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CHEMORECEPTOR FUNCTION AND REGULATION IN CULTURED
VERTEBRATE NEURONS(U) CONNECTICUT UNIV STORRS

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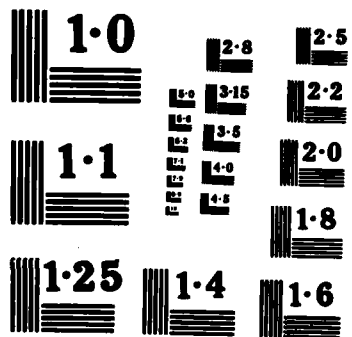
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of regulatory interaction may have broad significance to neuronal dynamics. The results to date pave the way to an understanding of receptor regulation at the molecular level.

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Final Technical Report
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FINAL REPORT
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MANUSCRIPTS IN PREPARATION:

Tuttle, J.B., Pilar, G., Lucas-Lenard, J., and Greene, C. Partial purification of Neuronal Survival Protein. (in preparation).

Tuttle, J.B. and G. Pilar Neuronal chemosensitivity in vivo following axotomy. (in preparation).

Tuttle, J.B. and Pilar, G. Mechanisms of parasympathetic neuronal chemosensitivity. Biochemical Pharmacology (in preparation).

Gray, D.B. and Tuttle, J.B. [3]ACh synthesis in cultured ciliary ganglion neurons: effects of myotube membranes (in preparation).

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Gray, D.B. and Tuttle, J.B. Target influences upon terminal morphology and ACh synthesis in cultured chick ciliary ganglion neurons. Soc. for Neuroscience 14th Ann. Meeting, 1984.

Tuttle, J.B. and Gray, D.B. Activity-dependent regulation of ACh synthesis in cultured ciliary ganglion. Soc. for Neuroscience, 15th Ann. Meeting, 1985.

SCIENTIFIC PERSONNEL and DEGREES AWARDED:

- 1) Ramon Nunez, M.D. Postdoctoral Fellow (Terminated after 1 month)
- 2) Eugene Casal, M.S. Technician
- 3) Christopher Greene, M.S. Awarded (Graduate student - left to attend medical school.)
- 4) D. Bruce Gray, M.S. Graduate student (Ph.D. expected July, 1985)
- 5) Michael May, B.S. Graduate assistant (research) M.S. awarded 1984.

- 6) Geraldine Crean, Ph.D. awarded June, 1983.
- 7) Zvi Goldman, M.S. Graduate assistant.
- 8) Masaharu Ogawa, Ph.D. Associate Professor (visiting fellow)
- 9) Guillermo Pilar, M.D. Professor
- 10) Jeremy B. Tuttle, Ph.D. Principal Investigator

SUMMARY of RESEARCH PERFORMED

NOTE: Many of the results gained are discussed in more detail in the semi-annual reports of progress submitted during the contract period and in the reports and abstracts published in scientific journals. This material will thus not be discussed in definitive detail in this report, and an interested reader is referred to these other sources (vide infra).

Questions Asked and Answers Gained

The central issue addressed by this body of research concerns the mode and details of the regulation of chemosensitivity by neurons. Rather than approach this issue in the daunting complexity of the nervous system of a whole organism, a reductionist path was chosen, utilizing a simplified model system of cultured vertebrate neurons. Isolated neurons in a well-defined system such as the ciliary ganglion in cell culture behave much the same as their counterparts in vivo, yet offer accessibility and experimental control. Chemosensitivity, the ability to respond in some detectable way to the presence of transmitter and perhaps to dynamic changes in transmitter amounts, is central to the normal function of the nervous system. Both the signalling functions of the nervous system and the complex interdependency shown by neurons with their synaptic partners seem to have a root dependence upon neuronal chemosensitivity. However, there was and is a paucity of information to aid our understanding of the cellular mechanisms for the regulation of neuronal sensitivity to transmitter compounds. In addition, the range and extent of modulation of chemosensitivity in response to perturbations is ill-defined and the basic repertoire of possible regulatory events shown by even one model neuron in a simplified system had not been explored. The research performed during the contract period represents several steps in the direction of understanding the cues neurons use to regulate chemosensitivity, the selectivity of transmitter receptor regulation, and preliminary work on the mechanism of

interactive contact-mediated regulation. In addition, the interactive regulation of the production of transmitter, a capability regulated by some of the same cues, was also examined.

SUMMARY of RESULTS:

Contact-Mediated Regulation of Neuronal Chemosensitivity

Avian ciliary ganglion neurons in culture form synaptic structures upon the cell bodies of other neurons in the culture (Crean et al, 1982), yet these synapses are non-transmitting when the neurons are cultured alone. The basis for this transmission failure is not due to a neuronal inability to synthesize or release acetylcholine (ACh) (Tuttle et al, 1983). Rather, the neurons in culture lose effective levels of sensitivity to the transmitter ACh (Crean et al, 1982). While this phenomenon is interesting in and of itself, it was further found that the neurons retained physiologically significant levels of ACh sensitivity if placed into co-culture with striated muscle, a normal target for the neurons and one upon which functioning neuromuscular junctions are formed. (Crean et al, 1982)

This situation was capitalized upon due to its potentially fundamental importance to an understanding of the process whereby neurons regulate the presence of appropriate chemoreceptors within the neuronal membrane. It should be recalled that the culture conditions used for the above referenced studies were such that ALL of the neurons plated survived, and levels of soluble trophic proteins in the culture medium were above saturation for growth and enzyme induction (Tuttle et al, 1980; Pilar and Tuttle, 1982). It was thus reasonable to ask if the co-culture with muscle target, resulting in retention of chemosensitivity, was providing a soluble factor to the neurons, the sustaining influence of synaptic activity, or if the event was mediated by membrane contact. Cultures of neurons were thus prepared with a substrate of lysed and washed muscle membrane remnants, and the neurons tested for retention or loss of chemosensitivity by iontophoresis of ACh during intracellular recording. The empty, washed membranes of muscle were nearly as effective as live muscle in sustaining neuronal chemosensitivity (Tuttle, 1983). Fibroblast membranes were ineffective, as were medium "conditioned" by muscle or fibroblasts, or their membrane remnants. These results strongly indicate that the neurons retain chemosensitivity as the result of direct contact with an adequate target membrane. Soluble trophic proteins and synaptic activity are not involved.

The next questions addressed in this regard relates to the mechanism of the interaction between the neuron and the muscle

membrane. First, cultures were prepared as before, and individual neurons under the three conditions (neurons alone, neurons on live myotubes, neurons on myotube membranes) were intracellularly injected with Lucifer Yellow CH. This brilliantly fluorescent dye diffuses to fill the entire extent of the neuron, revealing the structure of fibers and contacts. This revealed that the neurons on both the live muscle and the muscle membranes formed large terminal expansions at points of contact that were not found in the cultures of neurons alone (Gray and Tuttle, in preparation). Cultures were also prepared for viewing under scanning electron microscopy, with similar results at higher resolution. Thus it appeared that a specific interaction between the neuronal and muscle membrane occurred that lead to the retention of chemosensitivity and the formation of terminal expansions. These may have relevance to the regulation of ACh synthesis by the neurons (see below).

Myotube membranes were extracted with several treatments to reveal if a particular element in the membrane responsible for the retention of neuronal chemosensitivity was easily removed. High salt buffer and Triton X 100 (non-ionic detergent) did not remove the ability of the membranes to support chemosensitivity. However, a mild treatment with an ionic detergent (deoxycholate) was effective (Tuttle, in preparation). Triton X 100 in the concentration used (0.05% at 25 C) is known to solubilize all membrane proteins that are not anchored to the cellular cytoskeleton, nucleus, extracellular matrix or some other fibrous structural element of the cell. Thus the ineffectiveness of this treatment in removing the activity from the muscle membranes suggests that active agent is a transmembrane glycoprotein or glycosaminoglycan that is anchored to the muscle cytoskeleton. Furthermore, it suggests that some element in the neuronal membrane is capable of interacting with this muscle component and influencing the neuronal receptor dynamics. This sequence has broad parallel in the presumed sequence of events that serve to regulate the segregation of membrane proteins in epithelia. In addition, the ACh receptor in muscle has been found to be linked to the muscle F-actin cytoskeleton via a 43kD peripheral protein. Stripping of this 43kD protein from the ACh receptor in muscle does not affect its function in terms of agonist-mediated ionic flux, but does cause the receptor to become susceptible to degradative attack. Thus, a pilot experimental series was initiated (after the project activity moved to The University of Virginia School of Medicine) aimed at testing the hypothesis that the muscle membrane element was binding to the neuronal surface and causing receptor retention via neuronal cytoskeletal stabilization.

The initial stages involved developing a positive assay for the active agent in the muscle membrane. This was effected by

extracting the washed membranes with detergent, dialyzing the extract to remove the detergent, and then cross-linking the proteins to an artificial culture substrate and testing the effectiveness of the molecule(s) on ACh receptor retention. The muscle membrane extract remained active, and all of the more than 50 neurons tested by ACh application during intracellular recording responded to the transmitter, while the controls rapidly lost all responsiveness. This sequence demonstrates the feasibility of a purification attempt, and the feasibility of studying the mechanism involved. The pilot tests of the hypothesis have been very promising. Neurons were cultured on the active (extract-linked) substrate and on normal collagen surfaces, then fixed, permeabilized to allow penetration of protein markers, and incubated in antibody against bovine brain actin. After washing, a secondary antibody conjugated with flourescine was bound to the specific probe, and thus the neuronal pattern of actin distribution visualized. The cultured neurons retaining chemosensitivity had a bright cortical pattern of actin staining that the controls (having lost ACh receptors) lacked. These experiments, when continued with a probe specific for polymerized F-actin (MDB phalloidin) should prove that the loss of ACh sensitivity is associated with a disruption of the neuronal cytoskeleton, while the retention of chemosensitivity is signalled by a retention of neuronal cytoskeletal organization. Purification of the muscle membrane molecule that allows this retention of functional status may be a way to further examine the mechanism of its action.

Non-Cholinergic Chemosensitivity

The examination of the regulation of chemosensitivity in cultured ciliary ganglion neurons was extended to include other receptors. The neurons proved to be sensitive to GABA (gamma-amino butyric acid), glycine, leu-enkephalin and to show a slow (minutes) hyperpolarizing reponse to ACh quite distinct from the rapid excitatory ganglionic-nicotinic receptor's response. The neurons were insensitive to glutamate and norepinephrine, but extremely sensitive to small (0.5 pH units) changes in pH. The latter effect is mediated by a hydrogen ion-sensitive potassium conductance such that pH less than 6.5 depolarizes the neurons while pH greater than 7.8 hyperpolarizes the transmembrane potential. The significance of this peculiar K-conductance is not apparent.

The question pursued most vigorously was that of whether the loss of ACh sensitivity via the nicotinic receptor was accompanied by the loss of or change in any other transmitter sensitivities. The results proved that these receptors are regulated independently and most likely via different mechanisms. There was no alteration in any other sensitivity during the total

loss of all nicotinic ACh response. Thus, the loss of chemosensitivity was specific, and the effect of the muscle membrane on the receptor also specific. Preliminary experiments, not pursued during the contract period, suggested that the responses to the opioid peptide, leu-enkephalin, were also lost at a later time in culture (2 weeks) in the absence of muscle, but more work will be needed to confirm this.

Acetylcholine Synthesis

Parallel to the studies summarized above, the interactive influences on neuronal transmitter metabolism were also examined. A biochemical index of synthetic ACh metabolic capacity was developed and used to reveal the extragenomic cues leading to increased transmitter output and synaptic reliability (Pilar et al, 1981). Co-culture of the neurons with live muscle had a pronounced stimulatory effect upon transmitter content, synthesis, synthetic enzyme levels and release (Tuttle et al, 1983) by the neurons. This was subsequently shown to be a quantitative effect, not qualitative, expressed as an acceleration of development beyond that seen in the organism (Tuttle, 1985). Muscle membrane remnants, prepared as in the chemosensitivity studies above, had a partial effect upon the acquisition of synthetic capacity (Gray and Tuttle, in preparation). The remainder of the effect of live muscle in this interaction is accounted for by an influence of synaptic or electrical activity (Tuttle and Gray, 1985). These results demonstrate that cues similar to those regulating chemosensitivity also influence other neuronal parameters of function, as might be reasonably expected.

Pilot Experiments - New Questions and New Directions

In addition to the experimental series summarized above, several series of pilot projects were carried out as part of the normal course of the research and to identify "targets of opportunity". None of these were taken to a reasonable stage of completion during the project period, due to limitations on time, technical difficulties and due to the move to the new location. Many will be continued in the future. Because in total these efforts represented a significant outlay of time and project effort, it is proper that some mention of these be included.

Myoneural specificity - culture techniques were devised for striated muscle from three separate sources in the embryo, and co-cultures with neurons tested for short- and long-term neuromuscular junctional activity. The results suggested that the ciliary ganglion neuron in culture was able to distinguish between the differing muscle targets. The physiological sequence of this process will be described by future work, and perhaps the molecular basis of the variation in interaction examined.

Isolated adult neuron preparation - methods were devised to isolate ciliary ganglion neurons from hatched birds, and enable direct intracellular recording. This was part of an effort to test the effects of axotomy on chemosensitivity in vivo, and to develop the in vivo isolated neuron for patch-clamp recording. Sadly, once the techniques were developed, the effort had to be suspended due to a loss of funding in the laboratory with which collaboration was occurring.

Neurotransmitter release by cell soma - an experimental paradigm was developed that allowed investigation of somal release of ACh. Somal release is of interest in connection with the vesicular hypothesis of quantal release in that the soma does not possess aggregations of vesicles as seen at the usual junction, yet ACh release can be detected biochemically (Johnson and Pilar, 1980). Cultured myotubes were used as physiological detectors of release by freshly isolated ciliary ganglion neurons (see also above). Evidence of somal release was obtained during stimulation of the freshly plated neuron by direct intracellular current. However, technical problems prevented a rigorous determination of whether the release was quantal or non-quantal, and the effort was suspended until these could be solved.

Two-electrode voltage clamp - two electrodes were used for simultaneous recording from a single ganglion neuron in order to determine the feasibility of using a double electrode voltage clamp. While possible, this method requires a technical tour de force, and using it would have required the construction or purchase of clamp circuitry. This was not possible in the previous location.

SUMMARY

In sum, the research conducted has raised more questions than it has settled. Several potentially interesting phenomena have been described and investigated, and these results have generated much interest in the neuroscience community. The generality of the phenomena and the principles of regulatory physiology implied await further work for confirmation. Thus, the results serve to reinforce the supposition that a well-defined in vitro model system is an ideal object for study of the regulation of chemosensitivity by vertebrate neurons, and of related neurobiological topics. To date, the conclusions to be drawn highlight our lack of knowledge of the subject of transmitter receptor regulation, yet hold promise that an eventual understanding in molecular terms is within view and within our grasp.

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