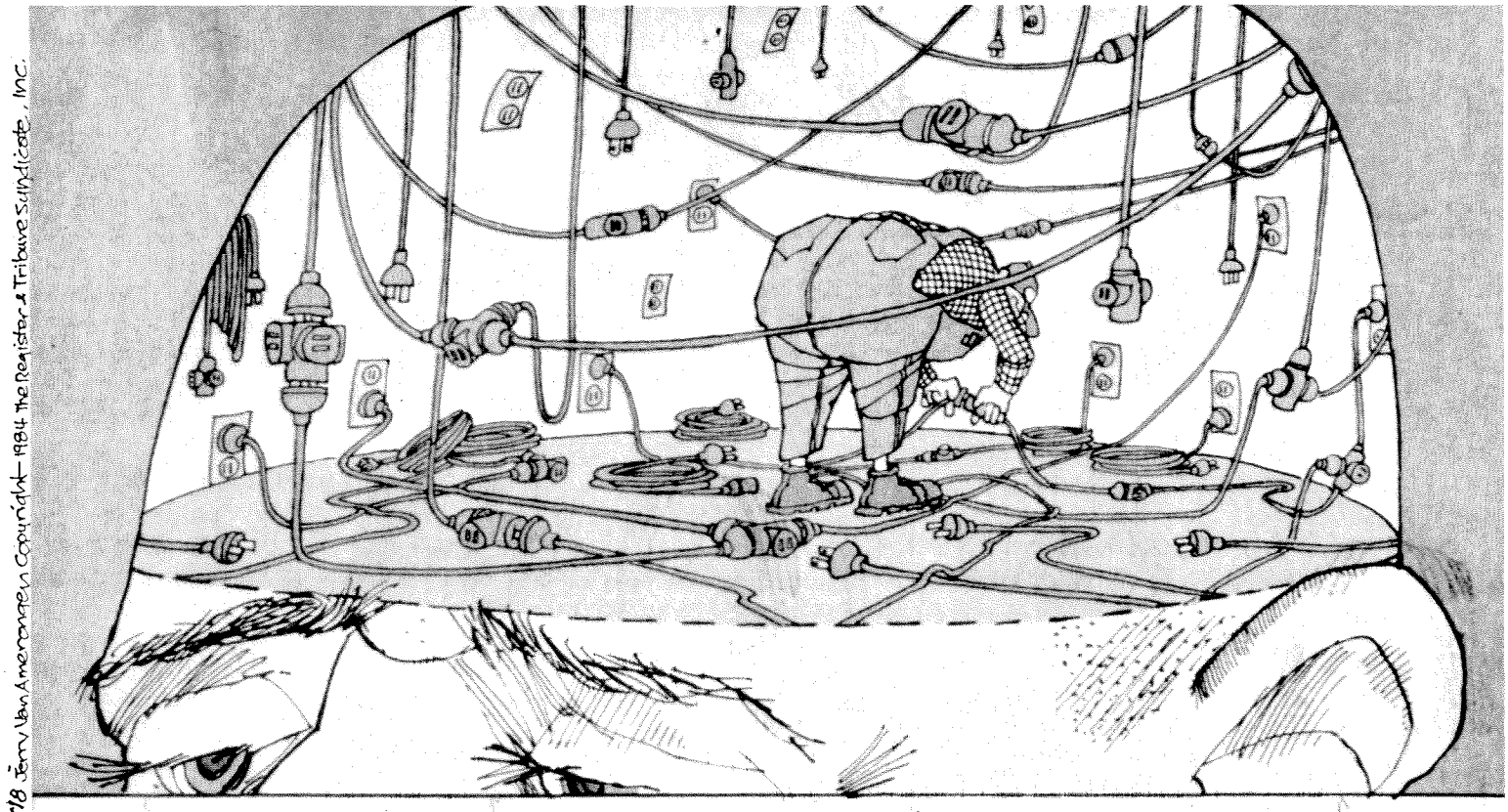


How the brain works.

Nature vs. Nurture in Building the Nervous System

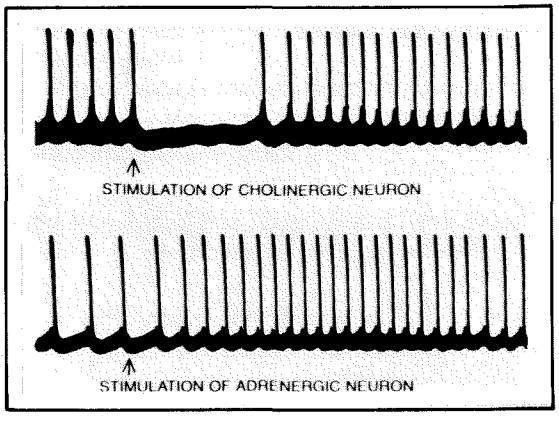
by Paul Patterson

GENERATION OF CELL DIVERSITY is one of the central problems of developmental biology. Every embryo starts as a single cell, the egg, and by maturity countless numbers of cells have been generated of many different types, from muscle cells to sperm cells. In the brain alone there are an estimated 10 billion nerve cells of thousands of different types. Added to this diversity is the fact that these nerve cells, or neurons, are interconnected in a highly complex way. For instance, a single motor neuron in the human spinal cord carries out the mundane task of controlling the contraction of a handful of muscle fibers in

the arm or leg. To accomplish this simple task, this single neuron receives some hundred thousand connections from other nerve cells.

The shape and size of neurons is also diverse. For example, the Purkinje cell, found in the cerebellum at the base of the brain and involved in controlling balance, contains an extremely elaborate dendritic tree, where it receives connections from other kinds of nerve cells. These connections, or synapses, are made on the numerous branches and tiny spines of the dendrites. At the other extreme are the neurons that control the contraction of the iris. These have no dendritic tree at all; all the inputs to these neurons are on the cell body.

In addition to their size and shape, number of branches, and other morphological features, neurons can also be distinguished from one another by their chemical composition. Most neurons make chemical synaptic connections with each other, and a variety of different transmitters are used at these connections. For example, adrenergic neurons use catecholamines as transmitters in their synapses on the heart, whereas cholinergic neurons release acetylcholine at their connections with the heart. We distinguish these two transmitters chemically because their



Stimulating a cholinergic neuron causes the spontaneous contractions of the heart muscle to stop temporarily (top), while stimulating an adrenergic neuron speeds up the contraction frequency (bottom).

effects on target cells are quite different. Acetylcholine inhibits the heartbeat, while catecholamines excite it. In the autonomic nervous system the balance of these two transmitters controls the rate of the heartbeat. Therefore, it is very important for a neuron to know what kind of transmitter to produce. Certain neurons should produce excitatory transmitters, and others should produce inhibitory transmitters. Mistakes in this chemistry would lead to a speedup of the heartbeat when it should rest and vice versa.

How do neurons decide which kind of transmitter to produce? Two explanations have been offered as to how this might come about. Sidney Brenner (of the Medical Research Council, Cambridge, England) has characterized these two extremes as the European plan versus the American plan. In the European plan the developing neuron would behave according to its ancestry or lineage; it would do what its family had always done. Neurons on the American plan, on the other hand, would do what their neighbors tell them to do. By this we mean that the local environment surrounding each cell would play a role in influencing its development. Recent work has shown that the chemical identity, or phenotype, of each neuron is surprisingly plastic, even in adult animals. Furthermore, neurons are influenced by local cues or signals in their environment, such as other cells, hormones, and so on.

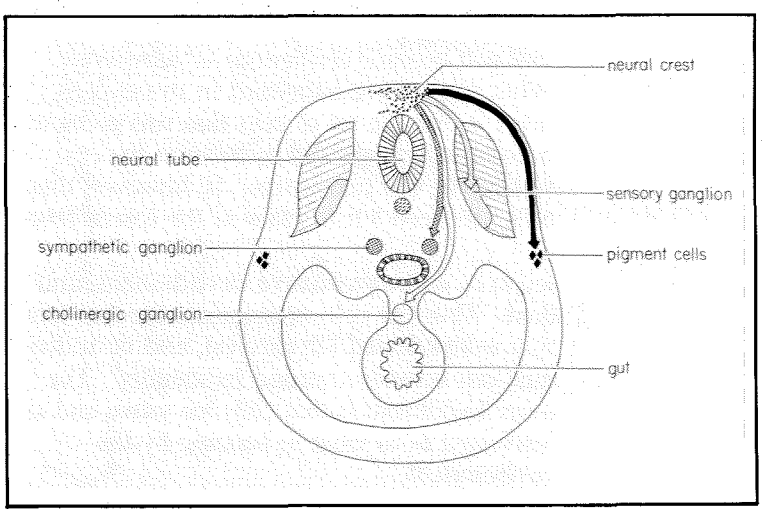
How does the nervous system develop during embryogenesis? After fertilization, folds rise up on the surface of the embryo and meet to form a tube. This simple neural tube will give rise to all the cells of the brain and spinal cord, which make up the central nervous system with its 10 billion neurons and supporting cells. In addition, there is a peripheral nervous system. These neurons come from a group of cells at the top of the neural tube, called the neural crest. The neural crest cells migrate to many different locations in the embryo and take up many different identities. Some migrate to the skin to become pigment cells; some migrate out and cluster to form ganglia or groups of neurons. Some of these are sensory neurons, which send one process out to the periphery in the skin, where they mediate sensations such as touch, heat, cold, or pain. Other neural crest cells migrate to still different locations and become the autonomic nervous system. These are the neurons that innervate the heart, the iris, various glands and smooth muscles, and thereby control blood pressure, pupillary dilation, and so on. The autonomic system is, in turn, composed of the sympathetic and parasympathetic systems, which use the transmitters mentioned previously — catecholamines and acetylcholine, respectively.

These various derivatives of the neural crest do not all come from the same axial level of the embryo. The adrenergic neurons of the sympathetic system come from the lumbar, or middle, region of the crest. The cholinergic neurons of the parasympathetic

system innervating the intestine come from the rostral and caudal regions of the crest. This arrangement suggests, in terms of how these cells decide what kind of phenotype they will take up, that perhaps the neural crest is made up of at least two populations of cells. Cells in the lumbar region may be predestined to be adrenergic sympathetic neurons. Cells from the rostral and caudal regions may be predestined to be cholinergic neurons innervating the gut. This possibility corresponds to the European plan. The other possibility would be that the neural crest cell population is homogeneous and naive. The cells migrate out passively and are led to locations appropriate for that axial level of the embryo. Cues in these locations then instruct the neuron as to its fate. This possibility corresponds to the American plan.

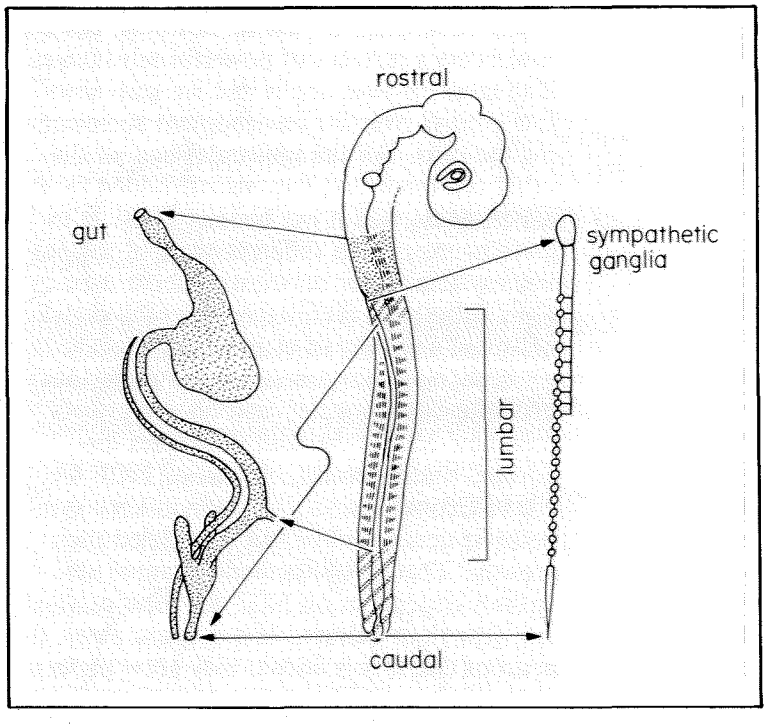
How do we go about investigating which of these plans is the correct description? One way — a very dramatic way of doing experiments on this system — is to take neural crest cells from the rostral region of one embryo and transplant them in a different embryo at the lumbar region, so that cells that would normally have become cholinergic are now put in a location where cells normally become adrenergic and vice versa. In order to do this experiment, of course, it is not enough just to transplant cells, but we have to keep track of where the cells go. We have to know the difference between donor cells and host cells, so that the fate of the donor cells can be determined. Nicole LeDouarin and colleagues in Paris have solved this problem by transplanting crest cells between two species, quail and chick.

Cells from each of these species can easily be distinguished from one another by simply looking at their nuclear staining patterns under the microscope. They transplanted the neural crest from a quail donor embryo into a chick host, effectively transplanting cells that would have become cholinergic neurons in the gut into a lumbar location in a different embryo. Do the quail cells now find their way to their original destination or do they migrate to the new location determined by the local environment? The result is that at least some of the cells do migrate to the new location appropriate for that axial level and become sympathetic neurons. Furthermore, some of these quail cells stain for catecholamines. Thus some of the cells in this population of crest cells that would, if they had been left in place, have become cholinergic neurons in the gut, when put into a new location migrate to an entirely new place and take up a new identity.



This cross section of a developing chick embryo shows the migration pathways of various derivatives of the neural crest.

This series of experiments shows that the neural crest cell population can be influenced as to the kind of phenotype, or chemical identity, they develop by the environment in which they find themselves. These important experiments raise several further questions. One question is: What happens at the level of individual cells? This experiment dealt with populations of cells — how do individual cells make their transmitter decision? Two very different hypotheses can be used to explain these results. It is possible that no individual crest cell is actually changing its identity after the transplantation; the crest population could be heterogeneous, contain-



Sympathetic neurons arise from different axial levels of the neural crest than do the cholinergic neurons of the gut. (Adapted from LeDouarin, 1977.)

ing two populations of predetermined cells that will become cholinergic or adrenergic. What might have happened in this experiment is that the set of cells that was going to become adrenergic did so because the environment was permissive. If, however, these cells had been left in place at the rostral level, they would have migrated out, found themselves in a nonpermissive environment, and died. Thus the cells appropriate for that location were selected for survival, and no individual cell actually changed its identity. The other hypothesis is that cells are naive and are instructed as to what to become by the environment.

The second question raised by the transplantation experiments is: What is the nature of the molecular cues that are instructing or controlling the survival of these cells? To answer these questions we turn to the technique of cell culture — growing cells *in vitro* in dishes. The problem with cell culture is that once we take cells out of the body and put them in a dish, we assume responsibility for their life and death. We have to provide all the functions that the body normally provides; we have to take over the role of the circulatory system by providing all essential nutrients, the role of the kidney by removing toxic wastes, the role of the lungs by providing an appropriate mixture of gases, and so on. If this is not done very precisely, cells become sick and die. Those of us working in the field of cell culture are very familiar with the phenomenon of cell death. If the appropriate conditions are found, however, cell culture offers a number of powerful advantages. The most obvious one is that we gain absolute control over the environment surrounding the cells. All the molecules and all the other cell types that they see are added by the experiments, and it has become possible to grow neurons from different parts of the nervous system under conditions where they grow entirely by themselves or in the presence of any of a variety of other kinds of cells.

Early on in the search for the molecules necessary for neuronal survival and growth, Rita Levi-Montalcini and colleagues discovered that a protein, christened NGF for Nerve Growth Factor, was able to keep sympathetic neurons alive and stimulated their growth. That is, sympathetic neurons are entirely dependent on NGF for their survival and growth in culture. If it is taken away, the neurons die; if it is added, they grow perfectly well. If an antibody against NGF is injected into an embryo to bind and inactivate the protein, the animal will grow up without a sympathetic nervous system. Furthermore, if extra amounts of NGF are injected into the embryo, it grows up with more sympathetic neurons than a normal embryo. This latter result was a profound finding. It has been subsequently found that many neurons die throughout the nervous system as a part of normal development. About one-half to two-thirds of all the neurons that are born in the embryo die before the animal reaches maturity. We now see this naturally occurring neuronal cell death as a kind of Darwinian struggle for survival; that is, the neurons appear to compete with one another for trophic or growth factors such as NGF. If extra amounts of this factor are added, then neurons that normally would have died can survive; take away the NGF, and all of the neurons die.

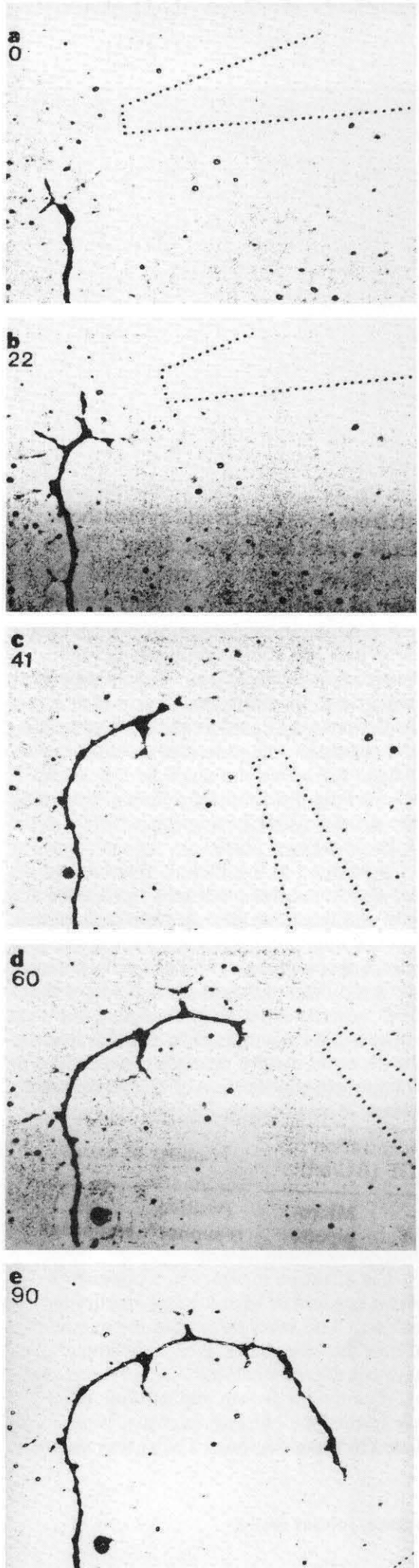
These observations raise the question of which cells produce NGF or trophic factors like it. It is now clear that target tissues toward which the neurons' axons are growing (heart cells, iris muscle, various glands, and so on) actually produce NGF. Wherever sympathetic neurons are found, it seems that the target cells synthesize and release this neural growth factor. What happens when a neuron actually gets to the source of the nerve growth factor? It binds it on its surface, internalizes it, and transports it from its growing tips back to its nucleus. In fact, neurons such as sympathetic neurons that are sensitive to NGF can actually change the direction of their growth in culture toward the source of NGF. The growing axon tips, or growth cones, can orient towards this soluble trophic molecule. It is also clear that the direction that nerves grow can be controlled by the surface on which they are moving. A good example of this comes from the work of Paul Letourneau at the University of Minnesota. He coated dishes with various pathways made of different kinds of artificial polymers and found that the growth cones move best on the surfaces to which they adhere well. Artificial substrates can support and guide growth; biologically relevant cells, such as muscle cells and glial cells, secrete a large protein complex into the culture medium, which will also stick to a dish and coat it. This biological substrate can also control the direction of axon growth just as the artificial polymer did.

Our group at Caltech and others are characterizing the molecular nature of this

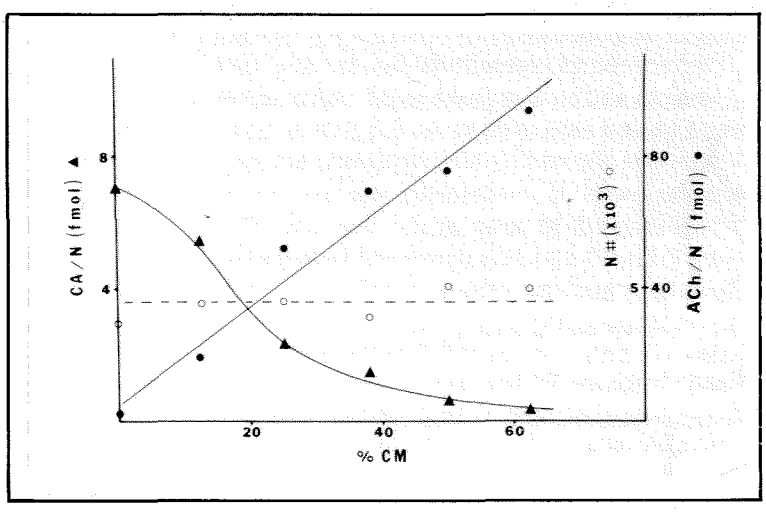
protein complex to try to see what molecules are involved in this guidance and growth stimulation. Using a novel method, we made an antibody that binds to this complex somewhere near its active site, so that it blocks the activity of the molecule. This antibody should be able to tell us something about the chemistry of the complex. Research fellow Arlene Chiu and graduate student Josette Carnahan are using the antibody to localize the molecule and have discovered it in places in the animal that are known to support regrowth of damaged axons. It was well known that when peripheral axons in the limbs and body cavity are damaged, they can regenerate back to their target sites, whereas once central nervous system axons (in the brain and spinal cord) are damaged, there is very little ability to grow back properly. Finding the molecule in places that are known to support regeneration suggests the possibility that this molecule itself could be one of the key factors in axon regeneration. Another way of stimulating regeneration, of course, would be through soluble molecules such as NGF.

A third way of looking at regeneration, or lack of it, has to do with the formation of scar tissue. Central nervous system neurons, damaged, for example, in an auto accident, may not be able to cross the simple physical barrier created by the scar where damage occurred. The same type of barriers do not appear to form in the peripheral system where regeneration can occur. We now know, however, that the growing tips of axons release enzymes, called proteases, that may help them get through such barriers. Proteases break down large proteins into small pieces. Research fellow Randy Pittman has used biochemical techniques to characterize the proteases released by growing neurons. He puts neurons in the central chamber of a culture dish and lets them grow their axons out into the surrounding chambers in a radial fashion so that the fine, distal processes and growth cones end up in the outermost chamber. He then collects the culture medium off the three different chambers — the cell bodies from the center, the axons from the middle, and the growing distal processes from the outer chamber — and analyzes it biochemically by electrophoresis to see what kinds of proteases are present.

In electrophoresis, proteins migrate in an electric field through a gel and separate according to their molecular sizes. Once



In an experiment by Gunderson and Barrett of the University of Miami, an axon with a growth cone on its tip turns toward a pipette (dotted line) with protein NGF. The numbers indicate elapsed minutes.



As the concentration of heart-cell factor in the conditioned medium is increased, the neurons' ability to produce acetylcholine rises with a corresponding decrease in their ability to produce catecholamines.

separated, the proteases eat little holes in the protein of the gel, so when the gel is stained for protein, there are bare spots where the proteases are located. In this way it is possible to analyze very small amounts of mixtures of proteases from, for instance, the growth cone. In other experiments Pittman found that one of these proteases has the properties of a collagenase. This enzyme breaks down the protein collagen which is the fibrillar material found in all of our extracellular spaces, as well as in hair and fingernails and so on. Collagenase might be very useful to a growing neuron tip to help the axon get through the extracellular space, which is a jungle of collagen fibrils.

In addition to this finding, Pittman has also discovered that other cells (such as heart cells — a target for these neurons) release a protease inhibitor. Thus, when the growing neuron is confronted with heart cells, it runs into a significant amount of an inhibitor that binds irreversibly to the protease and inactivates it. We're intrigued by the idea that growth could involve protease release and stopping growth could involve inhibiting that release. This discovery illustrates the idea that axon growth is not a one-way street; that is, axons don't just go wherever they are told. Rather, there seem to be many interactions. Growing axons react to soluble and surface-bound protein cues, and they can also modify their environment as well.

The direction in which neurons grow may play a key role in what kind of identity they take on. This takes us back to the question of how the cells choose their transmitter. We have considered whether NGF, a critical signal required for growth and survival, plays a role in the type of transmitter that is produced by these neurons. The answer seems to be no. Linda Chun and I did a series of experiments a few years ago showing that there is a different protein that can control the phenotype of these neurons. In particular, we found that a protein secreted by heart cells can control whether sympathetic neurons become cholinergic or adrenergic — whether they have an excitatory or inhibitory transmitter. It's a very simple experiment of growing heart cells in a dish, collecting the medium containing a variety of proteins of the heart cells, and putting it on the neurons in a separate dish. Neurons are then grown in various concentrations of this so-called conditioned medium, that is, a medium that has been incubated in heart cells.

When we assay the ability of the neurons to synthesize and store catecholamines versus acetylcholine, our two transmitters of interest, we see that adding more and more of the heart-cell factor increases the neurons' ability to produce acetylcholine, and there is a corresponding decrease in their ability to produce catecholamines. So there is a reciprocal change in the transmitter identity of these cells caused by a protein, which research fellow Keiko Fukada has been purifying. This heart cell protein, unlike NGF, does not affect the survival of the neurons. It does not affect their growth either; rather it instructs them as to what kind of neuron they're going to become — what kind of chemical identity they will adopt.

This heart cell conditioned medium experiment was also done with populations of neurons and, again, we wanted to know what happens at the level of single cells. To do that we needed to follow individual cells during development. To this end we have cultured single nerve cells on islands — called microcultures — of heart cells. We can impale the heart cell and the neuron with microelectrodes, stimulate the neuron, and see what effect this has on the heartbeat. By seeing whether the heart cells are speeded up or slowed down, we can determine if the transmitter released by the neuron is a catecholamine or acetylcholine, respectively.

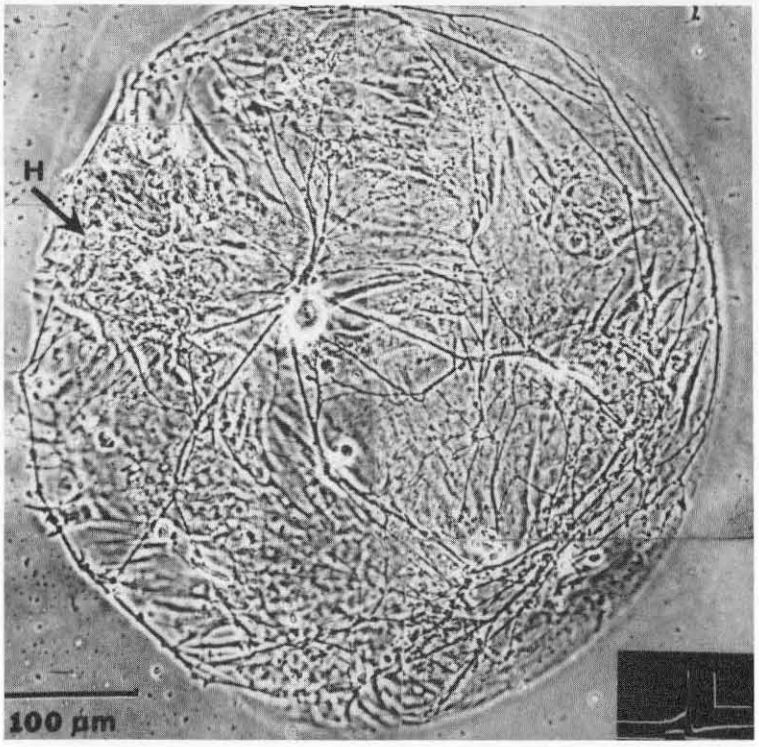
Edwin Furshpan and David Potter and their colleagues at Harvard have done this experiment on individual neurons. Recording from the heart cells showed that stimulating the neuron causes a substantial increase in the contraction rate of the heart cells. This is a classical effect of catecholamines, and the effect can be blocked completely by the drug propranolol, which is used in some cases to

control blood pressure. Thus this neuron started out the experiment as an adrenergic neuron using catecholamines as a transmitter. When the cholinergic factor from heart cells was added, stimulating the same neuron some days later speeded up the heartbeat again as it had done before, but there was also a slight inhibition. (This effect can be seen better if the excitatory effect is blocked by adding the drug that blocks adrenergic speedup.) The neuron had become dual- functional, seeming to release both acetylcholine and catecholamines. Some weeks later, stimulating the same neuron revealed no speedup of the heartbeat at all — just the inhibition. The neuron had begun the experiment with an adrenergic identity, midway through it produced both transmitters at the same time, and by the end it was completely cholinergic.

Studies like these demonstrate that individual postmitotic neurons can be made to change their identity by external signals. This is true also of neurons taken from adult rats. This kind of plasticity may be important in thinking about whether the results of the previously described experiments on transplanting neural crest cells were due to selection or instruction. It's possible in those experiments also, as in the culture one, that environmental cues instructed the cells as to which developmental pathway to follow.

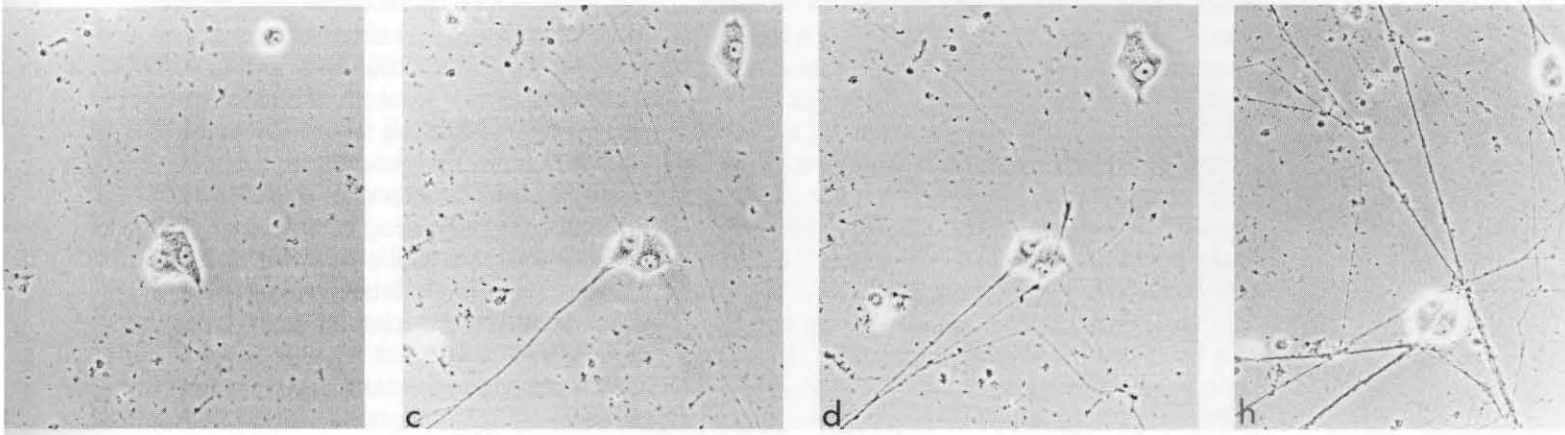
Cells also have another kind of phenotypic decision to make — whether to become a neuron or a non-neuronal cell. Klaus Unsicker and colleagues at the University of Marburg, Germany, have studied this phenomenon, as have Allison Doupe and myself — specifically the decision whether to become an adrenal chromaffin cell or a sympathetic neuron. These are two quite different cells — different in their morphologies, chemistry, and the antigens on their surfaces. Adrenal chromaffin cells have to be grown in the presence of the hormone hydrocortisone, which is very important in development. If the corticosteroids are removed from the culture medium and protein NGF is added, these chromaffin cells will grow processes. In a couple of weeks they become neurons; they get round and much larger; they grow long axons, and they lose all the characteristics of a chromaffin cell. Furthermore, we have shown that these neurons derived from chromaffin cells can be converted into cholinergic neurons by using the cholinergic protein from heart cells. Thus they can go the complete route of being changed from an adrenergic chromaffin cell to a cholinergic neuron.

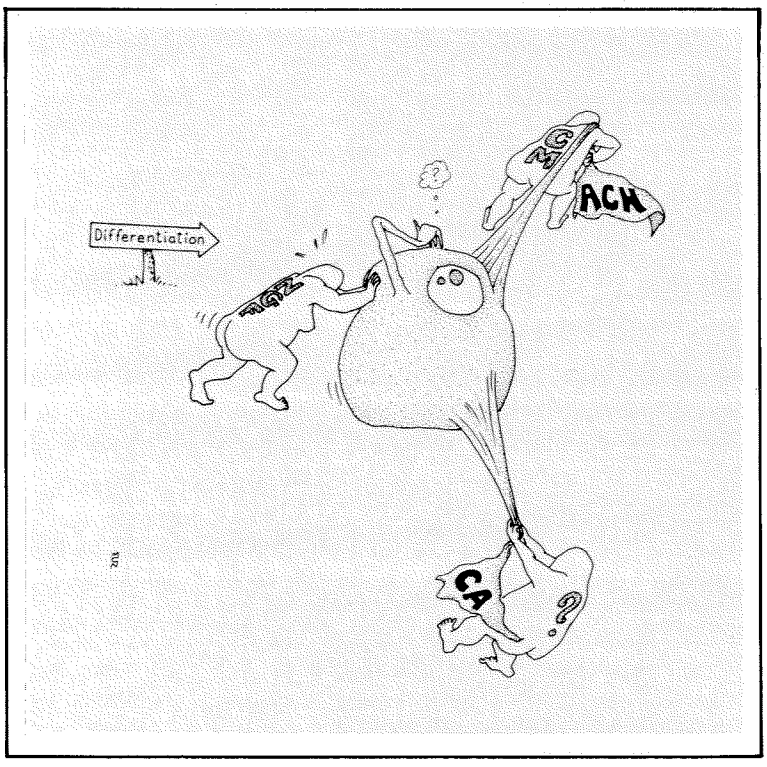
People often ask what the practical sig-



A single neuron extends its branches over a conditioned medium that has been incubated in heart cells.

An adrenal chromaffin cell (a), when corticosteroids are removed and protein NGF added, begins to grow processes (c, d), finally turning into a neuron (h).





When pushed by NGF, a neuron tries to decide which direction it should take. A conditioned medium of heart cells pulls it toward producing acetylcholine, while an as-yet-unknown factor is pulling it in the direction of catecholamines.

nificance of all this is. In the case of neuron survival, I've described very briefly some of the work in terms of characterizing signals that have absolute control over the survival of a given population of neurons. Why might this be important? There are pathological states that involve premature death of subpopulations of neurons. Alzheimer's disease is a classic case, in which neurons in certain parts of the brain die prematurely, leading to senility. In amyotrophic lateral sclerosis, motor neurons die prematurely, leading progressively to paralysis of the limbs and eventually to death. It would be interesting to know whether trophic factors that can keep those neurons alive in culture could be used to keep them alive in such disease states.

Second, a number of pathologic conditions are thought to be caused by chemical imbalances among groups of neurons in the brain. For example, the symptoms of Parkinsonism and schizophrenia can be characterized by imbalances between adrenergic and cholinergic functions of the brain. If we could get more information on the molecules that control that balance (such as the cholinergic protein signal from heart cells), it would be interesting to see if we could reform the balance in those diseases.

Third, work is now being done on rectifying brain lesions via transplantation. In certain cases it is possible to correct the behavioral deficit caused by lesions in the rat brain by transplanting fetal brain tissue from a rat embryo. In Sweden several transplants have been done with terminal Parkinson's disease patients, moving a piece of their adrenal medulla (containing the adrenal chromaffin cells) into the brain at the site of the lesion. This is of interest to us, because we're looking at the ability of cultured chromaffin cells to become neurons and what factors control the type of neurons they become.

Finally, we can now at least imagine solving the problem of axon regeneration in the damaged central nervous system. For example, the protein complex mentioned earlier, which promotes regeneration, is found only on the surface of peripheral cells, which are known to promote regeneration. Is its absence the problem in the lack of regeneration in central neurons? Or is it perhaps a problem of soluble trophic factors, such as NGF? Is there a problem that prevents central neurons from growing through particular parts of extracellular space, such as the lack of a particular protease? These are just idle speculations at this point, but at least there are now testable ideas available, and the appropriate experiments are currently under way.

Another point of interest is that we see that putting the brain together during development involves a massive struggle for survival — cells competing for essential survival factors. We've also seen that interactions between cells in the developing nervous system can lead to changes in the character of both participants in these interactions. It strikes me that all of this sounds a bit like what goes on at the level of the whole organism, both in the behavior of animals and in their evolution as well.

Perhaps we are beginning to make at least some modest progress toward answering a few of the questions posed at the turn of the century by the great Spanish embryologist, Santiago Ramón y Cajal. Ramón y Cajal posed most of the questions about neuroembryology that are discussed in this article, and he stated the problem of axon growth and synapse formation in his characteristically romantic fashion: "What mysterious forces precede the appearance of the processes, promote their growth and ramification, and finally establish those protoplasmic kisses, the intercellar articulations which seem to constitute the final ecstasy of an epic love story?"

We have at least made some progress on the foreplay. □