Helping Neurons Find Their Way

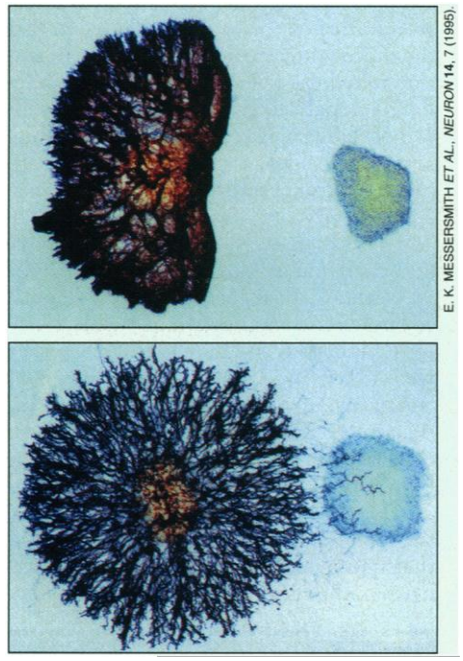
A flurry of recent papers suggests that the growing axons of neurons are guided to their targets by diffusible chemorepellents as well as by attractants

If you've ever been reduced to despair by a last-minute Christmas Eve effort to assemble a bicycle as a gift for one of your children, take heart. Your assignment could be worse. Much worse. Try assembling the nervous system of a developing embryo. While you would have more than one frantic evening to accomplish the job, in higher organisms the task requires making billions of precise connections between nerve cells, as well as between nerve cells and muscles and other body tissues. Often the axons, the nerve cell projections that make the connections, must travel distances of up to several feet—cosmic distances for cells—through a maze of many tissues to find their targets. And, as with bicycle assembly, the consequences of failure aren't trivial. As neurobiologist Lou Reichardt, a Howard Hughes Medical Institute (HHMI) investigator at the University of California, San Francisco (UCSF), puts it, "The nervous system doesn't work unless it's wired properly."

The obvious significance of axon guidance has made understanding the system a very high priority for neurobiologists. But its complexity has made it a difficult problem to crack. Indeed, until the past few years, the maze was almost impenetrable. But a flood of recent publications from labs in Europe, Japan, and the United States shows that researchers are finally beginning to find their way through the axonal guidance labyrinth. The latest developments are reported in a remarkable flurry of eight papers appearing in the May and June issues of *Neuron* and in the 19 May issue of *Cell*.

This recent growth spurt started last year, when axonal guidance research got a big boost from the discovery by a group led by Marc Tessier-Lavigne, also an HHMI investigator at UCSF, of netrin-1. This protein is secreted by neuronal target cells into the surrounding tissue, where it attracts the correct axons. Developmental neurobiologists had been looking for such a diffusible chemoattractant for decades, but this was the first molecule that seemed to fit the bill. The new flood of papers fills out the axonal guidance story by showing that secreted chemorepellents—proteins that tell growing axons to "stay away"—are every bit as important as those that say "come here."

Researchers believe that there may be many such diffusible proteins that repel the searching axons, complementing the many secreted attractive factors that they believe exist. So far they have found three proteins with chemorepellent activity. These include, ironically, netrin-1, which apparently serves a dual purpose in axonal guidance, along with proteins known as semaphorin II and semaphorin III (or collapsin), which belong to a different family. And they have found that chemorepellent molecules appear to be secreted throughout the developing nervous system, occurring in the brain as well as the spinal cord and influencing pathfinding by several different types of neurons.



E. K. MESSERSMITH *ET AL.*, *NEURON* 14, 7 (1995).

Selective. Cells secreting semaphorin III repel one type of sensory neuron *(top)*, while a second type is unaffected and can extend all the way to the cells.

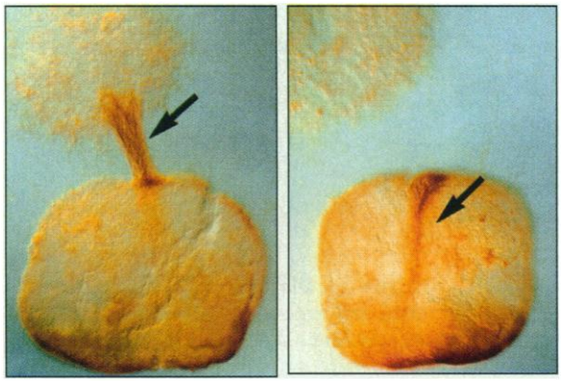
Adrian Pini of University College London, whose research 2 years ago provided the first evidence for diffusible chemorepellents, says that these latest discoveries "round off the possibilities for axonal guidance." He was referring to the fact that neurobiologists believe that there are four possible types of signals that could guide axons toward their targets: short-range repulsive and attractive cues provided by molecules on nerve cell surfaces, plus the diffusible chemoattractants and chemorepellents, which can act over longer distances.

The cell adhesion molecules involved in attraction were the first to come on the axonal guidance scene. In the early 1980s, researchers found that nerve cells carry molecules on their surfaces that can cause them to adhere to some other cells when they come into contact. The first evidence for short-range repulsion came later, toward the end of the 1980s, when three groups provided evidence that embryonic tissues carry membrane proteins that cause nerve cells to back away on contact. At the time, this finding was something of a surprise, according to axonal guidance researcher Corey Goodman, an HHMI investigator at the University of California, Berkeley. The reason: neurobiologists were "looking for 'turn-ons,' not 'turn-offs,' " he says.

Researchers soon succeeded in identifying some of the cell surface molecules involved in both short-range attraction and repulsion, and these discoveries focused attention on contact-mediated cues for guiding axons to their targets. "Molecules involved in guidance were thought to be acting through adhesion," says Jonathan Raper of the University of Pennsylvania School of Medicine, whose team was one of those that discovered that neurons may be repelled when they contact another cell.

Despite this emphasis on the short-range, contact-mediated cues, however, there was also reason to believe that axons might be guided by signals that act over much longer distances as well. Indeed, the suggestion that neuronal targets might release such guidance molecules dates all the way back to work performed a century ago by Spanish neurobiologist Santiago Ramón y Cajal. Finding these molecules was another matter, however, and neurobiologists didn't get their hands on a good candidate until last year, when Tessier-Lavigne's group came up with netrin-1 (*Science*, 28 October 1994, p. 568).

Their work showed that netrin-1 is made by the floor plate, a strip of tissue that runs along the lower (ventral) surface of the developing spinal cord, where it helps guide the axons of a particular type of spinal cord neuron: the commissural neurons, which relay information about pain and temperature to the brain. While these neurons are born in the dorsal (upper) surface of the spinal cord, their axons don't grow directly to the brain. They first project down to the ventral surface of the cord and cross the floor plate. Tessier-



PHOTOS BY S. COLAMARINO AND M. TESSIER-LAVIGNE/UCSF

Hostile territory. Trochlear motor neurons *(arrows)* emerging from a neural explant grow into control cells *(left)* but are repelled by cells secreting netrin-1 *(right)*.

Lavigne's group showed that netrin-1 may help guide them along their way by acting as a diffusible positive signal to draw the commissural nerve axons to the floor plate.

One more to go

With this discovery, researchers had bagged examples of three of the four possible types of axonal guidance cues. Still missing were diffusible chemorepellents. But those wouldn't be long in coming. Pini had provided evidence for their existence in 1993, in experiments Tessier-Lavigne describes as "seminal work." In that research Pini exploited a culture system devised by Andrew Lumsden of Guy's Hospital in London and Alun Davies of St. Andrews University in Scotland that had already shown its merits by demonstrating the existence of diffusible attractants. The system involves culturing two samples of nerve tissue some distance apart within a collagen gel. Because the tissue samples aren't in contact, the only way they can influence each other is by releasing a substance that diffuses through the gel. In the experiments of Pini and his collaborators, axons of some neurons actually turned and grew away from other neurons, indicating that the others were secreting repellents.

At the time, the specific identities of the repellents weren't known. But even as the Tessier-Lavigne group was showing that netrin-1 is a chemoattractant for commissural neurons, there were also hints that this molecule might be a chemorepellent for other nerve cells. The first hint came from analysis of netrin-1's sequence, which revealed that it is the chicken equivalent of a protein from the nematode *Caenorhabditis elegans*, UNC-6, whose gene had been cloned by Ed Hedgecock of Johns Hopkins University, Joseph Culotti of Mount Sinai Hospital in Toronto, and their colleagues.

Studies of neuronal growth during development in *unc-6* mutants indicate that the protein is involved in guiding axons of two classes of neurons that migrate in opposite directions. This suggested that UNC-6 attracts the one while repelling the other. The DNA sequence similarity suggested that the same might be true for netrin-1, and Tessier-Lavigne and UCSF colleague Sophia Colamarino set out to find out if that was in fact the case.

In the 19 May issue of *Cell*, they provide the answer—which is a resounding yes. For these experiments, the researchers chose embryonic trochlear motor neurons, which help control eye movements. Unlike the axons of commissural neurons, trochlear neuron axons grow away from the floor plate, not toward it, a growth pattern that suggests the floor plate might repel the axons of the trochlear neurons. And that is exactly what Colamarino and Tessier-Lavigne found when they put trochlear neurons into the gel culture assay with floor plate tissue. To prove that the effect was due to netrin-1, the researchers transferred the netrin-1 gene into a type of nonneuronal cell that otherwise has no effect on axon guidance. The altered cells also repelled trochlear neuron axons. "The bifunctionality [of netrin-1] mirrors the dual action of UNC-6," Tessier-Lavigne says. "In one fell swoop, we've identified a diffusible attractant and a diffusible repellent."

Netrin-1's dual guidance effects may not be limited to commissural and trochlear neurons. In the May and June issues of *Neuron*, a team led by Fujio Murakami of Osaka University in Japan describes results showing that axons of brain neurons respond to floor plate tissue much as spinal neurons do: Those that normally cross the floor plate during development are attracted by it, while those that don't cross it are repelled. "My guess is that many neurons share the [same guidance] mechanism," Murakami says. Further support for that idea comes from Sarah Guthrie of Guy's Hospital and Pini, who also report in the June issue of *Neuron* that the floor plate repels axons from certain motor neurons in the brain and spinal cord.

The Osaka team found that netrin-1 attracts the same neurons attracted by the floor plate in their studies, but haven't yet examined the protein's effects on the neurons that are repulsed. And while the London groups haven't done molecular studies to try to find out what was causing the effect they saw, the investigators predict that netrin-1 will be involved. "We haven't yet tested netrin-1 on the neurons, but we would be pretty surprised if it doesn't work," Guthrie says.

And netrin-1 isn't the only diffusible molecule that has a repelling effect on other axons. Semaphorin III/collapsin also plays that role, at least in culture. This protein has two names because it was discovered independently by two groups. Berkeley's Goodman and his colleagues originally discovered the first member of this family, semaphorin I, in the grasshopper as a cell surface protein that influences axon steering and inhibits axon branching. They then went on to identify genes for related proteins in the fruit fly and human. Two of these, semaphorins II and III, turned out to be secreted molecules, suggesting that they might be diffusible guidance molecules, although how they might function was something of a mystery.

Meanwhile, Pennsylvania's Raper and his colleagues had noticed that the growing tips of some axons from chickens collapse when they contact certain others in culture. Their assumption: The collapse of the growth cones, as the axonal tips are called, could well have been caused by some inhibitory substance on the axon they encountered. (This in fact was one of the early experiments indicating the existence of chemorepellent substances for neurons.) The Raper

ROLE OF NETRINS AND SEMAPHORINS DURING GROWTH CONE GUIDANCE

Protein	Organism	Type	Function
Netrin Family			
UNC-6	Nematode	Secreted	Guides ventral migrations (attraction?) and dorsal migrations (repulsion?)
Netrin-1	Chicken	Secreted	Attracts commissural axons and repels trochlear motor axons
Netrin-2	Chicken	Secreted	Contributes to attraction of commissural axons (?)
Semaphorin Family			
Semaphorin I	Grasshopper Fruit fly	Membrane	Regulates sensory axon steering and branching in limb bud
Semaphorin II	Fruit fly	Secreted	Inhibits synapse formation of a subset of motor axons
Semaphorin III Semaphorin III/D Collapsin 1	Human Mouse Chicken	Secreted	Repels axons of a subset of sensory axons

group set out to identify the molecule that causes the growth cone collapse, which they called "collapsin." They ultimately cloned and sequenced the gene in 1993, and its sequence revealed that collapsin is the chicken equivalent of semaphorin III, whose gene had just been cloned by the Goodman group.

In search of a role

While this work suggested that semaphorin III is a chemorepellent, the protein's exact role has been unclear. But another paper in the May issue of *Neuron* provides some answers. This work, a joint effort by the groups of Berkeley's Goodman, UCSF's Tessier-Lavigne, and Carla Shatz, also an HHMI investigator at Berkeley, shows that the protein specifically repels growing axons of one group of sensory neurons in the rat without affecting those of another. What's interesting, says Lumsden, is that the results are "thoroughly consistent" with the behavior of those neurons in the living animal.

The axons of both sets of neurons studied by the California team enter the spinal cord through the dorsal surface. After entering the cord, the axons of one set (which transmit sensory information about muscle stretch and position) proceed to the ventral region. The axons of the other group (which transmit pain and temperature information) stop in the upper dorsal region. Using the standard collagen gel culture assay, the three-way collaboration showed that semaphorin III repels the axons of this latter group but not those that normally penetrate the ventral cord. They also found that semaphorin III is made in the ventral half of the cord, putting it in just the right place to act as a "sieve" that can keep out pain and temperature neurons while letting the others in.

There is still a caveat about the roles proposed for both semaphorin III and netrin-1, however. Most of the work so far has been done with cultured neurons, and the ultimate proof of what the proteins do awaits the creation of animals in which the genes have been knocked out to see whether that produces the expected neuronal guidance defects.

In addition to the collaborative work on semaphorin III, evidence is piling up for other diffusible chemorepellents. The Goodman group, in a paper in the 19 May issue of *Cell*, presents data showing that semaphorin II has inhibitory effects on growing axons, although it apparently acts by a mechanism different from that of semaphorin III.

In this case, the evidence does come from studies in a living organism. Goodman and his colleagues genetically engineered fruit flies so that semaphorin II would be made in a muscle that does not normally express the protein. They found that this change blocked the ability of certain neurons to form synapses, the specialized connections between neurons and their targets, with the muscle. This suggests that semaphorin II acts much closer in than semaphorin III, which repels neurons before they get near the wrong place.

But these differences may only scratch the surface of the possible activities of semaphorin family members. For one thing, the family is growing by leaps and bounds. In the May issue of *Neuron*, Heinrich Betz and his colleagues at the Max-Planck-Institut für Hirnforschung in Frankfurt, Germany, report cloning the genes for four new mouse semaphorins, while Raper's team has cloned four new genes from the chicken. (The paper will be in the June issue of *Neuron*.) A search of the databases indicates that humans also have several semaphorins, Goodman says.

Although the researchers do not yet know what the new semaphorins do, they have some clues. The Betz and Raper teams have found that each semaphorin gene has a specific expression pattern, with different genes being turned on in different tissues. This suggests that each repellent helps guide a different set of neurons. "As soon as you see there is a big family [of semaphorins], you can build in a great deal of specificity with just these repellent molecules," Goodman says. Indeed, agrees Lumsden, discovery of this family of molecules "suggests this is going to be a major factor in governing the projection patterns of neurons."

What's more, because some of the semaphorin genes are expressed in tissues, such as the lung, and others may function in the immune system, both places where they might be expected not to function in neuronal guidance, the importance of the proteins may extend beyond the nervous system. "We are all focusing on growth cone guidance, but they could be functioning in a variety of other things," Goodman speculates.

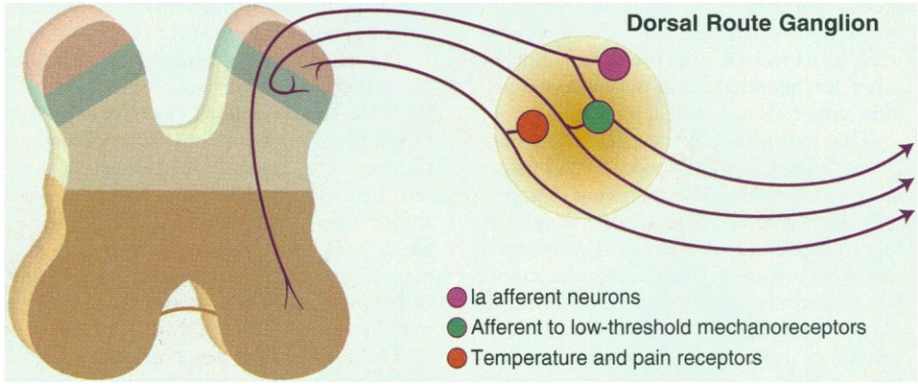
As the work progresses, researchers will try to decipher which neurons respond to which semaphorins, netrins, and other diffusible neuronal guidance molecules. They also want to know whether any of the semaphorins do double duty as netrin-1 does. Having such a system of diffusible guidance cues that repel, as well as attract, could be very useful to the developing nervous system, Pini suggests. If guidance works on such a "push-pull system," he says, "it would be a very efficient way of getting axons from one place to another over long distances."

An equally important question concerns the nature of the machinery by which neurons respond to the guidance molecules. This is especially true, Goodman notes, for molecules such as netrin-1 that can have opposite effects on different types of neurons. The reason for the differences must lie in the receptors and other components of the response machinery, as studies by the Culotti and Hedgecock groups already suggest. They have evidence that a protein called UNC-5 may be the receptor through which UNC-6 exerts its repellent—but not its attractive—effects on neurons.

And then there's the issue of what this growing repertoire of axonal guidance cues might mean clinically. Although the researchers are currently interested in the basic biology of the system, there has always been hope that understanding the molecules that guide neurons to their destination might lead to better treatments for people with spinal cord injuries and other nerve damage. Attractive molecules might be used, for example, to help direct regenerating neurons to their destinations. But researchers now know they will have to take the chemorepellents into consideration, too.

But despite the many mysteries remaining, the work has at least put diffusible guidance cues on a firm footing. And as Raper points out, the large number of unresolved issues has its own appeal. The work is "very exciting," he says, "because it raises a whole plethora of questions it will probably take years to answer." And, like diffusible molecules, those questions are drawing researchers over long distances toward the answers that are their targets.

–Jean Marx



SOURCE: E. K. MESSERSMITH *ET AL.*, *NEURON* 14, 7 (1995).

A molecular sieve? Semaphorin III produced in the ventral half of the embryonic spinal cord *(dark tan)* may repel the axons of temperature- and pain-sensory neurons while allowing in those of Ia afferent neurons that respond to muscle stretch.