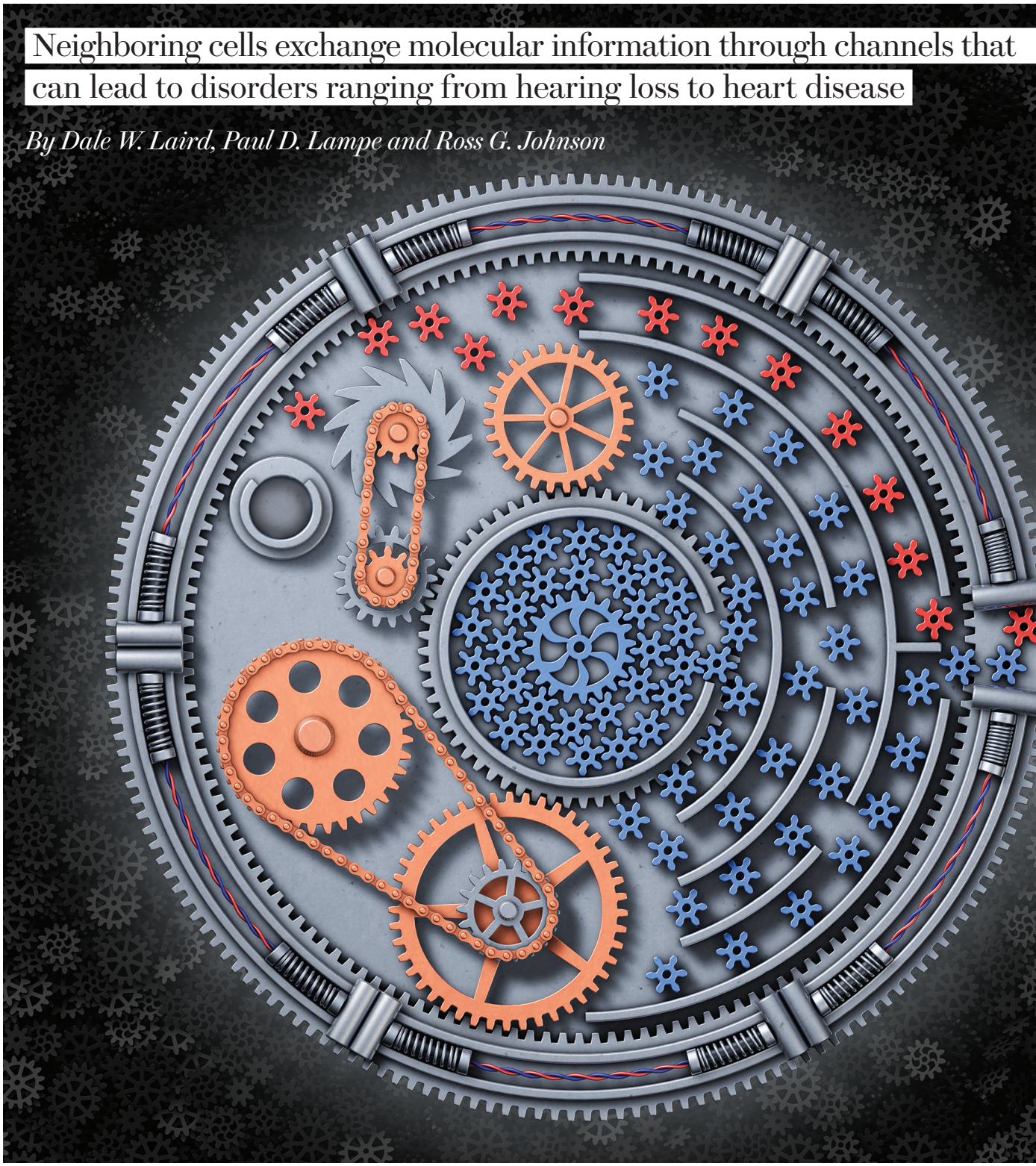
BIOLOGY

CELLULAR

Neighboring cells exchange molecular information through channels that can lead to disorders ranging from hearing loss to heart disease

By Dale W. Laird, Paul D. Lampe and Ross G. Johnson



SMALL TALK

directly connect them. Disruption of this signaling system

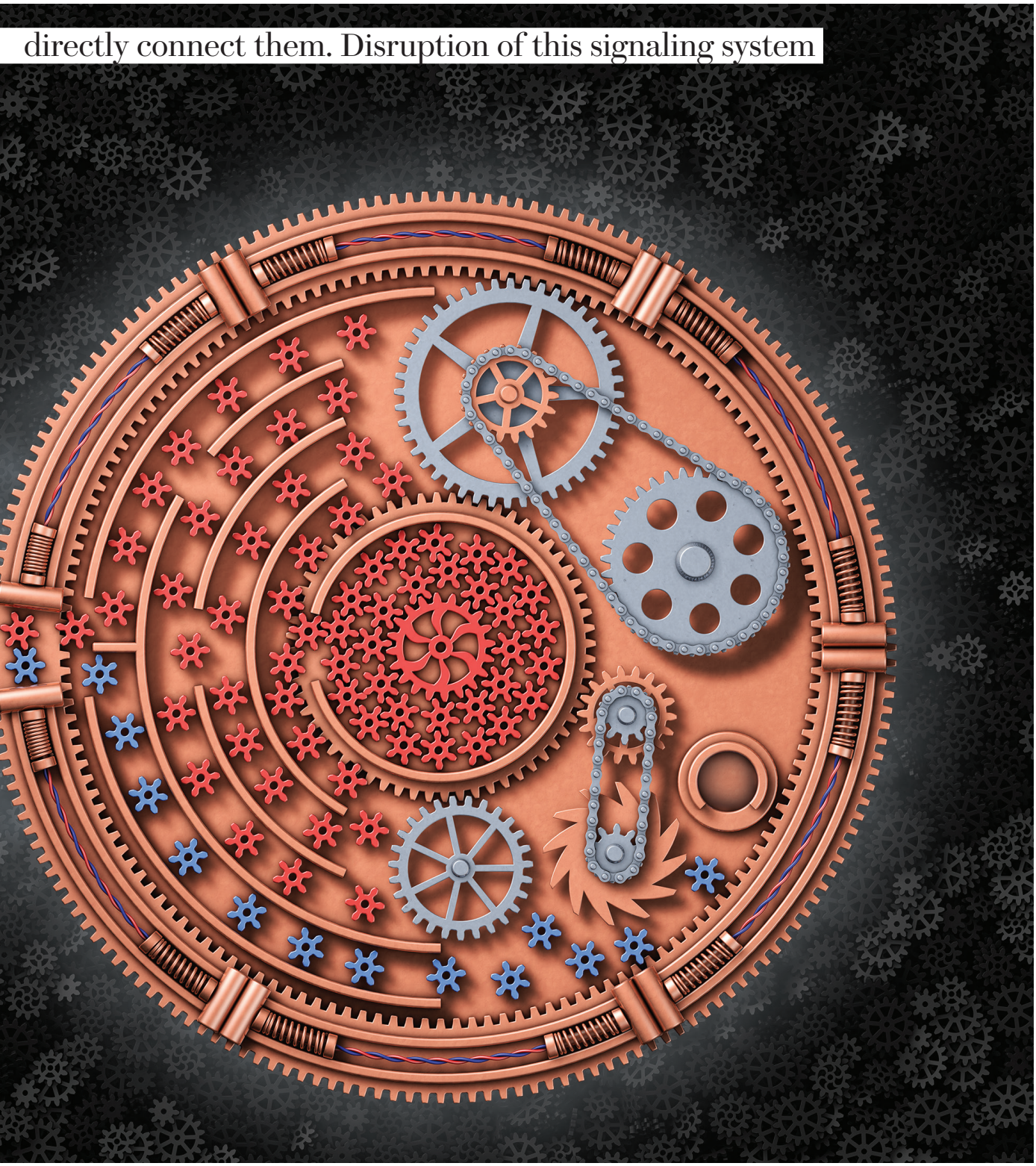


Illustration by Peter Kraemer

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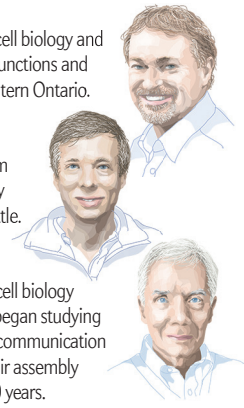
Ross G. Johnson is a professor emeritus of genetics, cell biology and development at the University of Minnesota. He began studying gap junctions in the 1960s, shortly after these cellular communication channels were discovered, and has been exploring their assembly and regulation with Laird and Lampe for more than 20 years.



LIKE PEOPLE WHO SHARE NEWS VIA TWITTER, FACEBOOK AND LINKEDIN, CELLS, too, make use of multiple modes of information exchange. Some send out hormones, which travel far and wide via the bloodstream; others emit neurotransmitters, which carry signals between one neuron and another. But virtually all cells, it turns out, network with their neighbors via extensive collections of channels that directly connect the inside of one cell with the inside of the next.

Investigators got a dramatic glimpse of this form of cell-cell communication in the mid-1960s, when they injected fluorescent dye molecules into an individual cell amid a sea of closely packed cells. Peering through a microscope, they saw the fluorescence spread rapidly from one cell to the next until sometimes hundreds of cells in the tissue were aglow. Previous studies had suggested that ions could convey electrical signals between neighboring cells. But observing the spread of dye molecules, which are small but larger than ions, confirmed beyond a doubt that cells harbor channels through which molecules pass in abundance between adjacent cells.

Biologists now know that these channels are everywhere. Collections of them occur in the tissues of all animals, including humans, where they participate in an extraordinary variety of functions. The collections, called gap junctions, help to synchronize the beating of muscle cells in the heart and the contraction of the uterus during childbirth. Gap junctions allow the eye to adjust to different levels of light. They even play a role in organ formation during embryonic development.

Over the past 20 years scientists have discovered that defects in the assembly or activity of gap junctions contribute to a range of human diseases, including hearing loss, cataracts, skin conditions, neurological disorders, heart disease and even certain cancers. A single mutation affecting a constituent protein of a gap junction in the inner ear accounts for hearing loss in up to 40 percent of individuals with inherited deafness. And new diseases linked to gap junctions are being discovered all the time—several in just the past few years, including a type of epilepsy that strikes children.

Now studies are providing exciting insights into how gap junctions are built, and they are beginning to reveal how disruption of gap junction assembly and activity precipitates disease. The findings should lead to new therapies for many disorders that result when cells can no longer share "inside" information.

BUILDING BRIDGES

INVESTIGATORS WERE NOT THINKING in terms of medical relevance back when the early dye injection experiments were first performed. In the 1960s and 1970s they were focused on uncovering further evidence of this mysterious neighbor-to-neighbor

IN BRIEF

Cells exchange information with their immediate neighbors through gap junctions—structures that directly connect one cell to another. These "conversations" are involved in everything from the synchronized beating of heart cells to our ability to hear.

Although gap junctions are complex and often made of more than 100,000 individual proteins, they are taken apart and rebuilt continuously. This carefully controlled restructuring allows cells to respond rapidly to injury or stress.

Mutations in the genes that encode gap junction proteins lead to a range of human conditions, including skin disorders, heart disease, epilepsy and deafness. Learning how these defects affect the assembly and activity of gap junctions should lead to new treatments.

communication between cells and on learning more about its properties. Before gap junctions were identified and named, physiologists found that this sharing of molecular information occurs in a variety of organs and organisms, from squid embryos and electric fish to an assortment of mammalian cells. And they confirmed that the molecules do move directly between cells at points where their membranes come in close physical contact.

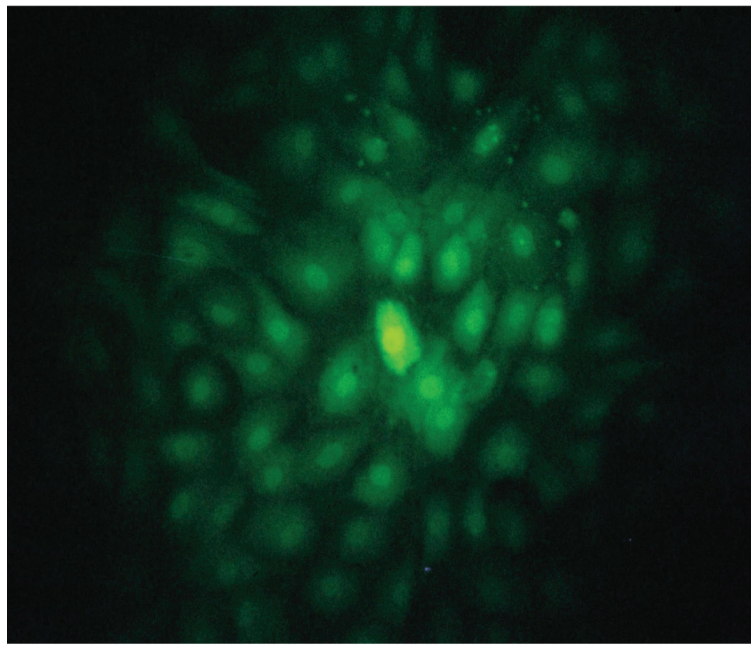
By the late 1960s scientists had their sights set on determining what the channels look like and how they form. Earlier microscopic studies had revealed the presence of large, flat patches where the membranes of two neighboring cells lie closely apposed. At these junctions, the cells appeared to be separated by a very thin gap, just a few nanometers wide, leading the structure to be called a gap junction. The name stuck even after investigators discovered that this narrow space is not empty but is filled with the parts of the channels that connect one cell to another.

To explore the role that these flat membrane patches play in the "cellular connectivity" seen in the dye and electrical experiments, one of us (Johnson) began studying what happens as these junctions are assembled. Along with his colleagues at the University of Minnesota, Johnson gently teased apart cultured cells derived from a liver tumor and then mixed them back together. Within minutes, flattened patches arose on the cell membranes but only where the two cells made contact. This observation confirmed a suspicion that gap junction assembly is a joint project that requires the collaboration of adjacent cells. As these flattened patches expanded and matured, the electric current passing between the cells also increased. These junctions, it seemed, could facilitate the exchange of ions.

Peeling apart the membranes of the connected cells to take a closer look, Johnson and his team saw what appeared to be large particles that had accumulated within the flattened patches. These particles, it later turned out, were the channels that are the very building blocks of the gap junctions [*see box on next page*]. Each channel is formed from molecules called connexins, which belong to a family of proteins identified in the late 1980s.

Six connexin proteins come together to form a doughnut-shaped structure called a hemichannel. This hemichannel gets inserted into the cell's outer membrane, where it can then interact with a hemichannel in a neighboring cell. When the connexins in these matching hemichannels interact, they form a continuous pore that connects the cells in a way that puts the cytoplasm of one cell in direct communication with the cytoplasm of another. This pore is, in effect, a single gap junction channel, hundreds or thousands of which aggregate to form each gap junction.

Building these enormous communications conglomerates is a massive undertaking for cells. A single gap junction can contain 10,000 channels. Because each gap junction channel involves two hemichannels, that would make a total of 120,000 connexins per junction. The heart alone contains billions of cells, each one of which interacts with several of its neighbors via gap junctions. The assembly of these colossal structures, in other words, is a marvel of molecular engineering.



DYE INJECTED into a cell in a culture (*center*) quickly crossed into other cells by way of gap junction channels that link neighboring cells.

COURTESY OF MICHAEL ATKINSON AND JUDSON SHERIDAN *University of Minnesota*

Even more remarkable is that gap junctions are not permanent or even long-lived but are continuously taken apart and rebuilt. It has been shown that half the connexins in a cardiac gap junction are replaced every two hours. Over the course of a day every single gap junction in the human heart is most likely torn down and replaced with channels that are newly assembled.

Given the complexity of these extraordinary structures, it seemed likely that systems must exist to ensure that their construction runs smoothly so that cell-cell communications are not lost. To get a handle on these regulatory mechanisms, the three of us, who were all studying gap junctions, chose to combine our expertise; in particular, we wanted to explore how the assembly and removal of these extensive communication channels are controlled.

COMING TOGETHER

WE HATCHED OUR PLAN to collaborate over coffee at a conference on gap junctions at the Asilomar Conference Center in Pacific Grove, Calif. It was 1991, and one of us (Lampe) was a postdoctoral fellow in Johnson's lab at the University of Minnesota, where the focus had turned to the regulation of gap junction assembly. The final member of our trio (Laird), then a postdoc in Jean-Paul Revel's lab at the California Institute of Technology, had just developed a set of antibody molecules that bound specifically to connexin proteins. These antibodies would allow us to interact with connexins and see which bits of the molecules are critical for gap junction formation and activity.

Laird's antibodies recognized one particular type of connexin: Cx43. Humans have genes that encode 21 different connexin proteins, and each cell type produces its own characteristic set of connexins. Skin cells, ambitiously, make up to nine different

connexins. Cx43, though, is the most widespread member of the family and is present in many organs, including skin, heart, brain, lungs and bone.

Cx43, like all connexins, consists of four membrane-spanning segments that anchor the protein in the cell membrane. The protein's tail, which dangles inside the cell, contains a variety of elements that we would later determine are involved in regulating its activity and assembly into channels and junctions. And two loops made by the protein as it weaves in and out of the membrane protrude into the space between the cells. Some of the antibodies that Laird had generated homed in on these extracellular segments.

Because the loops stick out from the surface of the cell, it seemed reasonable to think that they might function as Velcro-like hooks that enable connexins to latch onto one another. To examine that supposition, we again teased apart cultured cells and then mixed them back together—but this time we added in Laird's antibodies. Now gap junctions did not form at all; we saw no cell-to-cell transfer of injected dye and no flattened patches characteristic of developing gap junctions. By sticking to the loops, the antibodies had prevented the connexins in one cell from "docking" with the connexins in the neighboring cell.

Such antibody studies demonstrated that connexin attachments are critical to the construction of gap junctions. But a different technique was needed to watch connexins in real time as they made their way around a living cell.

FOLLOW THAT PROTEIN

IN 1994 THE THREE of us found ourselves together at another conference—this time the American Society for Cell Biology's meeting in San Francisco. Over late-night chats about the presentations we had heard, we became increasingly excited about green fluorescent protein (GFP)—a molecular tool whose usefulness would ultimately be recognized by a Nobel Prize. One speaker at the meeting described how she had linked this fluorescent marker to her favorite protein to track its travels in live cells. So we wondered whether our research groups could use GFP to monitor the movements of connexins.

We began by tacking GFP to the tail ends of the connexins. To our great delight, this approach worked well: the tagged connexins were inserted correctly into the cell membrane, where they assembled into functional gap junctions that exhibited nearly all the properties of those built from unmodified connexins. We now had a powerful way to observe the behavior of connexins inside cells—work that Laird continued at his new position at the University of Western Ontario.

Our very first observations were surprising. We initially took pictures of the cells containing tagged connexins every 10 minutes, thinking we could stitch these stills together to make a time-lapse movie highlighting connexin movement. But the connexins traveled so quickly that we could not tell which protein was which or where each had gone. We tried again every two minutes, but that time frame still was not good enough. To watch individual tagged molecules as they trucked around the cell, we ended up having to collect images every few seconds.

The resulting movies allowed us not only to follow connexins but also to watch hemichannels as they were transported inside cells along molecular tracks made of structures called microtubules. We and others saw that smaller gap junctions can

BASICS

The Making—and Breaking—of Gap Junctions

Cells continously and rapidly build up and remodel their gap junctions (*bottom right*). Buildup begins with the synthesis of proteins called connexins (*1*), their arrangement into structures called hemichannels (*2*) and insertion of the hemichannels into the cell membrane (*3*). If hemichannels encounter existing gap junctions, they can dock with hemichannels in a neighboring cell, forming new passageways between the cells (*4*). Meanwhile cells remove and degrade old gap junction channels (*5* and *6*).

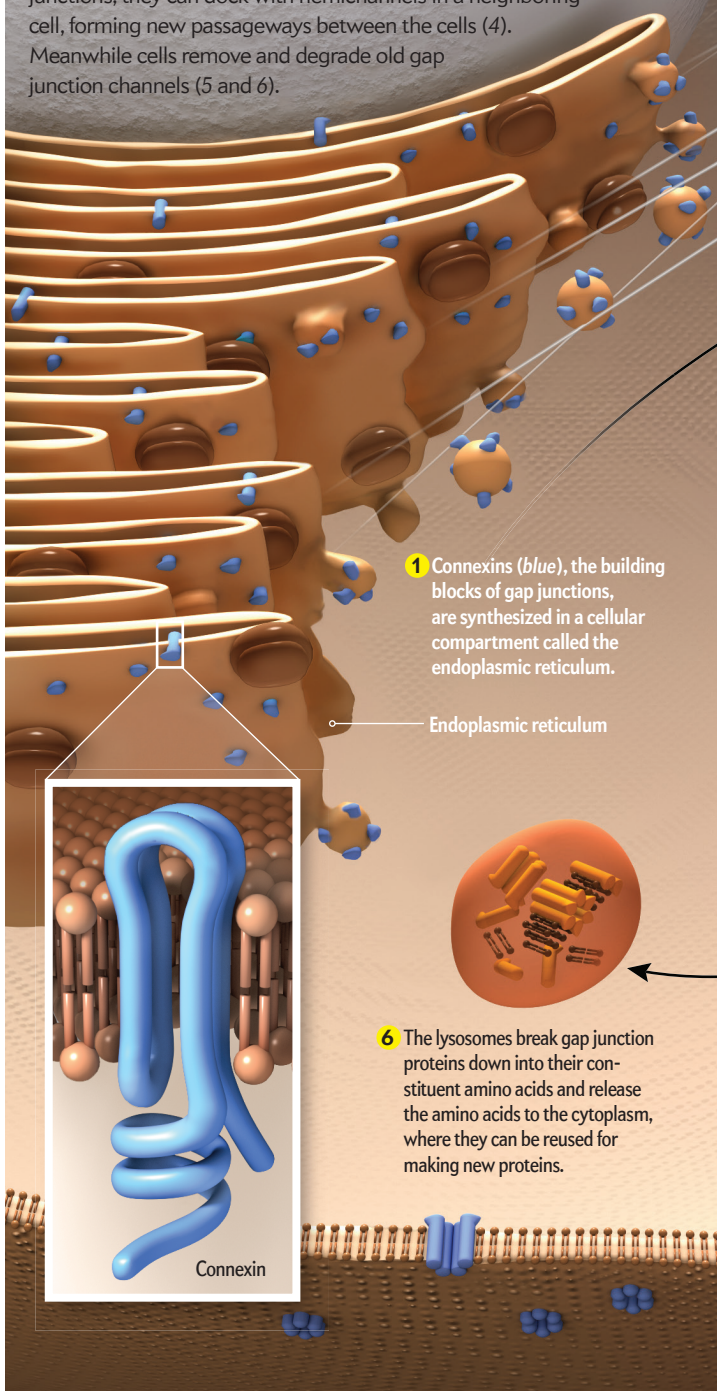
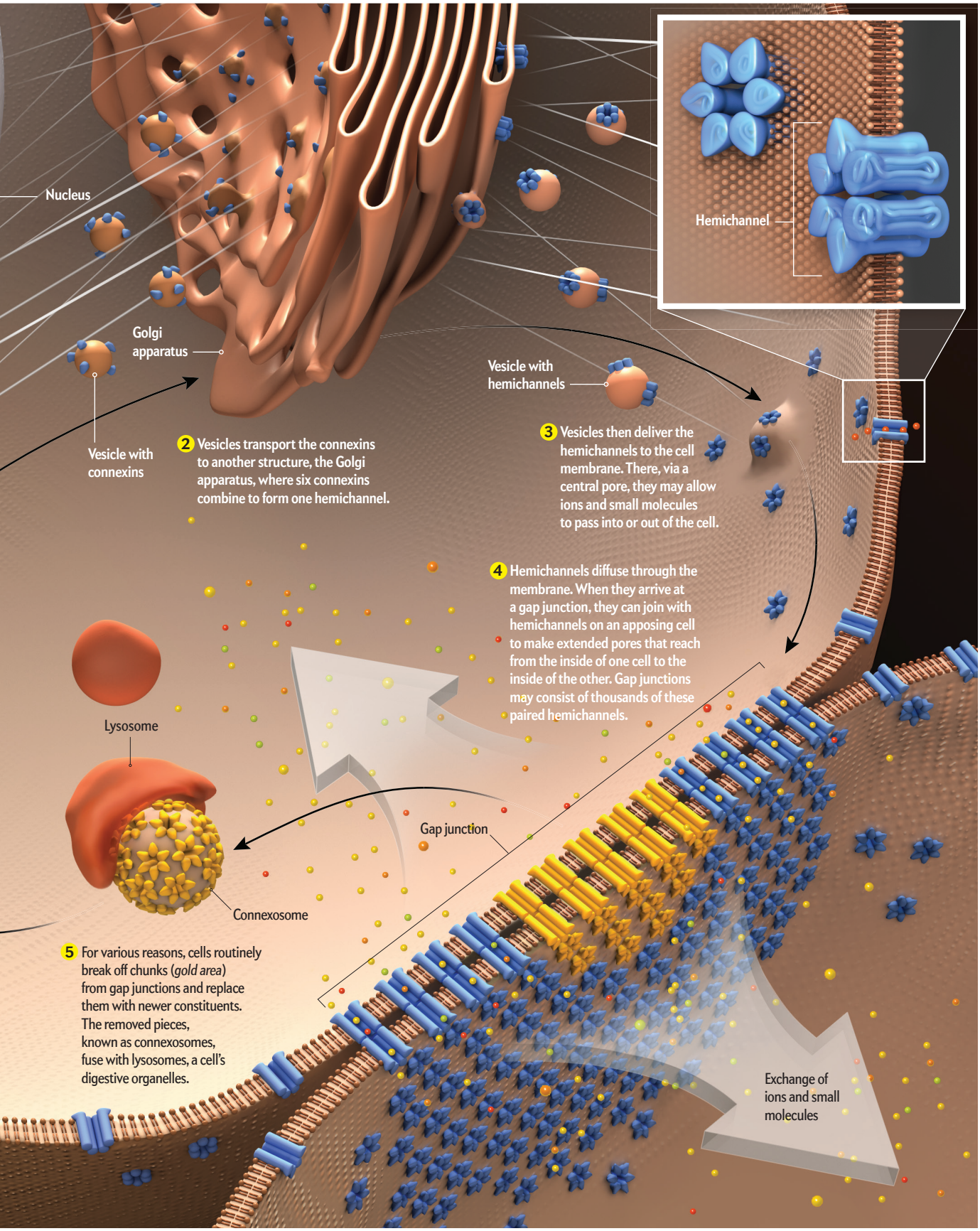


Illustration by Emily Cooper


Nucleus
Golgi apparatus
Vesicle with connexins
2 Vesicles transport the connexins to another structure, the Golgi apparatus, where six connexins combine to form one hemichannel.
Vesicle with hemichannels
3 Vesicles then deliver the hemichannels to the cell membrane. There, via a central pore, they may allow ions and small molecules to pass into or out of the cell.
Hemichannel
4 Hemichannels diffuse through the membrane. When they arrive at a gap junction, they can join with hemichannels on an apposing cell to make extended pores that reach from the inside of one cell to the inside of the other. Gap junctions may consist of thousands of these paired hemichannels.
Lysosome
Gap junction
Connexosome
5 For various reasons, cells routinely break off chunks (*gold area*) from gap junctions and replace them with newer constituents. The removed pieces, known as connexosomes, fuse with lysosomes, a cell's digestive organelles.
Exchange of ions and small molecules

combine to form larger ones—something we had gleaned hints of in our electron microscopic studies. We also demonstrated that larger gap junctions can break apart to form smaller ones, a kind of gap junctional remodeling that takes place as the cells grow, move, flex and divide.

Our colleagues devised more methods to tag connexins and found that gap junctions grow by the addition of hemichannels to their perimeter—so that the central part of a gap junction represents the "oldest" section of the plaque. These older components appear to be removed as gap junctions age, an observation that could explain how gap junctions remain established even as the connexins within them are lost; newer channels crowd inward to replace the channels that are older.

Perhaps the most striking finding from our live-cell studies was that gap junctions can also be reclaimed by the cell in large chunks, when one of the participating cells essentially takes a bite out of its neighbor, a mechanism others had proposed based on some earlier electron microscopic images. This radical maneuver ingests the junctional components contributed by both cells in one fell swoop—a process that may provide a fail-safe way to rapidly shut down communication between two cells when it is no longer desirable. Such large-scale elimination of gap junctions actually takes place in the uterus after childbirth, disabling the communication networks that have formed to coordinate contractions.

TAKING CONTROL

KNOWING THAT GAP junctions constantly get renewed, we next set out to explore how cells might supervise this massive molecular resculpturing. Early studies pointed to proteins called kinases as the regulators. By the simple step of adding phosphate groups to a target protein, kinases can alter that protein's activity or location within the cell.

Our challenge was to figure out whether protein kinases also regulate the behavior of connexins and, if so, what exactly this phosphorylation does. Lampe took the lead in this set of studies when he set up his lab at the Fred Hutchinson Cancer Research Center in Seattle in 1994. By taking apart Cx43 and examining the protein one small piece at a time, Lampe and his colleagues discovered that, over its life span, this connexin gets phosphorylated at up to 15 different places along its tail. This information allowed us to work out some of the regulatory code that controls the formation of gap junctions containing Cx43. When specific kinases act on certain parts of the protein tail, the modification enhances the assembly of Cx43-containing junctions; other kinases acting on different parts of the tail inhibit junction formation, activity or size.

Insights into the regulatory code are now making it possible for us to examine human tissue samples for clues to how changes in phosphorylation might alter the way gap junctions are assembled and function in response to injury or during disease. We and others have begun to ascertain, for instance, how communication through gap junctions changes when cardiac cells are trying to recover from oxygen deprivation after a heart attack or when cells in the skin mobilize to heal a cut or scratch. In both cases, Lampe's team found that phosphorylation increases on one particular site in Cx43's tail. This phosphorylation briefly boosts the size of gap junctions in these tissues by preventing Cx43 from interacting with a protein (studied by other groups) that restricts the ability of new connexins to integrate into existing junctions. The resulting increase in gap junction size serves to increase communication—which is critical in the first minutes after injury—and helps to preserve function in cardiac tissue and to facilitate the migration of the skin cells needed to close an open wound.

Knowing how phosphorylation affects connexin assembly and function—and how gap junction communication changes in response to injury or disease—has opened a door to the development of therapeutics that can promote or inhibit the activity of the relevant protein kinases. Such treatment strategies must be pursued with caution, however, because an increase in gap junctions during one stage of disease may prove harmful later on. For example, although gap junctions briefly enlarge immediately after an injury, they are later rapidly degraded to promote proper healing. In people who have diabetes, wound closure is delayed by a continued overproduction of Cx43. And when the cornea is scratched, connexins can promote inflammation and scarring rather than healing. In these cases, limiting the production or function of Cx43 in the cells surrounding an injury promotes the rapid repair of wounds without scars—an approach that is being pursued by several biotechnology companies.

Studying how mutations affect gap junctions could lead to therapies

To fully capitalize on our knowledge of gap junction biology for designing effective therapies, though, investigators need a deeper understanding of how connexins come together in different tissues under different conditions—and how their aggregation and activity go awry in the face of disease. The study of disease-causing mutations in the genes that code for connexin proteins is beginning to offer some useful insights.

INTERRUPTING COMMUNICATIONS

INVESTIGATORS UNCOVERED the first solid genetic evidence that connexins can participate in disease in the mid-1990s. Mutations in the gene encoding Cx32 were found to cause one form of a neurological condition called Charcot-Marie-Tooth disease. In this disorder, gap junctions disappear in the myelin sheath that insulates nerves, causing the myelin to degrade and leading to nerve degeneration; that loss, in turn, precipitates muscle atrophy and weakness, particularly in the limbs.

With the discovery that mutations in connexin genes have serious physiological consequences, the gap junction field witnessed an explosion of interest from researchers and clinicians intent on determining the genetic basis of these diseases. Additional studies turned up new connexin mutations, and today 14 different disorders are known to stem from defects in gap junction connexins.

The most striking thing about this collection of conditions is how different they are from one another. In addition to the neu-

rodegenerative Charcot-Marie-Tooth disease, mutations in connexins can underlie hearing loss, epilepsy, heart disease, skin ailments, cataracts and a variety of disorders that arise during embryonic development. As might be expected, mutations in different connexins contribute to different diseases. But in a more surprising finding, it turns out that mutant connexins do not necessarily afflict all tissues or organs equally; if a particular mutant is produced in two organs, it might impair function in one but not the other.

Many research groups are laboring to understand this phenomenon. One explanation could be that in certain tissues, other healthy connexins can compensate for a defective variant, allowing gap junction communication to continue at an adequate level. Such a compensation mechanism may occur in some tissues but not others. Or perhaps a particular connexin plays one role in one tissue but different roles in others, depending on which other connexins are present. The various connexin family members can also intermix, yielding hybrid channels that could facilitate the passage of different molecular signals—some of which are more important in one tissue than another.

that counteract these defects without triggering serious side effects.

For some connexins, however, defects do compromise multiple tissues. Take a disorder we study, called oculodentodigital dysplasia (ODDD). People with this condition, caused by mutations in the Cx43 gene, display a range of symptoms, including small eyes, underdeveloped teeth, skeletal deformities in the face and head, and webbing between the fingers or toes. As if that were not enough, some affected individuals develop a skin condition that produces thickened, scaly callouses on the palms of the hands and the soles of the feet. Recent studies on the life cycle of connexins have offered some hints as to why some people have a more severe form of the disease than others.

More than 70 mutations in Cx43 have been found in people with ODDD, and we began by exploring what these mutations do to the protein—and how they affect the construction of gap junctions. Laird and his colleagues have found that many of the mutations in the Cx43 gene result in a connexin that reaches the cell membrane but does not form a functional gap junction; dyes do not flow through these junctions from cell to cell, indicating that the gap junction channels are either not properly assembled or not allowing the molecular signals to pass. Either way, these mutations diminish cell-cell communication.

Other ODDD mutations prevent connexins from ever reaching the cell membrane. Patients harboring these mutations generally have the more severe form of the disease, including the skin condition, along with other defects. This finding suggests that connexin hemichannels might have a job beyond their role in building gap junctions and that when this job goes undone—as happens when connexins never get to the cell membrane—more severe problems arise. Perhaps, for instance, instead of pairing up to form channels, some hemichannels remain uncoupled, allowing cells to release signals or to take up molecules from their environment. These molecules may be different from the ones that normally pass through gap junction channels. Such hemichannel activity has been demonstrated experimentally, adding a new dimension to our understanding of the role that connexins play in cell communication. Further studies of mutant hemichannels could reveal new targets—including as yet unidentified molecules that pass through uncoupled hemichannels—for the treatment of ODDD or other connexin-based disorders.

TELLING SECRETS

STUDYING HOW MUTATIONS affect the construction and behavior of gap junctions could also lead to highly targeted therapies that counteract the effects of a mutation without triggering serious, unwanted side effects. Knowing, for example, that a particular mutation alters the assembly of a gap junction—but not the transport of connexins to the cell surface—could point the way toward a drug that might restore the ability of the connexin to form a functional channel. Such targeted therapies could provide a way to reestablish cell-cell communication without having to replace the mutant connexin entirely—a process that would involve gene therapy, an approach that is still risky and experimental.

Discovery of disease-causing mutations in connexins does more than provide promising therapeutic targets. It gives investigators a novel set of tools for studying the basic biology of gap junctions. We still do not have a complete understanding of the specific molecules that pass between cells via gap junctions, for example. In the case of heart cells, we know that the ions that flow through gap junctions carry an electrical signal from cell to cell. But we have little idea of what passes between cells to support the function of, say, the hearing apparatus in the ear or the wound-healing response in skin. By seeing how the connexin channels behave in different cells and how changes in their assembly and activity can invoke disease, we will finally be able to address the most fundamental questions about this intimate form of cell communication: What exactly are cells whispering to one another, and how do these molecular secrets govern the assembly and operation of complex creatures—including ourselves? SA

MORE TO EXPLORE

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