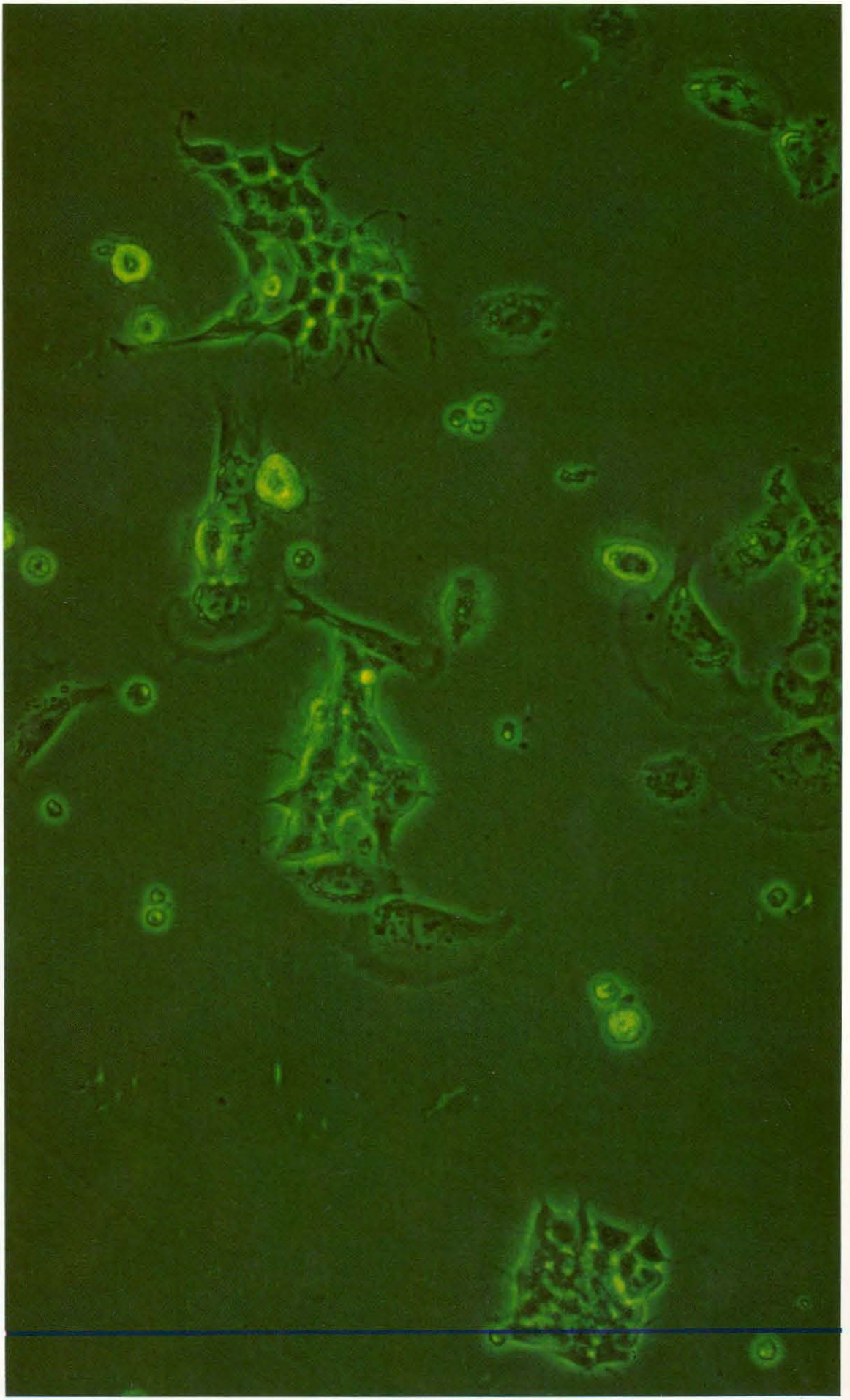


Career Choices for Developing Neurons

by David J. Anderson

How does a developing cell in the embryonic brain decide which type of neuron it's going to become?

These cloned immortalized precursor cells, with their characteristic nubbin-like protrusions, could become either sympathetic neurons or adrenal chromaffin cells. They have been co-cultured with the fan-like cells, visible on the right, that harbor the defective retrovirus that injects the immortalizing gene into the precursor cells.

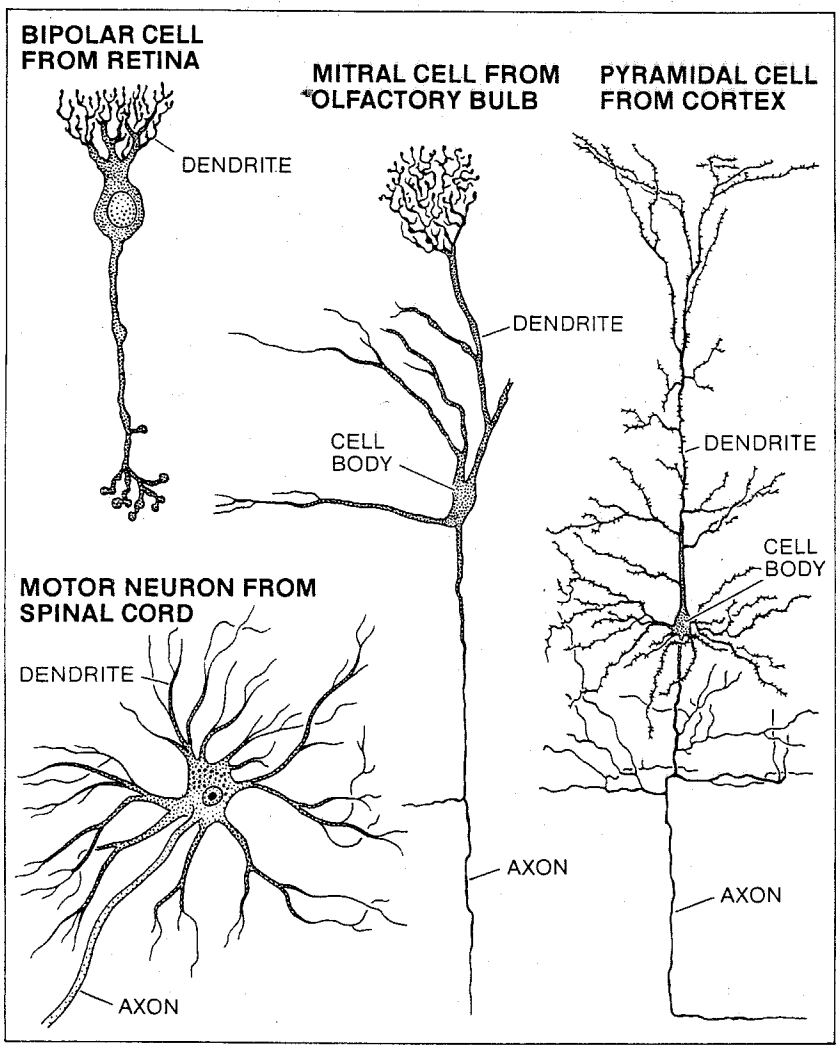
The study of the brain has become one of modern biology's last frontiers. We are fascinated by how we think, remember, and feel, and how such a complex machine capable of doing these things can assemble itself. It is the latter problem that I'm going to address—how the brain gets built. There are many aspects to this problem, but one of the central questions is the problem of neuronal diversity. The brain contains an enormous number of different types of neurons, not just one generic kind of nerve cell. The great neuroanatomist Santiago Ramón y Cajal recognized many of these different types of nerve cells almost a century ago. These neurons not only have different shapes, which were visible to Ramón y Cajal in the microscope, but are also specialized biochemically in ways that we can now observe with more modern methods. This great diversity of form subserves a diversity of function, which is extremely important for the way your brain works. Taken together, these different kinds of neurons amount to more cell types than are found in all the rest of the body combined.

How does a developing cell in the embryonic brain decide which type of neuron it's going to become? Does each precursor cell "know" intrinsically what type of neuron it is going to be, right from the start, or does it have a career choice? If precursors do have choices, what are they, and what mechanisms control the actual choice? To what extent are the choices reversible, once made? These are some of the specific questions that my laboratory has set out to address.

Our general plan of attack is quite straightforward. First we isolate the neuronal precursor cells. Then, under controlled laboratory conditions where we can vary the cells' milieu, we examine the developmental fates available to these cells. Thus we can determine the relative contributions of both the cell's local environment and its internal genetic programming, to its choice of developmental fate. In a sense, we are asking the nature versus nurture question at the cellular level.

Unfortunately, the brain is really just too complicated a structure to study in this reductionistic way. The brain contains between 100,000 and 1,000,000 different types of neurons, depending on how you define neuronal cell type. To attack this problem, therefore, we've had to break it up into bite-sized pieces that are relatively solvable. We've done that by making two simplifying decisions. First, we've decided not to work on the brain itself, but on another part of the nervous system called the peripheral nervous system. Second, we only work on two kinds of cells within that part of the nervous system.

The peripheral, or autonomic, nervous system is the part that lies outside of the brain and spinal cord, which together compose the central nervous system. The central nervous system controls conscious actions, such as deciding to call a friend, remembering the phone number, and picking up the telephone. The peripheral nervous system controls important, but unconscious, bodily functions such as heart rate, digestion, and reproductive activity. So even though you



Kuffler, Nicholls, and Martin, *From Neuron to Brain*, 2nd ed., (1984)

The central nervous system contains between 100,000 and 1,000,000 types of nerve cells. Many of these cell types were first recognized by their shapes almost a century ago by Ramón y Cajal. Dendrites collect incoming signals from other cells, while the axon carries the cell's electrical output. The cell body houses the nucleus and the cell's metabolic and secretory machinery.

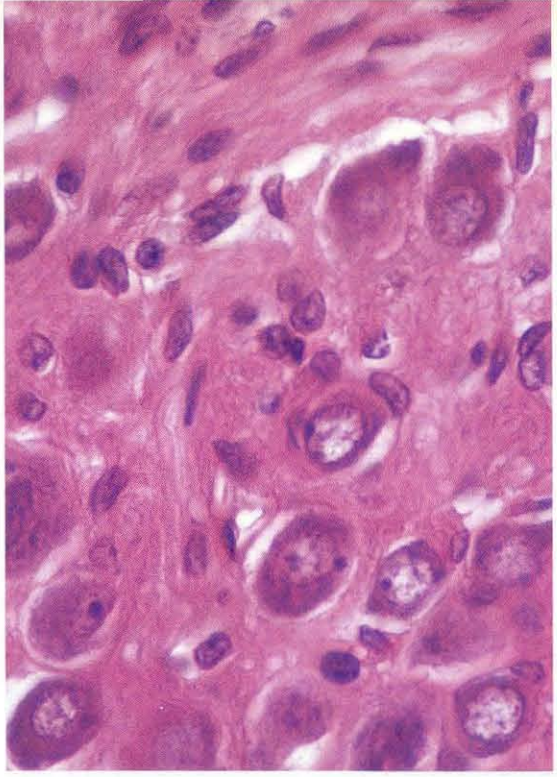
A chromaffin cell is a neuron manqué. It might have been a neuron, but during development it missed its calling.

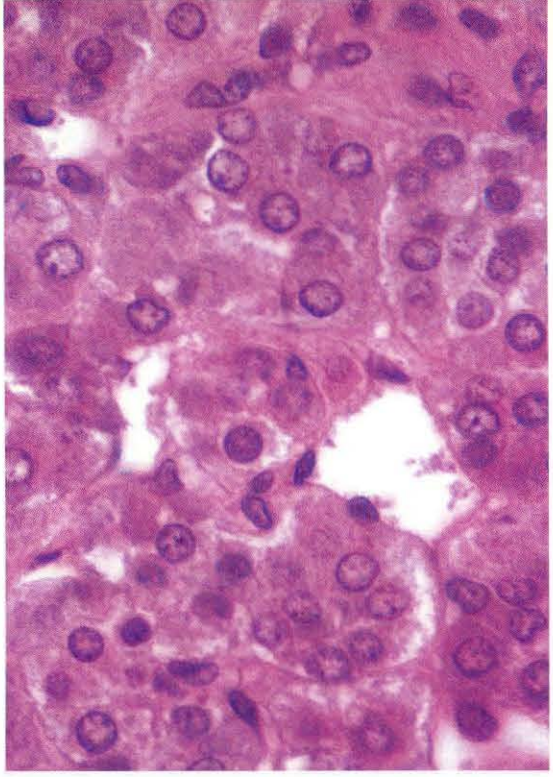
can't solve differential equations with these neurons, they're still very important to you.

Of the two particular cell types that we work on, one is the "sympathetic neuron" that exists in the little ganglia, or clusters of nerve cells, that run in two chains up and down either side of the spinal cord. Sympathetic neurons, like most other kinds of neurons, have both the long, thread-like structures ("processes") that are the wires of the nervous system, and the ability to make the connections ("synapses") that regulate the passage of nerve impulses from cell to cell. Each neuron actually has two types of processes—several comparatively short dendrites that collect incoming signals, and one very long axon that carries the cell's electrical output to its target tissue, such as the heart muscle.

The other kind of cell is an endocrine or secretory cell, called a chromaffin cell, found in the adrenal gland. The adrenal gland, a nutlike object that sits on top of the kidney, is really a gland within a gland. The outer zone is the adrenal cortex, which makes steroid hormones. The chromaffin cells exist in an inner zone called the adrenal medulla. They synthesize adrenaline, releasing it into our bloodstream when we're frightened or excited, accelerating our heart rate and preparing us to flee or fight. Chromaffin cells are little rounded cells without processes.

These two cell types appear very different superficially, but actually they are quite similar. Chromaffin cells, although they lack axons and dendrites, have much of the same molecular machinery as neurons—that involved in the synthesis, storage, and release of the catecholamines,

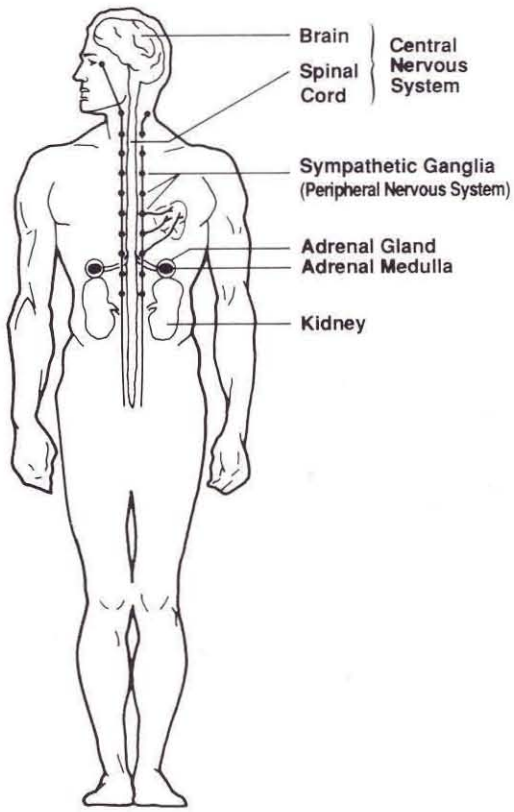




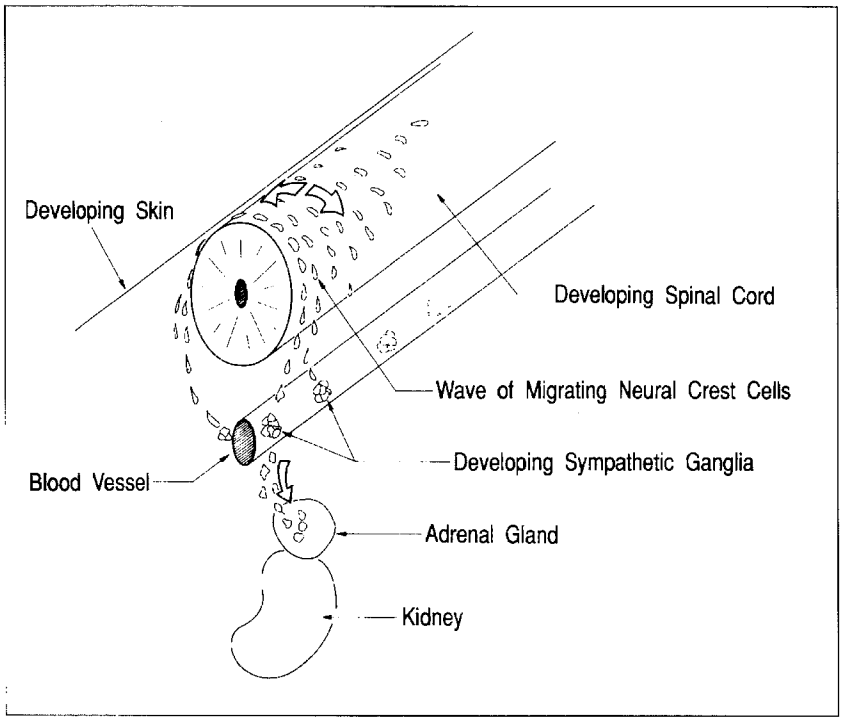
Right: A section through a sympathetic ganglion. The purple blobs are sympathetic-neuron cell bodies, and the whitish pepperoni-like structures within them are the nuclei. (The axons and dendrites aren't visible.) The stream of smaller cells crossing the top of the photo and proceeding down its left-hand side are the glial cells associated with a bundle of nerve fibers. Far right: A section through the adrenal medulla. The chromaffin cells are clustered around blood vessels (white voids) so that their adrenaline goes directly into the bloodstream. The cell bodies are indistinct, but the darker nuclei are clearly visible.

a biologically important family of chemicals. (Like a production pipeline in a chemical factory, this machinery makes dopamine, a simple catecholamine, from raw materials. Additional steps down the pipe convert dopamine into noradrenaline, and that into adrenaline. This pipeline occurs in several cell types. The cells open valves at various points along the pipeline to draw off different products—some central-nervous-system neurons make dopamine, while the sympathetic neurons produce noradrenaline, and the chromaffin cells secrete adrenaline.) Furthermore, chromaffin cells and sympathetic neurons both have electrically excitable membranes.

Thus it is accurate to say that a chromaffin cell is a neuron manqué. It might have been a neuron, but during development it missed its calling. In contrast to most other specialized cell types in the body, however, chromaffin cells retain throughout life the option to change their career in a way that many of us might envy. If these cells are taken out of the organism and exposed to a substance called nerve growth factor (NGF), they are able to drop their secretory-cell properties and acquire the properties of a nerve cell. They'll throw out processes—develop dendrites and axons—and make synapses. This involves not only a change in the shape and size of the cell, but also a change in the set of genes the cell turns on in its nucleus. This plasticity is an unusual phenomenon, and adds an extra element of interest to this developmental system. It may also be relevant, at some level, to understanding other forms of neural plasticity, such as regeneration and learning.



A road map for precursor-cell migration. The cells peel off the developing spinal cord in a wave. Some pull over and park along a nearby blood vessel to form the sympathetic ganglia, while others continue on to the adrenal gland.



The precursor cells peel off the neural tube and migrate downward through the embryo like tiny parachutists leaping from a moving airplane.

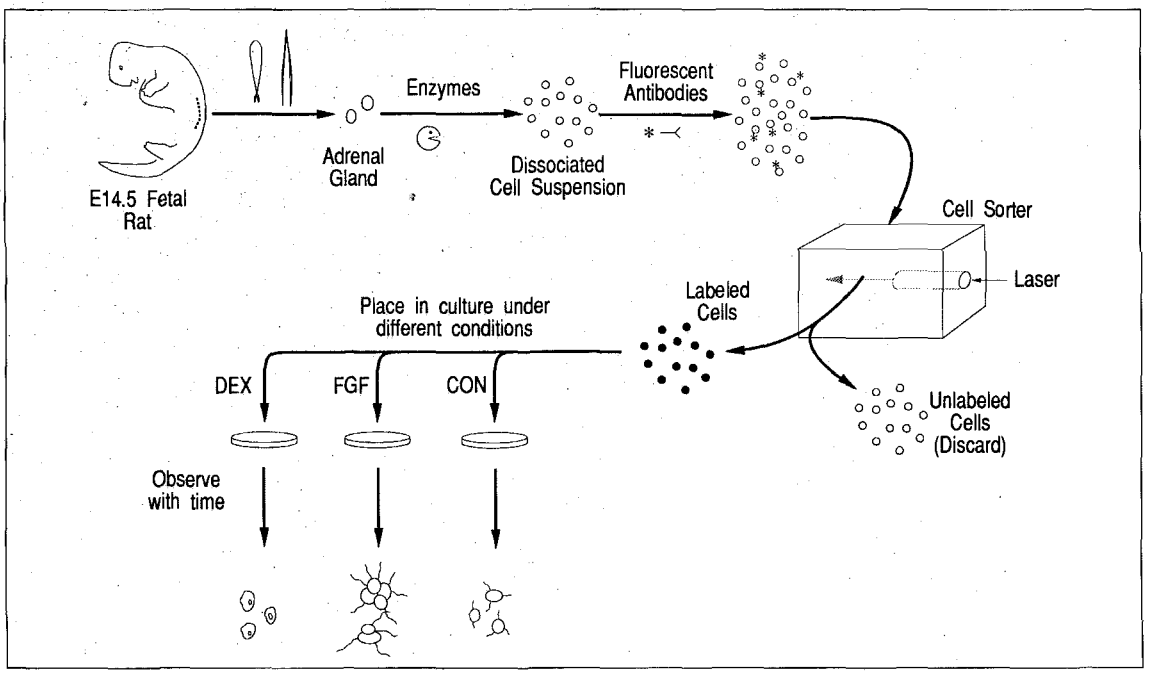
In the early 1980s, the functional similarities between sympathetic neurons and chromaffin cells, as well as the ability of adult chromaffin cells to convert into neurons, suggested to Allison Doupe, now a research fellow in biology, and Paul Patterson, now professor of biology (both then at Harvard) that these two cell types might arise from a common progenitor during embryonic development. We therefore set out to test this hypothesis in rat embryos.

According to classical descriptive embryology, the earliest undifferentiated precursor cells in this lineage can first be identified about midway through gestation. The cells become apparent when they detach themselves from the dorsal side of the neural tube (the embryonic spinal cord) to form a transient structure called the "neural crest." The neural crest first appears just behind the brain and propagates as a wave along the neural tube to the tail. The precursor cells peel off the neural tube and migrate downward through the embryo like tiny parachutists leaping from a moving airplane. Some of the cells stop migrating very quickly and form a chain of small clumps along a nearby blood vessel, where they eventually become sympathetic neurons. Others continue their migration downward to invade the developing adrenal gland, where they proliferate and become chromaffin cells. It's like the pioneers who came west—some stopped in Colorado, while others continued on to California.

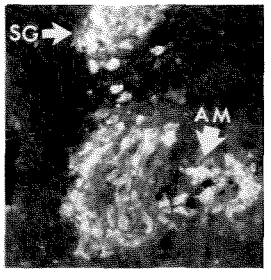
We can begin to see differences in the cells' development shortly after the migration ends. We and the Patterson laboratory have developed specific monoclonal antibody stains for these various cell types. Cells in the ganglia bind to antibodies specific for sympathetic neurons, whereas cells in the adrenal gland bind to antibodies specific for chromaffin cells. These anatomical observations support the idea of a common precursor, and suggest that the fate of a cell might be controlled by the environment into which it migrates.

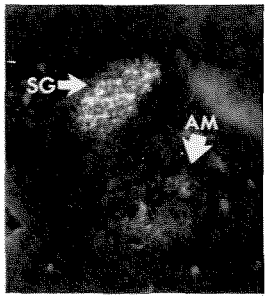
One way to test this hypothesis would be to transplant these cells to a different part of the embryo, and see how they developed there. However, this kind of experiment is very difficult to perform, especially in something as small as a rat embryo. As an alternative, therefore, we decided to isolate these cells from the embryo and put them in culture dishes. In that way we are able to watch them develop in an environment that we can control by adding different factors to the culture medium.

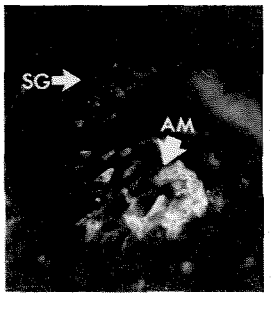
We isolate the precursor cells just after their migration into the adrenal gland. First, we dissect out the glands using very fine instruments.



The experimental scheme. "E14.5" is the number of days into gestation—a rat comes to term in about three weeks. "DEX" is a synthetic glucocorticoid that converts the precursors to chromaffin cells; "FGF" stands for Fibroblast Growth Factor, which drives the conversion to neurons; "CON" is a control culture to which neither substance is added and whose cells start to become neurons but don't go very far.



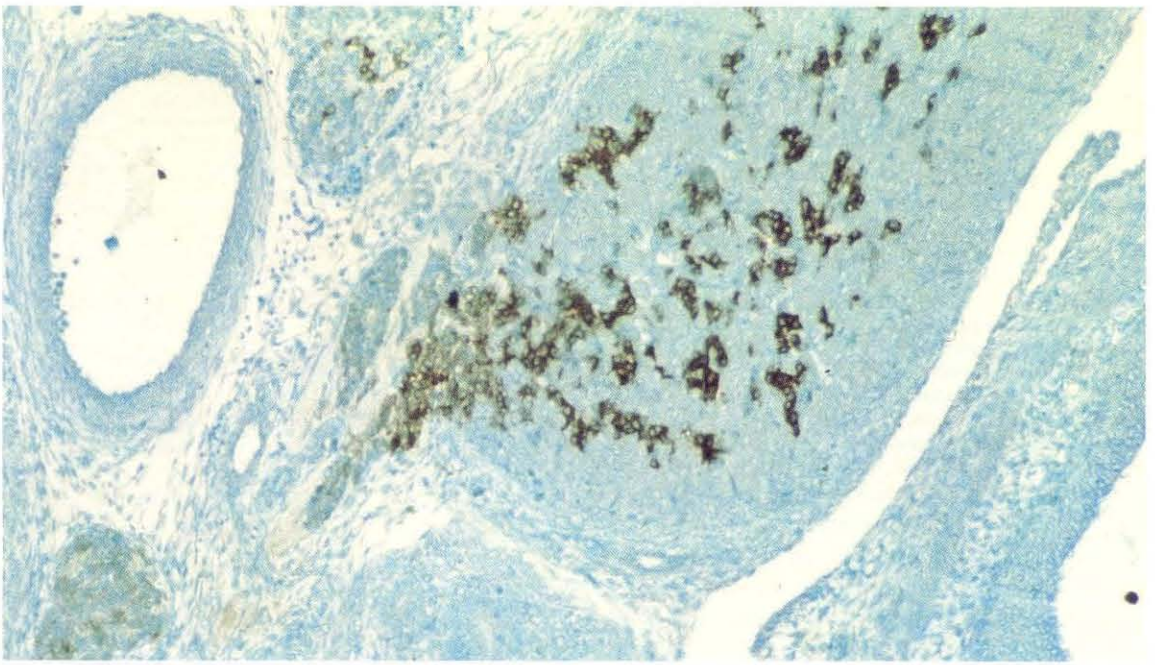




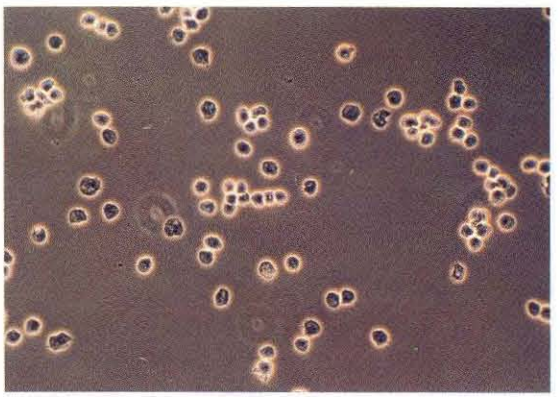
These sections through a rat embryo, magnified 70×, were made at embryonic day 14.5, about one day after migration begins. The migration will continue for three or four days longer, but differences can already be seen among the crest cells that have reached their destinations. Top: Tyrosine hydroxylase recognizes both sympathetic ganglia (SG) and adrenal medulla (AM) cells. Middle: B2, a monoclonal antibody developed by Jane Dodd at Columbia, recognizes sympathetic neurons only. Bottom: SA-1, a monoclonal antibody developed by Josette Carnahan and Paul Patterson at Caltech, is specific for chromaffin cells. The cells still look alike, and without these antibodies, it would be very difficult to tell them apart.

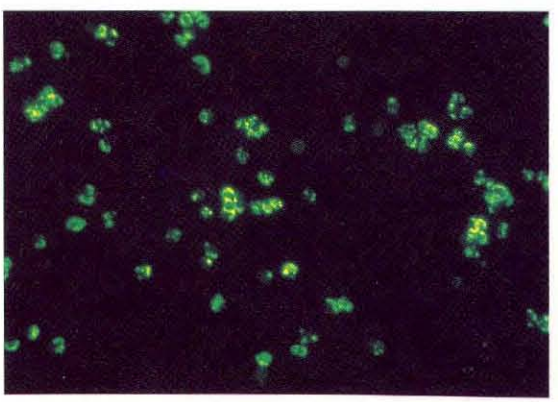
We then dissociate the glands into a suspension of individual cells, using proteolytic enzymes that cleave the bonds holding the cells together without damaging the cells themselves. This leaves us with a soup containing the cells we're interested in—the migrating precursors—mixed in with all the other adrenal cells. We use other specific antibodies to tag the precursor cells' surface with a fluorescent dye. We then run the soup through an extremely sophisticated and very expensive instrument called a Fluorescence-Activated Cell Sorter (FACS)—a part of the biology division's cell-sorter facility, which is run by Associate Professor of Biology Ellen Rothenberg. The FACS dribbles our soup through a laser beam. The soup is so dilute that each droplet contains, on average, only one cell. The laser excites the fluorescent dye, making the tagged cells glow. The other cells don't glow. If the machine senses that a tagged cell has been excited, it diverts that droplet into one tube. The other droplets fall into another tube. The precursors constitute about five percent of the starting cell population, so we obtain about a 20-fold purification.

We can now grow these isolated cells in a culture dish and watch how they develop under different environmental conditions. We divide the cells into different dishes whose culture media contain not only the nutrients and goodies necessary to keep the cells healthy, but also specific molecules whose influence on the cells' fate we would like to determine. We find that in order to get the precursors to develop into chromaffin cells, as they would have normally



Precursor cells, stained brown, caught in the act of invading the adrenal gland. These cells will set up housekeeping in the gland's center, completely surrounded by the cells of the adrenal cortex. The developing adrenal gland is the darker blue region in the center of the picture; the top of the kidney is visible below it. The white oval to the adrenal gland's left is the dorsal aorta, and a portion of the gut can be seen to the gland's right.





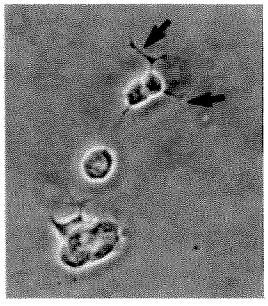
Above: A suspension of precursor cells from the FACS, as seen under phase-contrast microscopy. Below: The same cells with their fluorescent antibodies aglow. Virtually every cell in the suspension is a tagged precursor.

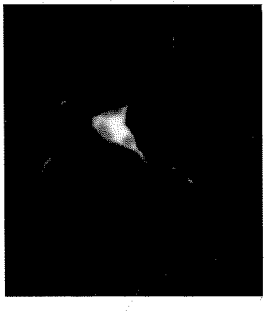
done in the embryo, we have to add substances called glucocorticoid hormones to the culture medium. If we don't do this, the cells begin to differentiate into sympathetic neurons, extending little nubbinlike processes, but they don't develop very far. We can push the cells all the way into bona fide neurons by adding two proteins called Fibroblast Growth Factor (FGF) and Nerve Growth Factor (NGF). This result strongly suggests that individual precursor cells have at least two possible alternatives—they can develop into chromaffin cells or into sympathetic neurons. Moreover, the choice does, in fact, appear to be influenced by factors in the cells' environment. In a complementary experiment, Patterson and graduate student Josette Carnahan exposed precursor cells taken from the sympathetic ganglia to FGF, NGF, and glucocorticoids and obtained similar results.

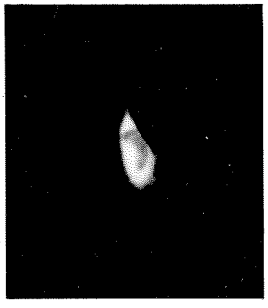
Why should glucocorticoid hormones be necessary for the precursors to develop into chromaffin cells? This result makes sense because these hormones are secreted by the cells of the adrenal cortex, through which the invading crest cells migrate en route to forming the adrenal medulla within. (I should point out that this process of migration, invasion, and proliferation is very similar to what happens when a cancer becomes metastatic and invades different parts of the body from a primary tumor. This is an example of how understanding fundamental biological mechanisms is relevant to understanding clinical specifics of disease.)

Glucocorticoids are a particular type of steroid hormone. The steroids are a family of fatty

Top: Undifferentiated precursor cells. The arrows point to the nubbinlike processes. Middle: Adding FGF changes the cells to neurons. Here three neurons have grown up in a cluster. The three gray blobs in the white body are the three nuclei. Thread-like processes are also visible. Bottom: Adding glucocorticoids produces a chromaffin cell. The gray banana shape is the nucleus. All three photos are magnified 250×. The top photo is of unstained living cells, but the cells in the other two photos have been fixed and stained with fluorescent antibodies.







On the one hand, the precursor cell is endowed with a limited repertoire of potential fates; on the other hand, it must choose one fate from among this repertoire.

molecules very similar to cholesterol. This family includes the sex steroids such as estrogen, and the anabolic steroids (used illegally by some athletes), as well as the glucocorticoids such as cortisol. Cortisol is the main glucocorticoid secreted by the adrenal cortex, and plays a number of roles in the adult, including controlling the inflammatory response. In the embryo, however, one of its functions is apparently to drive the neural crest cells that migrate into the adrenal gland down the chromaffin pathway of differentiation. And the precursors are migrating right into the belly of the beast—they're crawling right into the site where the steroid is synthesized, where its local concentration is extremely high. Thus the outer part of the gland-within-a-gland (the cortex) controls the development of the inner part (the medulla).

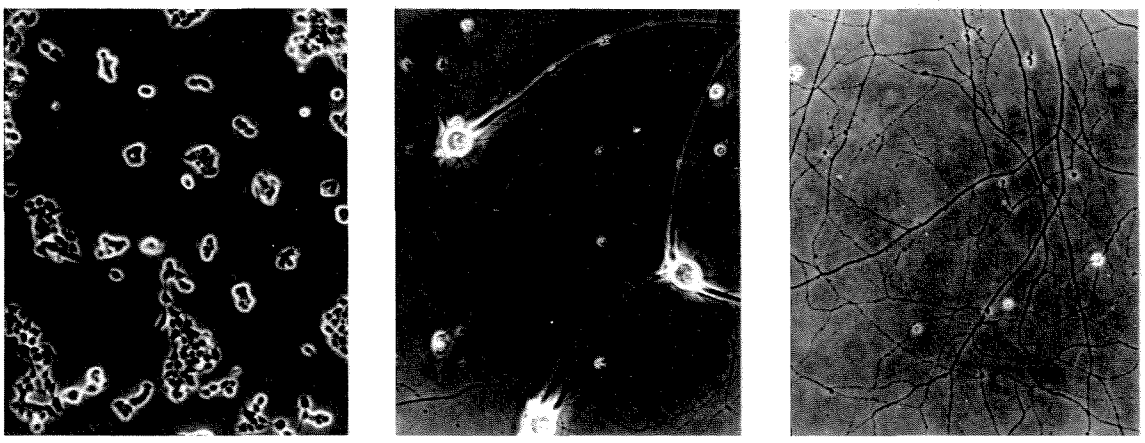
What about those cells that don't migrate into the adrenal gland, but become neurons in the sympathetic ganglia? We would like to think from our results that FGF is concentrated in the ganglia, in the same way that glucocorticoids are concentrated in the adrenal gland. The cells that stayed in the ganglia would therefore be exposed to FGF. However, we don't yet know if this is actually true. We are now using sophisticated molecular-biological techniques to see if FGF is really concentrated in the ganglia at the time that the neurons are differentiating.

This work shows how a peripheral-nervous-system cell can choose between two different fates, according to signals in the environment into which it migrates. We think that the same type of mechanism we have observed may well be true for central-nervous-system precursors that give rise to different kinds of neurons in the brain. However, this isn't the whole story.

Most other cells in the embryo—or even in the neural crest, whose cells also go on to become the bones in your face, the pigment-producers in your skin, the insulation in your nerves, and many other cell types—would not turn into sympathetic neurons or chromaffin cells in response to the hormones we've added to our culture dish. This is because the cells have to be conditioned, by their previous history, to respond to particular hormones in a particular way. Thus the cells that arrive at the sympathetic ganglia or the adrenal gland have already made, we believe, several earlier decisions that limit their choice of fate to only two final options. It's like going out for dinner—first you choose a particular kind of restaurant: Italian, Chinese, or Mexican; then, once you arrive there, you're committed to a particular *type* of food, but you can still choose from among the various dishes on the menu. The neuron-chromaffin decision is analogous to choosing among the different dishes—it's one of the final steps. To continue the analogy even further, the chromaffin cell's plasticity means that even after it has ordered dinner and started to eat, it can still send its choice back to the kitchen and order something else.

The neuron-chromaffin development decision involves an interplay between the cell's internal genetic programming and specific signals in the local environment. On the one hand, the precursor cell is endowed with a limited repertoire of potential fates; on the other hand, it must choose one fate from among this repertoire. What genes and proteins actually determine the specific repertoire of possible fates? What genes and proteins actually select a particular fate? These are the big questions we are pursuing in an effort to understand the molecular biology of this developmental system.

With the exception of the immune system, it is generally believed that every cell type in the human body, be it a neuron or a liver cell, contains in its nucleus the same set of 100,000 genes. Only a small fraction of those genes are active in any one cell type, however, and what distinguishes one type from another is the particular set of genes that the cell chooses to turn on. So at the molecular level, choosing the neuronal fate means that the precursor cell has decided to turn on some genes that are characteristic of neurons. Conversely, choosing the chromaffin fate means that the cell has decided to turn on chromaffin-specific genes. Our lab and others have cloned genes representative of each of these



Left: A culture of immortalized precursor cells. Middle: A culture of neurons made from the immortalized precursors. Right: Processes from these neurons grow to the farthest reaches of the dish. All three photos are magnified 100×.

Each gene is like a lamp with a dimmer switch.

two complementary classes. This enables us to study the ways in which these genes are turned on and off during development.

We have found that these genes appear to be regulated at two main levels. At one level, there is "all or nothing" control—in tissues unrelated to the nervous system, such as the liver, these genes are completely shut off. The second level of control is "more or less"—in chromaffin cells and sympathetic neurons, these genes are either turned up or turned down, according to the cell's local environment. For example, the glucocorticoid hormones that promote the differentiation of the precursor cell into a chromaffin cell act to both turn up the chromaffin-specific genes and turn down the neuron-specific genes. The converse is true for environmental signals that promote neuronal differentiation, such as FGF and NGF. A useful analogy might be that each gene is like a lamp with a dimmer switch. In the liver, the lamp is completely unplugged. In the chromaffin-neuron precursor, the lamp has been plugged in. Later, during differentiation, the lamp is turned up or turned down by the dimmer switch, depending upon which pathway the cell chooses. The environmental signals—the glucocorticoids, FGF, and NGF—control the dimmer switch.

One of the problems we've encountered in studying these precursor cells on the molecular level is that only a small number of cells can be recovered from rat fetuses. We've circumvented this problem by applying recently developed techniques to immortalize these cells. We use a defective retrovirus as a disposable molecular syringe to inject the precursor cells with a gene, called *v-myc*, that prevents them from differentiating and allows them to divide forever in the culture dish. (The precursor cells normally divide a limited number of times, and then stop for good when they differentiate.) Thus we get an endless supply of cells for experiments at any time, without having to first perform long hours of dissection on large numbers of rat fetuses. Fortunately, these immortalized precursor cell lines still appear capable of undergoing differentiation into sympathetic neurons when exposed to FGF.

These cell lines have taught us something very interesting. Developing neurons eventually come to need NGF to survive, and they acquire this chemical dependence only *after* they have first been exposed to FGF. Thus it takes at least two different factors, acting at different times, to make a neuron. I like to call this a "relay mechanism." FGF starts the cell down a pathway of differentiation and takes it through one stage, setting it up to respond to NGF. The cell can then bind to NGF, and this second factor moves it further down the pathway of differentiation into a full-blown neuron. We don't yet know if this is true in vivo, but this hypothesis makes testable predictions.

The reason that two factors may be needed is presumably that the first one is located in the ganglia, whereas the second one is located in the target tissues into which the axons grow—the heart muscle, for example. FGF starts the neurons growing their axons toward the targets, while NGF takes over after the axons arrive,

helping to maintain the synapses. Neurons whose processes don't reach an NGF-secreting tissue die, which is one way that the developing nervous system ensures that the body gets wired up correctly. This two-stage process is somewhat analogous to sexual differentiation in people. The decision as to whether you will be male or female is made very early in your development, but you still have to go through puberty after you're born. FGF, we believe, controls the initial decision, and NGF controls the later maturation that is the cellular equivalent of puberty.

Although we developed these immortal cell lines for basic research, they may turn out to have an unexpected clinical relevance as well. You probably know someone who is afflicted with Parkinson's disease. It's a relatively common disease that usually begins late in life and is characterized by an uncontrolled tremor in the extremities, sometimes referred to as a pill-rolling tremor. This symptom occurs because a group of neurons in the brain—called the substantia nigra, because its cells contain a black pigment —dies. These neurons normally provide a neurotransmitter, or chemical messenger, called dopamine to another part of the brain called the striatum. Without dopamine, the striatum neurons don't function and the tremor occurs.

This therapy may not be limited to Parkinson's disease.

Neurosurgeons have recently begun to pioneer a technique for treating Parkinson's disease in which the patient's chromaffin cells—the very cells you've been reading about this whole time —are grafted into his or her brain to replace the dead neurons. Chromaffin cells were chosen for two reasons: one, they can manufacture dopamine by opening the proper valve on the pipeline; and two, they ought to change into neurons in the brain just as they do in the culture dish. Although this technique appeared very promising at first, it now seems that the original idea of using the patient's own chromaffin cells doesn't work very well. However, all is not lost. Animal experiments have shown that the technique works much better when fetal cells rather than adult cells are used. Presumably the immature cells can adapt to the new environment of the brain more readily. Cells apparently get old and set in their ways, just as people do.

The routine therapeutic use of human fetal tissue isn't likely to happen in the foreseeable future for a number of reasons, ethical as well as technical. One way out of this dilemma would be to use immortalized fetal-cell lines such as the ones we have developed from rats. That is, if one could gain access to some human fetal adrenal glands just once, and immortalize those cells in the way that we've immortalized the rat cells, in theory one would have a continuous supply of human cells available for transplantation therapy; on tap, as it were. To this end, we're collaborating with Dr. Fred Gage of UC San Diego to test the viability and therapeutic value of our cell lines by transplanting them into rats that have been given a chemically induced form of Parkinson's disease. Interestingly, this therapy may not be limited to Parkinson's disease. Under certain circumstances, these cells can also secrete acetylcholine, another neurotransmitter. Patterson is trying to determine if this ability can be exploited in analogous transplants to treat Alzheimer's disease.

In this article, I have tried to describe our approach to investigating an important question in neurobiology—how the different kinds of neurons in the brain form during embryonic development. We have seen that approaching this question requires making some decisions to simplify the problem, and then combining a variety of techniques at both the cellular and molecular levels to find out what's going on. In this case, the answer to the question of nature versus nurture is "a little of both." Nature, or the genetic programming of the precursor cell, endows the cell with a limited set of developmental options. Nurture, or the signals from the environment into which the cell migrates, helps it to choose from among these options. At the molecular level, this choice involves a complex network of regulatory genes that we now only dimly understand. These developing cells, therefore, make choices in their lives just as we do, as we grow up. How satisfying, then, that understanding these little cells and their microworld may help us to one day treat diseases that make it so hard for many people to live their later years fully, when they should be enjoying the benefits of their own career choices.□

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