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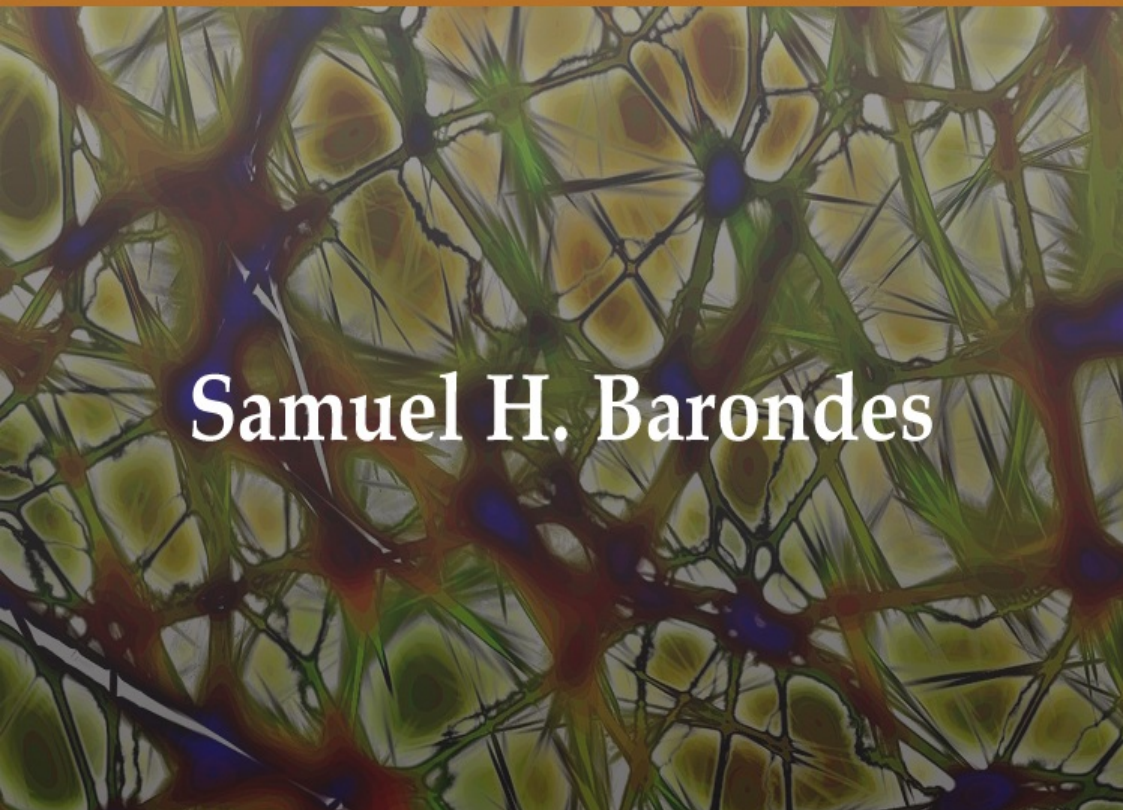
Establishing and Modifying Neuronal Interactions:

Some Experimental Approaches



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ESTABLISHING AND MODIFYING NEURONAL INTERACTIONS: SOME EXPERIMENTAL APPROACHES[1]

Advances in biology in the past decade have greatly clarified the mechanism of regulation of the metabolism of individual cells. This in turn has set the stage for analysis of cellular sociology—how certain cells come to associate with others and how neighbors regulate each other. Solution to these problems is particularly important for an understanding of the most societal of organs, the nervous system. The goal of this essay is to provide the reader with a sampling of several selected aspects of this work.

There are two areas of investigation which I will discuss briefly: (1) studies concerned with various aspects of the genetically determined "wiring diagram of the nervous system"; (2) studies concerned with establishment of new functional interneuronal relationships with learning. Since only a superficial discussion can be given here, it is hoped that the bibliography will be consulted. All that I will to do is to present work in several areas that is not usually called to the attention of psychiatrists. I will also emphasize the usefulness of various lower organisms for studies of this type. The least this will do is to remind us again of our humble origins.

Genetic Control of Nervous System Structure and Behavior

There is an obvious and striking constancy in the behavior of all

members of a species and in the gross structure of their brains. Both reflect the genetically directed neuronal wiring diagram. Recent studies have been concerned with analysis of (1) the degree of precision of the connections between one nerve cell and another, and (2) the magnitude of the genetic change required to produce an observable change in behavior.

Work on these problems has been largely confined to organisms whose life history or nervous system structure makes them relatively easy to study. Although we will ultimately want to know the answers to these questions as they relate to man, favorable biological preparations are a prerequisite to any serious investigation. The following will provide some examples of what is being done.

Constancy in Structure of an Identified Neuron

Through extensive neurophysiological and behavioral studies it has been possible to identify the specific and often unique function of a number of identified neurons in the nervous systems of several marine organisms. Favorites for study are crustacea and mollusks. In both these groups of organisms there are a number of discrete ganglia, each of which contain relatively small numbers of nerve cells that can be identified in the living state. Identification is made both by the appearance of the neurons under the dissecting microscope and by characteristic electrophysiological properties

that can be determined after introduction of a microelectrode into the nerve cell body. These ganglia are rich in interneurons and analogous to our central nervous system in that they perform complex integration. One, the stomatogastric ganglion of the lobster, contains only thirty neurons; yet it controls complex movements in the breaking up and transporting of food. A number of interneurons in such systems have been shown to play a critical role in integrating inputs and directing outputs that control specific behavior.

Given the availability of such well-defined preparations, attempts have been made to determine the constancy of the microstructure and connectivity of specific neurons. Analysis of this type has been done by injection of Procion yellow, a fluorescent dye, into the soma of the same nerve cell in a number of specimens. The dye spreads through the axons and dendrites of the cell and permits direct visualization of the three dimensional structure of its branches. These studies showed that a specific neuron has very similar microstructure from animal to animal, which reflects its synaptic connections with other neurons. Such anatomical constancy demonstrates the relationship between microstructure of a specific neuron and its participation in the regulation of a specific behavior.

Higher resolution studies of structural variability of an identified neuron have recently been initiated, using the small crustacean, *Daphnia.* This organism was chosen since it can reproduce parthenogenetically. It is thus

possible to study many animals that are genetically identical. Analysis was done by reconstructing the precise structure of the branches of an identified neuron after electron microscopy of serial sections. Although there were slight variations in the precise morphology of the terminal ramifications, a striking constancy in the branching of a single identified nerve cell was found. This is further evidence of the degree of genetic control of an identified neuron s precise structure.

Behavioral Genetics in Drosophila

The types of studies I have just mentioned seek to analyze organisms that are genetically identical, although they may ultimately be applied to analyzing the effects of genes on specific neurons. In contrast, genetic analysis has been concerned with the question: how small a genetic change can produce a grossly observable change in behavior? Whereas it is well known that different inbred strains of mammals may differ strikingly in their behavior, it is not known whether or not these strains have extensive genetic differences. A more precise analysis of the genetics of behavior is presently being conducted in fruit flies. These organisms are a favorite of geneticists because they reproduce quickly and have many progeny. In addition, there is a vast technology that has been developed for analysis of genetic changes in this species. There are maps and markers that allow one to precisely determine where the genetic change is and how many genes might be

affected.

In recent years an extensive series of behavioral mutants of *Drosophila* has been identified and investigated. Some, with abnormalities in locomotion, have been named “sluggish” or “hyperkinetic.” Some have an abnormal response to a perturbation. For example, the mutant called “easily shocked” responds to a mechanical jolt with something like a seizure. Still others exhibit abnormalities in courtship such as the male “savoir-faire” mutants.

One striking finding of these studies is that a mutation in a single gene can produce distinct behavioral changes. Although it is not yet known what modification of the nervous system is produced by these mutations, techniques are now being developed so that it may someday be possible to relate the effect of mutation in a single gene to morphological changes in the nervous system that mediate the altered behavior.

Mechanisms for Development of Specific Intercellular Interactions

The studies thus far described have helped to identify the precision of connectivity of identified neurons and the susceptibility of gross behavioral systems to mutation at a single genetic locus. Another line of investigation has been concerned with the developmental mechanisms for establishing specific neuronal relationships. It has made use of embryological or biochemical techniques.

Experimental Embryology of the Retino-Tectal Connections in Amphibia

The retino-tectal connections of the amphibian are a particularly favorable system for studying how developmental forces determine specific interneuronal connections. The organism is a good choice since embryogenesis occurs outside the parent. This simplifies observation and manipulation. As for the system, it readily lends itself to investigation because of the relatively simple geometric arrangement of the retinal ganglion cells. These cells receive information about light from the photoreceptor cells and then transmit it directly to the contralateral optic tectum. There is a precise geometric correspondence between the location of a ganglion cell in the retina and the tectal region where it projects. The system is therefore ideal for experiments designed to determine how this correspondence is established during embryogenesis. Another favorable feature of this system is the ability of transected axons of the retinal ganglion cells to regenerate and reestablish functional synaptic connections with cells in the tectum, even in adulthood.

Sperry was the first to take advantage of the system in an attempt to determine the mechanism for the establishment of retino-tectal connections. First he demonstrated that a transected optic nerve in the adult frog regenerated retino-tectal connections and that normal vision was reestablished. Regeneration also occurred when the eye was rotated 180 degrees after transection. In this case the retinal ganglion cells again

established connections with exactly the same tectal cells they had sought out in embryonic development. Consequently, the animal now had inverted vision in the nasal-temporal and dorsal-ventral directions. If an object was presented to its dorsal visual field, it responded as if the object has been presented to its ventral visual field.

This experiment showed that the retinal ganglion cells connected with the tectal cells, for which they had a specific embryologically determined association. Despite the fact that this was now maladaptive to the organism, these connections persisted and the animal could not learn to respond normally to his visual environment. Clearly then the retinal cells and/ or the tectal cells were immutably specified to make connections with each other.

To determine the mechanism of the specification of retinal and tectal cells, Gaze and Jacobson rotated the primordial eye at one of a number of times during embryogenesis. If rotation was done early in embryogenesis, normal vision was found in the adult. This indicated that the retino-tectal system had not been specified at this stage of embryogenesis. If rotation was done late in embryogenesis, the results were the same as those in the adult. By varying the precise time of rotation, the critical developmental period when “specification” occurred was determined. It was also found that, after rotation at a specific stage in the adult, vision was normal in one dimension but inverted in the perpendicular dimension. This indicated that specification

occurred first in one dimension and then in the other.

These findings have stimulated extensive work and speculation on the mechanisms of specification of cells during embryogenesis. Two sequential processes may be operative. First it is proposed that there are foci within the embryo that release “inductive” substances to which retinal and tectal cells can respond. Cells are presumed to respond as a function of their relative proximity to this inductive source. Second it is proposed that the “inductive” substance specifies the type and number of surface molecules on the retinal and tectal cells that mediate their selective affinities for the surfaces of other cells. Both these notions and many others are considered in two recent books on this subject. Speculations about the nature of putative cell surface molecules that mediate specific intercellular recognition have also been reviewed. The use of other experimental preparations for evaluation of this problem is considered below.

Molecular Bases of Intercellular Recognition

Analysis of this problem has been begun in a variety of systems including the brain. Extensive experimentation indicates that the surfaces of vertebrate cells contain substances that favor association of cells from the same organ as opposed to cells from different organs. For example, retinal cells will self-associate, but they separate from liver cells when the two cell

populations are mixed together. More recently, cell extracts from different areas of the brain have been shown to promote association of cells from these areas as opposed to cells from other areas. Thus far, however, purification and characterization of the molecules involved have proceeded more rapidly in simpler systems. For example, association of male and female yeast is apparently mediated by a cell surface glycoprotein. The role of specific molecules in cellular associations of sponge and slime mold is beginning to be understood. The relative simplicity of the structure of these organisms greatly facilitates this work.

Factor Mediating Association of Sponge Cells

Early in the century it was shown that when two species of sponge (whose cells happened to differ in color) were mixed together, the cells of each species self-associated but rejected association with cells of the other species. These results suggested that there were substances on the surfaces of the two species that mediated the selective association. Evidence for the existence of such substances was provided by the immersion of sponges in sea water from which magnesium and calcium had been removed. It was found that this led to the dissociation of the sponge cells and the appearance of a soluble factor in the medium. When this factor was added to dissociated cells suspended in plain sea water, large aggregates were formed. The factor was species specific in that adding it to its own species promoted aggregation

of that species but not that of other species. The soluble factor has since been purified, although the mechanism whereby it mediates specific cellular aggregation is unclear. Since the factor mediates a species-specific intercellular aggregation, it may prove to be a model for similar substances in higher organisms.

Studies with Slime Mold

The cellular slime mold is a particularly favorable organism for studying cellular association, because it exists in both social and unsocial states. In the presence of abundant food (bacteria) the slime mold cells show no interaction. However, when food is gone, the cells cohere and form a multicellular organism containing a stalk and a spore cap. When the spore caps are disseminated to a bacteria-containing medium, they de-differentiate and become single amoeboid cells again. Because of these properties it is possible to study the development of the factors responsible for cell cohesiveness by inducing this phenomenon through the removal of food. In addition, as with sponge, a number of species of cellular slime mold will, in the absence of food, segregate when mixed together.

The presence of a factor on the surface of slime mold cells that mediates their self-association was demonstrated by making an antibody to slime mold cells. When this antibody was broken down into univalent fragments, it

bound itself to the surface of the slime mold cells and blocked aggregation. More recently a carbohydrate binding protein that could mediate this association has been isolated from slime mold cells. The protein is made only when slime mold cells are deprived of food. It can be assayed by its ability to agglutinate sheep erythrocytes. This reaction occurs because the protein binds to specific sugars on the surface of the red blood cells and acts as a protein bridge between them. The nature of the binding is indicated by the fact that addition of N-acetylgalactosamine, a specific sugar that is a common constituent of cell surface glycoproteins, blocks the erythrocyte agglutination produced by this protein. The protein has been purified by affinity chromotography and its molecular characteristics have been studied. Although it has not been proven that this factor mediates cohesiveness of slime mold cells, the evidence is highly suggestive. Like the sponge factor, it may prove to be a useful model for similar substances in higher organisms.

Memory: Modification of Functional Neuronal Connections

Although the nervous system is wired up under the influence of genetic forces, it retains the capacity for stable modification through the development of memory. The mechanisms whereby functional interneuronal relationships are modified for long-term memory storage are not presently known. As in the cases I have already discussed, it would be ideal if a simple preparation were available for the study of this process.

A relatively simple system that has been intensively investigated is the neuromuscular junction. It has been shown that repetitive stimulation of this junction at high frequency produces an increased sensitivity to subsequent stimulation. This phenomenon, called post-tetanic potentiation, has been known for a number of years and is of considerable interest since the change in synaptic efficacy may last for up to several hours. Studies of changes of synaptic efficacy as a consequence of other forms of stimulation have been conducted in a variety of nervous systems, most notably the abdominal ganglion of the marine mollusk *Aplysia*, which contains a small number of readily identified cells. It remains to be determined what the relationship is between relatively short-lived changes in such identified synapses and those which mediate long-term memory in mammals.

Because of the anatomical complexity of the mammalian brain, it is difficult to analyze modifications of the efficacy of specific synapses with learning. For this reason attempts have been made to design studies that will elucidate aspects of the mechanism of memory storage without a knowledge of the specific synapses involved. For example, it has been possible to test the hypothesis that memory is just another type of long-lasting cellular regulation; and that it is therefore mediated by the synthesis of proteins that specifically facilitate the synapses involved in a specific behavioral event. Critical evaluations of this work have been published.

This approach was made possible by the existence of specific antibiotics that inhibit protein synthesis in the nervous system as well as other cells. Administration of such drugs inhibits cerebral protein synthesis extensively for several hours with no long-term adverse effects for the animal. Since all cells in the nervous system are affected by these drugs, it is possible to examine the relationship of cerebral protein synthesis to memory in mammals without a knowledge of the specific neurons involved in the memory storage process.

In a typical experiment mice are injected with cycloheximide, a potent inhibitor of protein synthesis, and trained half an hour later at which time their cerebral protein synthesizing capacity is reduced by 95 percent. The effects on learning and on memory at various times after learning can then be examined. When mice treated in this way are trained to escape shock by choosing the lighted limb of a T-maze, their learning curves are indistinguishable from mice injected with saline. Furthermore, in a typical experiment normal retention can be detected several hours after training. This result suggests that cerebral protein synthesis is not required for learning or for memory for hours after. If other groups of mice, trained in this manner, are tested for retention one day or seven days after training, they are found to have markedly impaired memory. Therefore, it appears that cerebral protein synthesis during training is required for a long -lasting memory. The critical cerebral protein synthesis presumably occurs during training or

within minutes thereafter since injection of the drug thirty minutes after training has no effect on memory measured a day or seven days later.

The results of an extensive series of experiments of this type suggest that there are two processes in memory storage—a short-term process that lasts for hours and is independent of cerebral protein synthesis; and a long-term process that is dependent on cerebral protein synthesis. For the purposes of this discussion these experiments are also of interest because they indicate how tools developed for general biological studies may be applied to analysis of this special problem of mammalian brain function.

Conclusion

The goal of the essay is to provide a sampling of contemporary research on the establishment, consistency, and modifiability of functional neuronal wiring diagrams. Unlike studies of neurotransmitter metabolism or of psychoactive drugs, such work has not as yet provided any practical tools for psychiatry. It is concerned primarily with relatively primitive organisms and with general problems of differentiation and biological regulation. In a sense this work underscores our ignorance of the most fundamental processes involved in creating and molding the nervous system. It remains to be seen how analysis of these problems will influence our ability to predict or control behavior.

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Notes

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