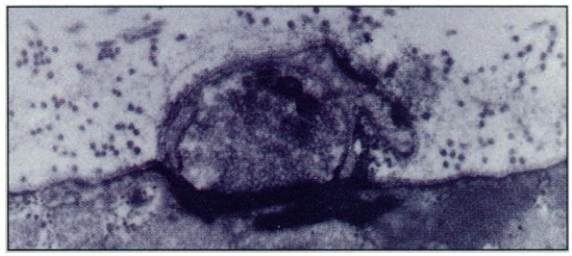
Getting It Together at the Synapse

A protein called agrin, released by nerve cells, helps trigger the formation of machinery by which nerve and muscle cells communicate; it may also play a role in learning and memory

Over the past several years, neurobiologists have begun to assemble a detailed picture of how nerve cells communicate with one another and with other targets such as muscle. They have sketched in dozens of the chemicals that neurons use to transmit their signals, and they have started roughing out the pathways through which these neurotransmitters exert their effects. But a crucial area of the canvas has remained hazy: No one has been able to detail just how nerve cells and their targets establish the specialized connections, known as synapses, where the communication takes place—until recently, that is, thanks to advances in understanding the neuromuscular junction, the synapse that links motor neurons to muscle cells.



Filling the gap. The dark stain shows agrin concentrated in the synaptic cleft between the nerve terminal *(above)* and the muscle cell membrane.

N. REIST *ET AL., J. CELL BIOL.* 105, 2457 (1987)

Researchers working in several labs have shown that when nerve cells make contact with muscle cells, they release a protein that triggers a complex set of changes in the muscle cell membrane. The result: the organization of an intricate piece of synaptic machinery that allows muscle cells to receive and respond to neuronal signals. What's more, researchers are concluding that this protein, known as agrin, may help fill the gaps in a much bigger neuroscience picture: Recent evidence suggests that agrin may contribute to synapse formation in the brain, in which case it might be involved in memory and learning.

But until now, most of the work on agrin has concentrated on the neuromuscular junction, primarily because this synapse is so easy to study. The drawback of brain is the density and complexity of its synapses; even a tiny area can be crammed with thousands, from many different types of intermingled neurons. This has made it difficult to figure out what any one type of synapse is doing. In contrast, each individual muscle cell is innervated by a single nerve terminal—a simplicity that has made the neuromuscular junction what neurobiologist Jack McMahan of Stanford University calls "the synapse of choice" for understanding the fundamental biology of neuronal connections for more than six decades. "We know more about that synapse than any other synapse," he says. Referring to the bacterium that's proved so valuable for understanding molecular biology, he adds: "It's sort of the *E. coli* of synapses."

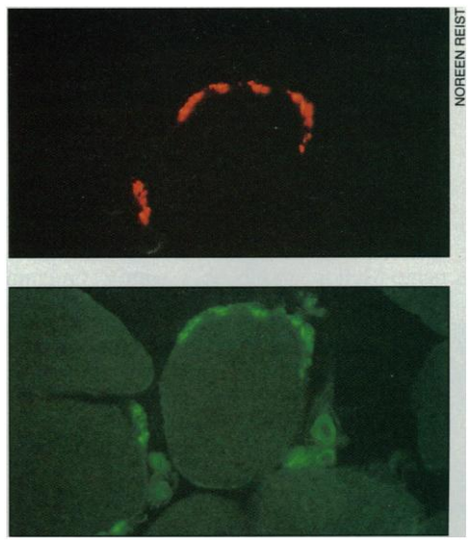
As long ago as the 1930s, work on the neuromuscular junction led to a seminal finding: When a motor neuron fires, the terminal, which is the part that makes contact with the target cell membrane at the synapse, releases the neurotransmitter acetylcholine. The acetylcholine then diffuses the short distance —about 50 nanometers—from the nerve terminal to the muscle cell membrane, where it binds to specific receptor molecules and triggers an impulse that results in muscle contraction.

Although this sequence of events sounds simple enough, some delicate molecular choreography is required to produce a synapse. As McMahan points out: "In order for synaptic transmission to occur, there has to be a lot of machinery for that purpose." On the part of the nerve cell, the machinery includes tiny acetylcholine-containing sacs assembled just inside the nerve terminal so that the neurotransmitter can be instantaneously released when the nerve fires. And on the target cell side, several proteins—including the acetylcholine receptor itself—are highly concentrated at the synaptic site. The receptor density at the synapse, for example, is 10,000 per square micron—roughly 1000 times higher than elsewhere on the muscle cell membrane. The synapse also contains high concentrations of the enzyme acetylcholinesterase, which is needed to break down the acetylcholine and terminate the muscle cell contraction once the nerve stops firing.

Agrin's long history

Researchers got their first clue to what causes the assembly of this synaptic machinery with the discovery some 15 years ago that an interaction between the nerve terminal and the membrane of the target cell is involved. As neurobiologist Stanley Froehner of the University of North Carolina in Chapel Hill notes, the question then was: "How does the nerve signal the muscle cell to assemble the synapse?" Now researchers finally have the answer: by releasing agrin.

Although a direct demonstration of agrin's role in establishing the neuromuscular junction has come only in the past year, the initial experiments pointing to its involvement were performed in the late 1970s by McMahan and his colleagues Steven Burden, Peter Sargent, and Clarke Slater. The group was studying the regeneration of frog muscle cells that had been damaged and consequently had lost their neuronal connections. The basal lamina, a tough sheath of proteins that surrounds every muscle cell, remains intact after the cells degenerate, and new cells can grow back into the old sheaths. When the Stanford workers looked closely at the regenerated cells, they noticed an intriguing pattern: Acetylcholine receptors clustered on the new cells in exactly the same places they had occupied on the old ones—even though there were no nerve terminals to tell them where to form. This suggested that some substance, which had localized on the basal lamina at the original neuromuscular junction locations, remained bound there when the muscle fibers degenerated, and induced the new clusters to form at those precise places. McMahan and his colleagues then set out to identify the substance.



NOREEN REIST

Two for one. Double staining of muscle fibers shows acetylcholine receptor *(above)* and agrin clusters at the same sites.

To accomplish this job, they turned to the electric organ of the marine ray *Torpedo*

californica, which has the same kind of synapses as muscles do, but in much greater numbers. By the mid-1980s, their efforts had paid off: They purified a *Torpedo* protein that induced the clustering of acetylcholine receptors on chick muscle cells grown in lab cultures. Because the protein causes receptor aggregation, they named it "agrin."

Over the next several years, the McMahan team, including Stanford colleagues Lee Rubin, Earl Godfrey, Ralph Nitkin, and Bruce Wallace, built a strong circumstantial case that agrin is one of the major signals used by motor neurons to induce the formation of acetylcholine receptor clusters and other components of the synaptic machinery at the neuromuscular junction. They found, for example, that the protein is made in the cell bodies of motor neurons and is transported down to the terminals of the long axonal projections that the nerves send out to the muscles they innervate. And antibody staining showed high concentrations of the protein in the synaptic cleft, the tiny gap between the nerve terminal and the muscle cell membrane.

But the case for the "agrin hypothesis" wasn't air tight. One problem was that other tissues, including muscle, make agrin too. That led some researchers, such as former McMahan postdoc Justin Fallon, who's now at the Worcester Foundation for Experimental Biology in Shrewsbury, Massachusetts, to raise the possibility that muscle agrin, rather than agrin made by nerve, is what tells the acetylcholine receptors to cluster. Fallon notes, for example, that muscle agrin is also localized at the neuromuscular junction. If muscle agrin does induce the assembly of the synaptic structures, then the researchers would have to go back to the drawing board to find out what the true neuronal signal is.

Work over the past year has, however, removed any doubts about the role of agrin from nerve cells. One piece of evidence came in the spring, when McMahan's group cloned the gene encoding chick agrin. Analysis of the gene sequence showed that the protein has a complicated structure. It contains more than 1900 amino acids and is divided into several subregions or "domains" whose sequences resemble those found in other proteins, including protease inhibitors, epidermal growth factor, and laminin, a protein present in the basal lamina. But especially intriguing was the discovery that the agrin gene could produce slightly different protein products, depending on whether short amino acid sequences were present at two positions in the protein or were left out.

Such variants typically arise as a result of "alternative splicing" in which the coding sequences specifying the amino acid sequences are sometimes cut out of the messenger RNA —the molecule that ultimately directs the protein synthesis—and sometimes left in. Alternative splicing can produce protein variants that differ in activity, and that appears to be the case for agrin. McMahan group members Karl Tsim, Markus Ruegg, Gérard Escher, and Stephan Kröger found that only the form with the inserts at both positions in the protein can induce acetylcholine receptor clustering on muscle fibers. What's more, McMahan says, motor neurons produce this active form while muscle cells don't, which means that nerve agrin appears to be the agent that triggers receptor clustering during the formation of the neuromuscular junction.

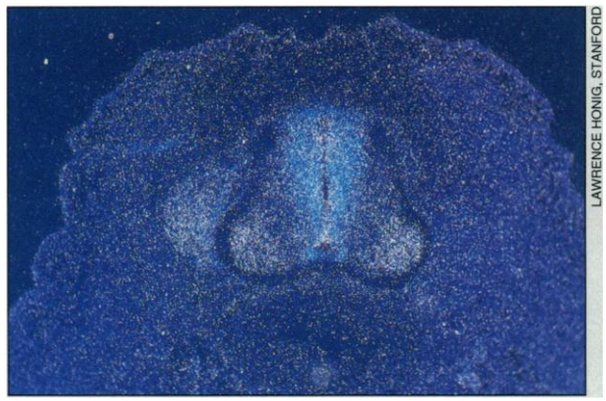
This finding didn't quite clinch the case, however. While parallel research on the rat, by a team led by Stanford neurobiologist Richard Scheller, gave results similar to those with the chick, the distinction between active and inactive rat agrins wasn't quite so clear cut. Scheller's group had previously cloned the rat agrin gene and found that the rat protein closely resembles the one from chick. And in collaboration with Zach Hall's group at the University of California, San Francisco, the Scheller team also found that alternative splicing can give rise to different agrin variants. But the catch is that although those with the inserts in the two positions are the most active in inducing acetylcholine receptor clustering, all the variants have some activity in rat muscle cells, which makes it harder to attribute the clustering to any one particular form.

The clinching evidence that neuronal agrin, rather than the muscle protein, triggers the clustering needed for synapse formation at the neuromuscular junction came from antibody studies performed by two groups. In one set of experiments, McMahan, with Stanford colleagues Noreen Reist and Michael Werle, grew chick muscle fibers in lab culture with either chick or rat motor neurons, both of which can trigger acetylcholine receptor clustering on the chick muscle fibers. The researchers then exposed the cultured cells to an antibody that blocks the activity of chick agrin only. The result: The antibody prevented the receptor clustering in muscle cells cultured with chick neurons, but not in those cultured with rat neurons. In other words, the neuron, not the muscle fiber, was the source of the agrin needed for the clustering.

In a somewhat similar set of experiments, Monroe Cohen of McGill University in Montreal and Godfrey, who is now at the Medical College of Wisconsin in Milwaukee, came to the same conclusion. For example, when they grew embryonic neurons from the frog *Rana pipiens* with muscle fibers from the clawed toad *Xenopus laevis*, and then stained the cells with an antibody to *Rana* agrin, they could clearly see the staining at the synapses. What's more, Cohen says, the staining showed that the agrin was present at the earliest detectable nerve-induced clusters of acetylcholine receptors. "Agrin is supplied to the synapse by the nerve," he concludes. That leaves open the question of what muscle agrin does, although one possibility is that it comes in later to maintain the neuromuscular junction.

Brainy speculations

Now that these experiments have clearly indicated that neuronal agrin helps to establish the neuromuscular junction, researchers are beginning to speculate that the protein could play a broader role. "One wonders," says Scheller, "is agrin involved in organizing neuron-neuron synapses as we think it's involved in organizing the neuromuscular junction? If it is involved in some way, it certainly could be involved in learning and memory." Preliminary evidence suggests that it may be.



LAWRENCE HONIG, STANFORD

Right location. In this cross-section of a 6-day-old chick embryo, agrin gene activity, indicated by darker blue, is high in spinal cord regions containing motor neurons.

McMahan's group, for one, has detected signs that the protein is made in embryonic chick spinal cord and brain cells at a critical period during development when many synapses are forming. And not only do neurons other than motor neurons contain agrin, they also have the active form with the inserts. "This raises the possibility that agrin is needed for synapse formation throughout the nervous system," McMahan says.

Moreover, other cell components needed for agrin activity also appear to be present in the brain. Evidence from researchers including Wallace, who's now at the University of Colorado Health Sciences Center in Denver, and Fallon indicates that agrin must bind to its own receptor on muscle cells in order to trigger the clustering of the acetylcholine receptor and other synapse proteins. That means that researchers can at least consider the possibility that agrin's action isn't limited to the neuromuscular junction, Fallon says: "If it bound directly to the acetylcholine

receptor you could think it was a special case. But the evidence is building for an effect in brain." Indeed, with Worcester colleague Mary Nastuk, Fallon has detected agrin receptors on some spinal cord neurons and on brain "synaptosomes," which are membrane preparations with a high synapse content. "The actors—agrin and its receptor—are there [in brain] now," says Fallon, although he cautions that they haven't yet been shown to contribute to synapse organization in brain.

The finding that agrin works through its own receptor also provides some clues to how the protein brings about the assembly of synaptic structures. Since it doesn't interact directly with the acetylcholine receptor, it apparently exerts its effect through its own intracellular signaling pathway. One clue to what this pathway might include comes from Wallace and Rick Huganir of Johns Hopkins University School of Medicine.

They've found that one of the protein subunits that make up the acetylcholine receptor is rapidly phosphorylated on the amino acid tyrosine when chick muscle fibers are treated with agrin. Such tyrosine phosphorylations are a common way of controlling the activity of proteins, and in this case the phosphorylation appears necessary for the clustering of the receptor. Wallace and Huganir suggest that the tyrosine phosphorylation may be what enables the acetylcholine receptor to bind to the cell skeleton proteins, which are thought to anchor the receptors in place at the neuromuscular junction.

The researchers still have a long way to go to work out how agrin exerts its effects. But if the protein proves to be as important for formation of neuron-neuron synapses as it is for the neuromuscular junction, it's a safe bet that agrin will wind up with a prominent place in the emerging picture of nerve cell communications.

–Jean Marx

Additional Reading

M. J. Ferns and Z. W. Hall, "How Many Agrins Does It Take to Make a Synapse?" *Cell* **70**, 1 (1992).

U. J. McMahan *et al.*, "Agrin Isoforms and Their Role in Synaptogenesis," *Current Opinion in Cell Biology* **4**, 869 (1992).

F. Rupp *et al.*, "Structure and Chromosomal Location of the Mammalian Agrin Gene," *Journal of Neuroscience* **12**, 3535 (1992).

BIOCHEMISTRY

From 'Hunter Magic,' a Pharmacopeia?

Every time Peter Gorman looks at his scars, he remembers the time 6 years ago when, near the Peru-Brazil border, a Matses Indian introduced him to "hunter magic." After burning Gorman's arm with a white-hot stick, his mentor spit onto a stash of dried frog secretion to make a paste and rubbed it into the burns. For 15 minutes, the amateur anthropologist and writer from New York City vomited, defecated, urinated, and drooled while his heart beat furiously and sweat streamed from every pore. "I was hoping and praying that I would die," he recalls. After this wrenching episode, he remembers falling into a day-long listlessness. Finally he woke up feeling transformed. "When you wake...you feel god-like," he says, as if your senses have been sharpened and your strength enhanced.

GORMAN/LAVALLE

The magic touch. Before releasing this frog into the forest, a Matses Indian harvests hunter magic.

To the Matses Indians of Peru and several other indigenous groups in western Amazonia, that's exactly how you want to feel before a hunting expedition, and they have perpetuated the practice of "taking frog"—secretions scraped from the skin of the large green frog *Phyllomedusa bicolor*—for generations. Now it seems that the Amazonian potion might work some high-tech magic as well. In the 14 November *Proceedings of the National Academy of Sciences (PNAS)*, bioorganic chemist John Daly of the National Institutes of Health and seven coauthors report finding in the concoction a peptide, 33 amino acids long, that may provide a key to manipulating cellular receptors for the ubiquitous biomolecule adenosine.

The possibility is of more than academic interest. Preliminary animal studies by researchers at the Warner-Lambert Co. have hinted that those receptors, which are distributed throughout the brains of mammals, could offer a target for treating depression, stroke, seizures, and cognitive loss in ailments such as Alzheimer's disease—"it goes on and on," says Daly. As a result, the discovery of a natural compound that interacts with the receptors is catching the attention of Daly's pharmacologist colleagues. "It represents what appears to be a new class of pharmacological mediator," remarks biochemist Michael Zasloff, who recently founded Magainin Pharmaceuticals Inc., which develops drug leads from the vast, barely catalogued library of animal chemical defenses.

The first hints that the frog secretions might contain such a peptide, dubbed adenoregulin in the *PNAS* paper, showed up about a year ago when Daly began experimenting with samples donated by Gorman and Katharine Milton, an anthropologist at the University of California, Berkeley, who, like Gorman, had observed Indian groups in the Amazon jungle taking frog. Daly injected extracts of the frog secretion into a mouse, a common practice for testing whether substances have biological effects. "I saw a profound behavioral depression," Daly recalls, one that resembled what he and others had seen before in mice injected with adenosine and its derivatives.

Daly's team set out to look for the suspected adenosine-like ingredient in the samples of hunter magic. After fractionating extracts to separate the brew's many chemical components, the researchers found one fraction that stood out. Other fractions inhibited the binding of adenosine-receptor agonists—molecules, such as adenosine itself and its analogues, that bind to and activate the receptor. But the adenoregulin-containing fraction enhanced the binding, presumably by subtly altering the receptor's chemical personality to make it more receptive to the agonists. That property makes the compound an exciting pharmacological lead, Daly thinks. After all, he notes, the successful anti-anxiety drug Valium acts on a different class of receptors by much the same mechanism.

If adenoregulin doesn't turn out to be a pharmacological boon, other possibilities may be lurking in the frog potion. As pharmaceutical chemist Vittorio Erspamer at the University of Rome and his co-workers wrote in a 1985 analysis of peptides in *Phyllomedusa* skin, "No other amphibian can compete." Their list totaled about 19 peptides, but the Italian group and other workers including Daly's group have since pushed the number up to several dozen.

Something—maybe a lot of things—in that brew took Gorman and his Matses drug mentors on their pharmacological wild ride. But Milton isn't sure the concoction always lives up to its name, at least among the Mayoruna people she studied. She's never observed its effect on hunting. But she did notice that the Indians tended to perform the ritual on rainy days, when hunting was at best a second string activity. "It seemed more like something to do," she remarks.

–Ivan Amato