Receptors Find Work As Guides

New evidence suggests that a large new family of receptor protein tyrosine kinases helps developing neurons to make the right connections in the brain

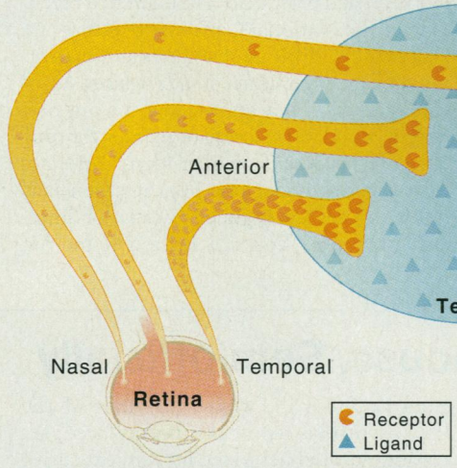
Several years ago, when Genentech biologist Ingrid Caras was casting about for a new research project, she considered collaborating with Friedrich Bonhoeffer at the Max Planck Institute in Tübingen, Germany. He was trying to clone the gene for an elusive protein that helps guide neurons from the retina to make appropriate connections in the brain. But Caras decided instead on a different project: a hunt for new members of a family of proteins called receptor protein tyrosine kinases (RPTKs), cell surface molecules that might themselves play important, although largely unknown, roles in the brain. Now, much to her surprise, Caras's project and Bonhoeffer's have converged.

In results described in the May issue of *Neuron*, Caras and her colleagues found a brain-specific receptor tyrosine kinase and another protein called AL-1 that activates the receptor. Meanwhile in Tübingen, Bonhoeffer's group identified its mysterious guidance protein. When their report came out in the 11 August issue of *Cell*, Caras recalls, a Genentech colleague who had previously collaborated with Bonhoeffer on his search came into her office and said, "Do you remember that Bonhoeffer protein? They finally cloned it, and guess what it is?" The answer: It turned out to be none other than AL-1.

That convergence provides the first concrete evidence of a function for the intriguing new family of RPTKs to which Caras's molecule belongs. These receptors, which are concentrated in the nervous system, have several unique characteristics that set them apart from other RPTKs. But until recently they had been "a family in search of a function," says developmental neurobiologist Marc Tessier-Lavigne, of the Howard Hughes Medical Institute at the University of California, San Francisco (UCSF). The Caras and Bonhoeffer results begin to fill that functional void, by adding the receptors to the long list of molecules that help guide developing axons, the threadlike projections of nerve cells, to their targets.

Other early clues suggest the receptors play a role not only in guiding axons, but also in guiding cell migrations. What's more, they may do so by tapping into a different set of internal signaling molecules than other RPTKs, perhaps triggering a chain of events that directly alters the structural proteins that make cells move. "It is clear," says Salk Institute neurobiologist Greg Lemke, "that these receptors work in quite different ways from your garden-variety receptor tyrosine kinases."

This group of maverick RPTKs is known as the Eph family, named after its first member, discovered in the mid-1980s in Fumimaro Takaku's lab at the University of Tokyo. Takaku's group was looking for proteins with a structural signature characteristic of tyrosine kinases, enzymes that trigger changes inside cells by adding phosphate groups to key intracellular proteins. Takaku's team identified the gene for a new RPTK, which they called Eph, for the erythropoietin-producing hepatic (liver) cell line in which it was found.



Unfriendly territory. A gradient of Eph receptor ligands may steer temporal neurons away from the posterior tectum.

As other groups found other receptor tyrosine kinases with a similar makeup, the Eph family began to reveal its distinctive features. In the late 1980s, Richard Lindberg, then a postdoc with Tony Hunter at the Salk Institute, found the second family member, which he named Eck for epithelial cell kinase, and shortly after that, Tony Pawson's team at Mount Sinai Hospital in Toronto cloned a third family member, Elk, for Eph-like kinase. With Elk, Pawson's group saw signs of what would be a recurrent theme for this family: "Its expression was almost exclusively restricted to the brain and the nervous system," Pawson says.

As the RPTK gold rush continued, the Eph family grew to include more than a dozen members, becoming "by far the largest receptor tyrosine kinase subfamily that people have characterized," says Salk's Lemke, whose lab discovered several members. And most of these new proteins showed up in the nervous system.

Besides their novel distribution in the nervous system, another clue that these receptors operate differently than other RPTKs came from Pawson's group at Toronto in 1993. The researchers performed on the Elk receptor a standard test of new RPTKs: inducing connective tissue cells known as fibroblasts to make a receptor by giving them an active copy of the gene and then activating the receptor to see what happens to the cells. Because the Elk receptor's natural activating molecules (known as ligands) had not yet been found, the Pawson group genetically engineered the Elk gene so the Elk protein could be activated by epidermal growth factor instead.

A new kind of receptor biology

RPTKs are most commonly known for the important role many of them play in cell growth. And even those RPTKs that do other things, such as controlling cell survival or differentiation, trigger growth in this test. But when Pawson's group conducted the test on the Elk receptor, the fibroblasts didn't grow. That suggested that Elk and other members of the Eph family tie in to a different set of internal signaling proteins than other RPTKs. That revelation gave researchers the feeling that "if we could find the ligands, we would get into a whole new area of biology," says Harvard developmental biologist John Flanagan.

And now that feeling is being borne out. Last year a flurry of ligand findings began to hit the press, beginning with the discovery by Lindberg, now at Amgen Inc. in Thousand Oaks, California, of the ligand for the Eck receptor. Before the dust settled, multiple labs had added ligand discoveries to the list, bringing the number of known Eph family ligands to seven, many of which—like the receptors themselves—were independently discovered by several labs.

The ligand discoveries brought more surprises. Several groups showed that the ligands for Eph receptors remain bound to the membranes of cells that produce them,

unlike most other RPTK ligands, which are released into the extracellular milieu. And while other RPTKs can be activated by free-floating ligands, George Yancopoulos and his colleagues at Regeneron Pharmaceuticals in Tarrytown, New York, showed that ligands of the Eph family must be membrane-bound to turn on their receptors efficiently.

The requirement for Eph ligands to stay put would make them useful in providing very specific spatial cues to axons, which crawl along the surfaces of other cells to reach their targets. In fact, Pawson's group had already suggested that Eph receptors might be involved in axon guidance, based on their discovery last year of an Eph family member they call Nuk on developing axons.

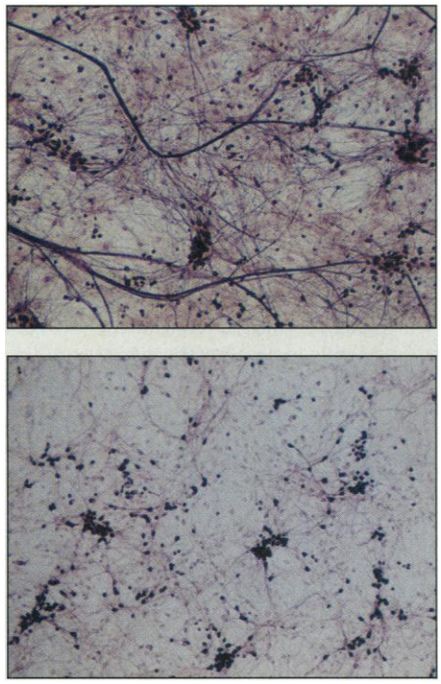
The first concrete support for that role arrived with the report in *Neuron* from Caras, John Winslow, Klaus Beck, and their colleagues at Genentech. The Eph-related receptor they call REK7 is found exclusively on neurons, including neurons from the developing cerebral cortex of fetal rats. They found the REK7 ligand, AL-1, on brain cells called astrocytes, which provide support for growing neurons. Those findings suggested that REK7 and AL-1 might help guide the growth of the neuronal axons.

To test that idea, the Genentech workers cultured the cortical neurons on layers of astrocytes. Under those conditions, the axons grow together in ropy bundles. But when the team blocked the action of either REK7 or AL-1, the axons no longer formed bundles, instead fanning out over the astrocytes. Because the Genentech team was using a simple assay in a culture dish, it is not clear just how their result translates to neurons growing in the brain. But, says Yancopoulos, "they very clearly observed a functional effect." REK7 activation somehow caused the axons to form bundles, and that suggests a role for REK7 and AL-1 in axon guidance.

A new insight into what that role may be came with the *Cell* paper from Bonhoeffer's group. Bonhoeffer's team had spent years studying a region in the chick brain called the optic tectum, which is the destination for axons growing into the brain from the retina. They had several lines of evidence showing that something on tectal membranes guides certain axons by repelling them from places where they shouldn't grow.

The tectum is a two-dimensional oval surface running from back to front along the side of the chicken's brain. In the developing animal, axons from the temporal side of the retina (the side farthest from the nose) avoid the rear of the tectum, growing into the front of the tectum instead. Bonhoeffer's group found that membranes taken from the rear of the tectum repel temporal, but not nasal, axons. To better understand this repelling activity, Bonhoeffer's group set out to purify the protein responsible for it.

Team member Uwe Drescher pulled out a protein last year that seemed to fit the bill and named it RAGS, for repulsive axon guidance signal. When he then went on to clone and sequence the RAGS gene, he found it is the chicken version of AL-1. RAGS has



PHOTOS BY JANET VALVERDE/GENENTECH

Losing their grip. Keeping an Eph receptor from binding its ligand *(bottom)* prevents bundling by cortical neurons on astrocytes.

the right biochemical characteristics to be the unknown factor; it is also arrayed across the tectum in an increasing gradient from front to back, and it repels retinal axons. One problem is that unlike the membrane preparations from the tectum, it repels nasal axons almost as well as temporal axons. Bonhoeffer's group is on the trail of another protein that may increase RAGS's specificity for temporal axons.

Neuronal riches from RAGS

The finding that RAGS repels axons sheds new light on the Genentech result. It suggests that when the AL-1 on the cultured astrocytes activates REK7 on the axons, it causes the axons to be repelled from the astrocyte surfaces. If that is true, says Caras, "it could be that when two axons meet [in the culture dish], they decide they are happier to grow on each other, which is a way of avoiding the repellent substrate." That may explain why the axons form bundles.

Although RAGS's exact role in the tectum is not resolved, one thing is clear: RAGS isn't the only Eph receptor ligand in the optic tectum. In last year's wave of ligand gene-cloning, Hwai-Jong Cheng, a graduate student in Flanagan's group at Harvard, found a molecule called ELF-1 that is a ligand for Sek and Mek4, two other Eph-family receptors found in the nervous system. Cheng subsequently discovered high concentrations of ELF-1 in the optic tectum of chicken embryos. That suggested that ELF-1 may play a role in guiding retinal axons, and when he looked at the neurons of the developing retina that send axons to the tectum, he found that they have the corresponding receptors, Mek4 and Sek.

Moreover, as Cheng, Flanagan, and their co-workers report in the 11 August issue of *Cell*, when they checked the distribution of receptors and ligands, they found that receptors for ELF-1 (which could be Mek4, Sek, or other receptors that bind ELF-1) were most concentrated in axons from the temporal part of the retina, and ligands for Mek4 (which may include ELF-1, RAGS, and perhaps others) were most concentrated at the rear of the tectum.

That discovery fulfills a long-standing prediction that such gradients would be found in the tectum, where they would presumably help guide axons, says UCSF's Tessier-Lavigne: "There have been tremendous efforts to identify ligands distributed in gradients on the tectum whose receptors are also distributed in gradients on the retina, and this is one of the first instances where that approach has borne fruit." Researchers must do more work to pin down the exact function of these Eph receptors and their ligands, but it's a pretty safe bet that they are helping the axons reach their targets, says Tessier-Lavigne. One possibility is that such gradients on the tectum provide precise position coordinates to the axons, giving them the equivalent of "latitude and longitude" and helping them find their destinations on the tectum.

The gradients found by Flanagan's and Bonhoeffer's groups may provide the equivalent of latitude. But that raises the question: What gives the axons their longitude? Recent work suggests that answer may be other members of the Eph family.

Yancopoulos and his colleagues at Regeneron showed last fall that the Eph ligands fall into two classes, those that are stuck to the membrane by linkage to a molecule called GPI, and so-called transmembrane ligands that thread right through the membrane. In a paper in the July *EMBO Journal*, developmental biologist Rüdiger Klein of the European Molecular Biology Laboratory in Heidelberg, Germany, and his colleagues show that each group of ligands has a separate set of receptors, and there is little or no cross-reactivity between groups.

ELF-1 and RAGS are both GPI-anchored ligands. To set up the other axis on the tectum, Klein offers, "it could be that you use a transmembrane ligand with its corresponding receptor." That turns out to be more than mere speculation. In a paper in press in *De-*

velopmental Biology, Elena Pasquale of the La Jolla Cancer Research Foundation in California and her colleagues report finding Cek5, an Eph-family receptor that has a transmembrane-type ligand, arranged in a top-to-bottom concentration gradient in the retina, at right angles to the nasal-temporal Mek4 gradient. To try to complete the picture, Pasquale has begun looking to see whether Cek5's ligand is arranged in a gradient along the dorsal-ventral axis of the tectum.

The story of the Eph receptors goes beyond the optic tectum; family members are popping up throughout the nervous system, and in non-neuronal tissues as well. What they are doing there is unknown, but some researchers are finding evidence of a common theme. For example, Greg Lemke's group at the Salk Institute has found Mek4 on motor neurons in the spinal cord whose axons grow out to trunk muscles. They have no evidence yet, but Lemke suggests that the protein may be helping guide the axons to their target muscles.

Other researchers think the receptors do things besides guide axons. David Wilkinson at the National Institute for Medical Research in London and his colleagues have found certain Eph-related receptors in specific segments of the developing hindbrain. In a paper in press in *Development*, they show that Eph family member Sek-1 is needed in fish and frogs to keep the segment boundaries sharp. Sek-1 may do its job by preventing cells from drifting across the boundaries, says Wilkinson, but he favors another explanation in which Sek-1 instead controls the segmental identity of cells. It is too early, he cautions, to propose a single "unified role" for the Eph receptors in directing cell movements.

Biochemist Vishva Dixit of the University of Michigan supports the idea of a unified role and offers additional evidence from his lab. In the 28 April issue of *Science*, his group reported an example of an Eph receptor guiding cell movements outside the nervous system. They showed that activation of Eck on endothelial cells during inflammation causes those cells to migrate into areas where new blood vessels will form. A hint of how the receptor might affect cell movement came last year, when Dixit's group reported that the Eck receptor stimulates phosphatidylinositol 3' kinase, an enzyme that has been implicated in controlling cell movements by causing changes in the cytoskeleton.

Such findings, tantalizing as they are, amount to small bits of a jigsaw puzzle that is far from completed. But considering all the provocative clues emerging about these intriguing receptors, it will not be long before more pieces are fit into place.

–Marcia Barinaga

MALARIA RESEARCH

Inbred Parasites May Spur Resistance

Inbreeding can be dangerous, as everyone from population geneticists to historians of royal families knows, because it can fix deleterious traits such as hemophilia in a population. Now on page 1709 of this issue, Karen Day and colleagues at the University of Oxford suggest another evil role for inbreeding: It may speed the development of drug resistance in populations of malaria parasites in certain parts of the world. Although conventional wisdom blames the evolution of resistance on the widespread use of anti-malarial drugs, Day and colleagues suggest that natural geographic variation in parasite mating habits may also play a role.

These results could have major implications for efforts to protect the 2.1 billion people regularly exposed to malaria, says Donald Heyneman, a parasitologist at the University of California, San Francisco. "General strategies to control malaria in whole regions don't hold much promise," he says, because control programs may have to accommodate the geographic peculiarities of the disease.

MALARIA

Country	Bites per person per year	Parasite genotype per person (avg.)
Papua New Guinea	40–200	1.8
Tanzania	300–3000	3.2

SOURCE: K. DAY, D.WALLIKER

Day and colleagues at Oxford's Wellcome Centre for the Epidemiology of Infectious Disease in England embarked on their project in 1991 in tandem with David Walliker at the University of Edinburgh, who was analyzing the population structure of *Plasmodium falciparum*—the most lethal species of malaria parasite—in Tanzania; his study was published last year. Day and graduate student Rick Paul suspected that parasite mating habits might vary around the world, so they packed off to Papua New Guinea to do a similar experiment with researchers at the PNG Institute of Medical Research.

Their work involved 4 years of hard labor, in part because of the parasite's complicated life cycle. *P. falciparum* moves between *Anopheles* mosquitoes and humans, producing its male and female gametes inside the swollen gut of a mosquito that has just had a blood meal. When gametes of different parasitic strains mate and their genes recombine, novel, multilocus traits—such as drug resistance—can emerge. Day and colleagues dissected 16,000 New Guinean mosquitoes and analyzed the parasites inside the infected ones, studying three genetic loci. They also examined parasites from nearly 400 people—and found striking differences between their results and Walliker's. Only about 15% of the Papua New Guinea parasites were heterozygotes, compared to 65% of the Tanzanian parasites, suggesting much more inbreeding in the New Guinean population. Evolutionary biologist Francisco Ayala of the University of California, Irvine, calls this finding "one of the most important insights on the population structure of *Plasmodium* to date."

Both Walliker and Day say the key reason for the different mating patterns appears to be the local rate of transmission of the disease. In Tanzania, transmission from mosquitoes to humans is very high—people receive between 300 and 3000 infected bites per year. A single human host often carries many different strains of malaria at once, and mosquitoes are likely to take up mixed strains, allowing frequent cross-mating. But although this orgy of genetic mixing may favor the appearance of new multilocus traits, it doesn't help establish such traits in the population, explains Day. The new combinations of genes can be destroyed as often as they are created and may not hold together from one generation to another.

In contrast, in Papua New Guinea, the transmission rate of malaria is 10 times lower, which means more mating between parasites of the same strain. But there's still enough cross-mating to occasionally generate new, multilocus traits. And when these traits do arise, they are more likely to be fixed in the population, Day says. She predicts that where transmission intensity is low and parasites are inbred—as in Papua New Guinea—drug resistance may spread rapidly.

All this has the surprising result that efforts to decrease the transmission rate of malaria may inadvertently accelerate the evolution of drug resistance, says Daniel Hartl, a population geneticist at Harvard University. But, he says, "we will need to learn a lot more about the population biology of the parasite to assess the magnitude of this effect." It remains to be seen how the inbreeding effect compares with the well-known effect of drug pressure in accelerating the spread of resistance, says Walliker. Ayala agrees, saying that similar types of experiments should be conducted in other parts of the world to test Day's predictions. But not without another several thousand mosquito dissections.

–Karen F. Schmidt

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