9-21-78

ANNUAL REPORT OF THE
LABORATORY OF BIOCHEMICAL GENETICS
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1977 through September 30, 1978

Two model systems were used to study the process of synaptogenesis: clonal cell lines of neural origin and neurons dissociated from embryos and cultured in vitro.

Twenty-three neuroblastoma or hybrid cell lines were tested for their ability to synthesize and release acetylcholine and form synapses with rat striated muscle cells and clonal muscle cells. Six cell lines form synapses with muscle cells with high frequency; whereas, 17 cell lines were found which have defects in synapse formation. Of the 19 cell lines found that synthesize and release acetylcholine into the medium, six cell lines form synapses with high frequency, whereas three cell lines form few or no synapses. Rates of acetylcholine synthesis were 34-463 pmol acetylcholine/min/mg/homogenate protein; intact cells incubated with [^{3}H]-choline contained 55-1,600 pmol [^{3}H]-acetylcholine/mg/protein and released 80-6,400 fmol [^{3}H]-acetylcholine /min/mg protein into the medium. Five kinds of defects were detected with cell lines that form few or no synapses: (1) little or no acetylcholine synthesis, (2) large dense core vesicles present but not clear vesicles, (3) clear vesicles present but not large dense core vesicles, (4) little or no action potential Ca^{++} ionophore activity, and (5) a defect in another step required for stimulus-dependent acetylcholine secretion.

NG108-15 hybrid cells which form synapses with muscle cells release into the medium a macromolecule which results in aggregation of nicotinic acetylcholine receptors of cultured striated muscle cells. The factor also is formed by neuroblastoma parental cells, but not by parental glioma cells, fibroblasts, or HeLa cells. The factor increases the aggregation of acetylcholine receptors without altering the number of receptors, and the action of the factor does not depend on myotube protein synthesis. The factor was partially purified and was shown to be a heat-labile macromolecule, with a molecular weight of > 10,000. A role for the acetylcholine receptor aggregation factor in synaptogenesis was hypothesized; namely, that the factor stimulates the accumulation of nicotinic acetylcholine receptors on myotube surface membranes at sites of contact or interaction between neurons and muscle cells.

The ability of hybrid cells to form synapses was found to be regulated. Growth of hybrid cells in the presence of dibutyryl-cAMP increased the concentration of intracellular acetylcholine, the abundance of vesicles, the amount of acetylcholine released from cells in response to excitory stimuli, the efficiency of synaptic communication, and the number of synapses formed. These effects also were obtained with cAMP, but not with cGMP.

The effects of putative neurotransmitters and hormones on intracellular cAMP or cGMP levels, cell membrane potential, and acetylcholine secretion from cells were determined. At least 10 species of receptors were detected on synapse competent cell lines. Activation of receptors for serotonin,

$PGF_{2\alpha}$. acetylcholine, bradykinin, neurotensin, or angiotensin resulted in secretion of acetylcholine from cells. Receptor mediated increases in cAMP or cGMP levels had no immediate effect on acetylcholine secretion from cells. However, growth of hybrid cells for 24 or more hours in the presence of ligands for receptors that are coupled to the activation of adenylate cyclase and/or exposure of cells to other inhibitors of cyclic nucleotide phosphodiesterase resulted in increases in cAMP levels of cells and mimicked all regulatory effects of dibutyryl-cAMP on acetylcholine storage and release from cells. These results show that cell lines with and without synapse defects can be generated and that receptor mediated reactions that activate adenylate cyclase and elevate cAMP levels of cells regulate the storage and stimulus-dependent secretion of acetylcholine, thereby regulating synapse formation and the flow of information across synapses. Synapses were turned on or off slowly over a period of days, which suggests that cAMP, directly or indirectly, regulates the acquisition of components that are required for synaptic activity.

We previously showed that activation of NG108-15 opiate receptors or muscarinic acetylcholine receptors results in inhibition of adenylate cyclase and that exposure of cells to morphine or carbamylcholine reduces cAMP levels of cells initially, but gradually, over a period of 24-48 hours, cAMP levels increase and return to the control value due to a compensatory, long-lived increase in the specific activity of adenylate cyclase. Subsequent studies have shown that NG108-15 cells possess presynaptic α_2-receptors and that exposure of cells to 1 μM norepinephrine similarly reduces cAMP levels initially, and that cAMP levels slowly return to the control value over a 10 hour period as the specific activity of adenylate cyclase increases. The cells then are dependent on norepinephrine to inhibit the elevated adenylate cyclase activity. Withdrawal of norepinephrine or blockade of the α-receptors results in a 4-9 fold increase in intracellular cAMP. Approximately 8 hours are required for the elevated enzyme activity to return to the control value. Cyclic AMP levels are elevated during the withdrawal period, and cells are supersensitive to ligands for other species of receptors, such as PGE_1, that activate adenylate cyclase. The demonstration that 3 species of receptors which mediate inhibition of adenylate cyclase also evoke persistent increases in adenylate cyclase activity suggests that the phenomenon is a general one, and that other species of receptors that inhibit adenylate cyclase also may act as dual regulators of the enzyme.

The hypothesis that activation of adenylate cyclase may lead, conversely, to a reduction in adenylate cyclase activity also was tested. Incubation of NG108-15 cells with PGE_1 for 12 hours resulted in 60-80% decreases in basal adenylate cyclase specific activity and NaF-, Gpp(NH)p-, 2-Cl-adenosine, and PGE_1-stimulated activities. Basal and PGE_1-stimulated adenylate cyclase activities of cells exposed to PGE_1 decayed exponentially with half-lives of 6 hours. On withdrawal of PGE_1, adenylate cyclase activity slowly increased and returned to the control value over a period of 24 hours; cyclohexamide inhibited the increase in adenylate cyclase activity > 90%. These results show that activation of adenylate cyclase leads to a loss of enzyme activity and that the recovery of enzyme activity to the control value requires protein synthesis and approximately 24 hours of incubation.

These long-lived, receptor-mediated effects on adenylate cyclase activity, acetylcholine storage, stimulus-secretion coupling, and the demonstration that synapses can be turned on or off by regulating acetylcholine release, have properties that resemble those expected for simple forms of learning and memory, such as habituation, tolerance, dependence, and sensitization but whether synapse plasticity in a cultured cell system is related to behavioral phenomena is not known.

Synapse competent cell lines possess serotonin receptors which mediate cell depolarization and stimulate secretion of acetylcholine into the medium. Serotonin also stimulates adenylate cyclase in homogenates of one, but not other, hybrid cell lines. The concentration of serotonin required for half-maximal activation of adenylate cyclase (Ka) is 0.5 μM. Hill and Eadie-Scatchard analyses suggest a simple, bimolecular interaction between serotonin and the receptor. D-Lysergic acid diethylamide (D-LSD) also stimulates adenylate cyclase activity (Ka=12 nM); however, the increase in enzyme activity is less than half that produced by serotonin. In contrast, D-LSD neither mimics nor antagonizes the effects of serotonin on cell depolarization and acetylcholine secretion. These and additional studies on serotonin receptor specificity for agonists and antagonists suggest that serotonin receptors that are coupled to activation of adenylate cyclase are postsynaptic serotonin receptors, whereas, those mediating cell depolarization and acetylcholine release are presynaptic serotonin receptors. One cell line exhibits both pre- and post-synaptic serotonin receptor functions; other cell lines have only presynaptic serotonin receptor function.

Cultured neurons dissociated from chick embryo retina and spinal cord also were used to study the process of synapse formation. Both intact chick retina and cultured retina cells were shown to have high choline acetyltransferase activity and abundant nicotinic and muscarinic acetylcholine receptors. ^{125}I-Labeled α-bungarotoxin and 3-[^{3}H]-quinuclidinyl benzilate, which bind with high affinity and specificity to nicotinic or muscarinic acetylcholine receptors, respectively, were used as probes to determine the properties of the receptors, the number of binding sites, and their distribution within the retina during embryonic development. Most of the nicotinic acetylcholine receptors and all of the muscarinic receptors of chick retina were localized in layers within the inner synaptic layer of the retina; 11 layers were distinguished within the inner synaptic layer of chick retina on the basis of muscarinic and nicotinic acetylcholine receptor concentrations and acetylcholinesterase activity. The layers appear in an ordered sequence during development with respect to temporal and positional relationships. These results and those of others show that neurites of the same type sort out from neurites of other types on the basis of species of receptor, transmitter, or enzyme of transmitter metabolism. A possible mechanism for generating sets of stratified or columnar neurons with similar properties and relating one set to another by cross-linking neurons of the same type to one another via synaptic connections was proposed.

An α-bungarotoxin-peroxidase conjugate was synthesized and used to study the distribution of nicotinic acetylcholine receptors in the developing chick retina at the ultrastructural level. Five percent of amacrine neuron synapses and 14-20% of bipolar neuron synapses in the inner synaptic layer of the

retina were labeled; high concentrations of nicotinic acetylcholine receptors are restricted to these synaptic sites. These results suggest that some bipolar neurons and amacrine neurons in chick retina synthesize acetylcholine and synapse with ganglion neurons or amacrine neurons which possess nicotinic acetylcholine receptors.

The specificity of synapse formation by dissociated chick embryo retina neurons was examined by culturing retina cells with inappropriate synaptic partner cells, such as striated muscle cells which possess nicotinic acetylcholine receptors. The results show that neurons are generated in chick embryo retina that are able to form synapses with striated muscle cells and then lose the ability to form synapses with a half-life of 21 hours. These neurons first appear in chick retina on the sixth day of embryo development, and are most abundant on the eight day, comprising perhaps 8% of the retina cell population. Almost all myotubes were innervated after coculturing retina and muscle cells for only 2 hours. However, the mismatched synapses between retina neurons and muscle cells are transient and slowly disappear over a period of 8 days. Neurons lose the ability to form new synapses by the 16th day, but not the ability to synthesize and secrete acetylcholine. Cultured retina neurons also form synapses in abundance with other retina neurons (approximately 1×10^9 synapses/mg of protein), and synapses between retina neurons were found after all synapses between retina neuron and muscle cells had been terminated.

Preparations of neurons from spinal cord, which presumably contain motorneurons that normally innervate striated muscle cells, also formed synapses with muscle cells in vitro but the number of synapses remained constant during subsequent culture. Thus, spinal cord neuron either form stable, long-lived synapses with muscle cells or attain a steady state wherein rates of synapse formation and termination are equal. These results show that populations of cholinergic neurons from retina and spinal cord differ in the rate of synthesis of synapses with muscle cells and probably also the rate of termination and that populations of synapses can be selected on the basis of differences in synapse turnover rates. The results suggest that part of the specificity of synaptic circuits may be acquired after synapses form by a process of selection.

A factor extracted from chick embryo retina and spinal cord was found to agglutinate rabbit erythrocytes in vitro. The amount of agglutinin activity varies markedly during embryonic development in the spinal cord, rising to a peak on the 10th day of embryonic development and then decreasing 7-fold by the time of hatching. The factor was first detected in 10 day embryo retina and increased in concentration until the 16th day in ovo. Despite differences in hemagglutination activity and the patterns of development, both the retina and spinal cord lectins exhibit the same specificity for saccharides. Lactose was the most potent inhibitor hemagglutination found (half-maximal inhibition with 2×10^{-5} M lactose).

To identify retina molecules required for synaptogenesis or communication across the synapse, we have used the technique recently introduced by Milstein and coworkers of monospecific antibody synthesis by clonal spleen cell x myeloma hybrid cell lines formed by fusion of clonal mouse myeloma cells with mouse spleen cells immunized against retina cells. Large quantities of