal root ganglion neurons. Using the patch clamp technique, the neurons were found to be sensitive to GABA as expected from other studies with DGR neurons from other species. However, these $GABA_A$ receptors are insensitive to extracellular acidification. These findings are in contrast to others that show the same pH reductions markedly reduce the currents resulting from glutamate activation of receptors on central neurons. These findings suggest that acidification plays a protective role in preventing excessive excitation, not only by decreasing glutamate responses, but also by leaving the inhibitory $GABA_A$ response intact (Vyklicky *et al.*, 1993).

These studies have also shown that several populations of neurons can be identified by their electrophysiological properties and these properties can be easily correlated with their morphological appearance. Small and intermediate sized neurons, which appear dark and are seen to contain granules when observed under bright field illumination, are sensitive to 5-HT. 5-HT induced a rapidly desensitizing inward current with a reversal at 0 membrane potential. This sensitivity was not observed in the large pale neurons which do not contain granules. In contrast, the large pale neurons showed fast inactivating inward currents induced by a rapid decrease in pH which was not seen in the population of smaller neurons

which contained granules. These results allow us to speculate that the 5-HT_3 receptors and channels which generate fast inactivating proton induced currents are expressed independently in DRG neurons which may represent distinct nociceptors. Also, both 5-HT and low extracellular pH indiscriminately inhibit high threshold voltage dependent calcium currents (Philippi *et al.*, 1994).

Experiments are now under way to characterize the receptors on the cultured motoneurons. The results of these experiments so far show that these neurons have NMDA receptors.

(4) Do muscle fibers and denervated nerve pathways release diffusible factors that promote neuron survival and process outgrowth *in vitro*?

Release of neurotrophic factor/s from denervated nerve tube and muscle fibers.
The cells of the peripheral nerve contains mRNAs for several neurotrophic factors: nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and ciliary neurotrophic factor (CNTF). However, the physiological role of these neurotrophins when released from the cells of the nerve tube and their influence in promoting and directing process outgrowth from cultured neurons needs to be further examined.

We have found that pieces of denervated peripheral nerve do release neurotrophic factor/s that significantly increase process outgrowth (ca. 10 fold) from sensory neurons (Kuffler and Megwinoff, 1994). Although not fully characterized, the factor is a protein with a molecular mass between 100 and 300 kD, is functionally and immunologically related to nerve growth factor and influences process outgrowth from sensory neurons other species (Dobretsov, Dobretsov and Kuffler, 1994). Release of the factor is upregulated following nerve section and reaches a maximum, and apparent plateau of release, by 7 days and it is released for more than 4 months. Experiments to provide further characterization of the factor are being carried out.

Muscle fiber - neuron co-cultures.
Sensory neurons have been co-cultured with intact adult skeletal muscle fibers. These neurons extend processes that are two times longer than those of control neurons. In addition, factors released from the muscle fibers influences both the number and morphology of the processes extended. These observations are not as dramatic, but parallel those observed for the modification of process number and morphology induced by neurotrophic factors on dorsal root ganglion neurons.

Although processes of the neurons have been seen to grow over the membrane of the muscle fibers, no synapse has yet been observed. These results indicate that although the muscle fibers release neurotrophic factors that influence process outgrowth, some membrane factors are missing for triggering synapse formation.

Motor neuron-muscle fiber co-cultures have been started. A neurotrophic influence of these fibers on the neurons has been seen and is presently being analyzed.

(5) Can extracellular matrix molecules be isolated that promote process outgrowth and trigger synapse formation?

The cells of the nerve tube have been shown to release a unique form of the extracellular matrix molecule (ECM) laminin in the their culture medium (Kuffler and Luethi, 1992). This laminin induces a significant increase in process length when used as a substrate on which to plate the neurons. Although laminin does promote process outgrowth, it is not the primary molecule released from the cells of the peripheral nerve tube that promoted process outgrowth. This can be ruled out by the finding that the neurotrophic influence has a maximum molecular mass of 300 kD where as the laminin molecule has a molecular mass of ca. 1000 kD.

The frog laminin has been isolated and characterized (Kuffler and Luethi, 1992). Several polyclonal antibodies have been generated against both the alpha and beta chains of the frog skeletal muscle laminin. These antibodies have been used at the light microscopic level to demonstrate that laminin is present in the ECM of both innervated nerve and muscle. Further, the synthesis of this laminin is rapidly and significantly up-regulated in both nerve and muscle following nerve section. A high resolution electron microscopic examination of the distribution of the laminin has now been initiated.

(6) Develop long-term cultures of intact adult muscle fibers.

Intact muscle fibers of the lumbricalis can now be isolated and maintained *in vitro* for up to 5 months (Broesamle, 1994). During this time they retain their ability to contract when electrically stimulated. They also maintain a normal morphology as seen in the electron microscope. Muscle fibers have been labeled with alpha-bungarotoxin at the time of isolation. The original synaptic sites on these fibers can be localized by the presence of labelled alpha-bungarotoxin for up to 4 weeks *in vitro*, in spite of having their extracellular matrix removed by the dissociation technique. Thus, the original synaptic acetylcholine receptors do not totally disperse following muscle fiber isolation, as has been reported for other preparations. Using the pulse-chase technique with alpha-bungarotoxin labelled with two different labels, we are presently examining how long the original acetylcholine receptors remain at the old synaptic sites and whether receptor turnover and the insertion of new receptors continues in the absence of the nerve terminal and the molecule agrin that is normally present in the synaptic extracellular matrix. These results suggest agrin is not essential for the continued insertion of new receptors into the synaptic site and that other molecules, such as those of the cytoskeleton, are important in the maintenance and turnover of the receptors.

(7) Do macrophages interact with motoneurons?

Macrophages, whose principal role has generally been considered to be phagocytic, have been co-cultured with adult motoneurons. Interactions have been observed between migrating macrophages and motoneuron growth cones. As a result of these interactions, as the macrophage continues to migrate, the growth cone and its related process elongate in association with the macrophage. Such interactions lead to directed process outgrowth over distances of hundreds of microns. As a result of these interactions the macrophages can bring about the establishment of networks of motoneurons. Experiments are planned to examine the nature of these interactions.

(8) Can process outgrowth can be directed *in vitro*?

Novel tissue culture chambers have been designed that allow the establishment and maintenance of long-term and long-distance gradients of diffusible factors (Dobretsov and Kuffler). These dishes are being used to test whether factors released from the cells of the nerve tube can direct process outgrowth as is seen *in vivo*. One critical point in these experiments is, if there is directed outgrowth, does it result from diffusible or substrate bound molecular gradients?

Using a totally different approach to address this question, medium, containing the nerve released neurotrophic factor, is being pipetted onto growth cones of DRG neurons. The concept of this approach is to establish local concentration gradients in the region of a single growth cone to determine whether they respond to them. This technique is slow because it allows us to only test the influence of a factor on a single growth cone at a time, as opposed to the above discussed method. However it should allow us greater control of the steepness of the concentration gradients established.

In initial experiments, growth cones have been seen to turn and grow up the concentration gradient of the nerve tube released factor. Although these experiments are in their early stages they indicate that factors released by the cells of the nerve tube can both promote, as well as direct, the outgrowth of elongating processes.

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List of participating scientific personnel

Undergraduates Students

Yari Vale	Diana Balsalobre	Daniel Hernandez
Robert Lewis	Brenda Ramos	Rene Paez
Olga Megwinoff	Nivia Perez	Armando Oliver
Josef Galib	Grace Berrios	

Graduate Students
Christian Broesamle
Will receive his masters degree in March, 1994.

Medical Student
Rene Baez

Postdoctoral Fellows
Jesus Jose Garcia Colunga
Maxim Dobretsov

Research Associates
Anna Dobretsov

Visiting Scientists
Ladislav Vyklicky
Serge Skachkov