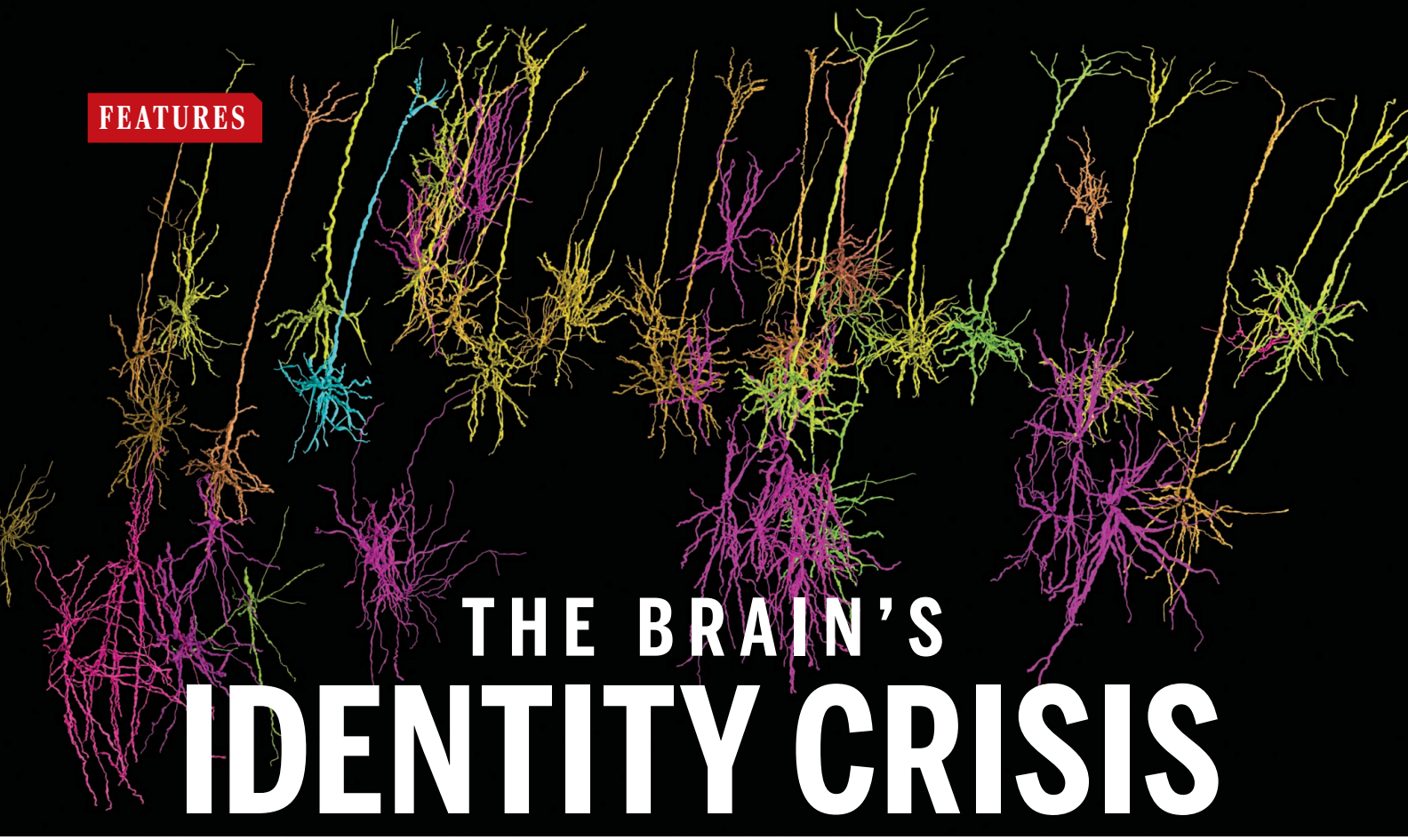

THE BRAIN'S IDENTITY CRISIS

Will new tools for classifying neurons put a 150-year-long debate to rest?

By **Emily Underwood**

The tiny Spanish town of Petilla de Aragón boasts no Marriott or Hyatt conference center. The medieval red tile-roofed village has about 30 year-round residents, and the nearest hotel that can accommodate a large group can only be reached via a winding, roughly hour-long drive along the southern slopes of the Pyrenees mountains.

Still, when Rafael Yuste, a veteran brain researcher at Columbia University, and Javier DeFelipe of the Cajal Institute in Madrid were searching for a place to gather some of the world's top neuroscientists in 2005, the town seemed an ideal location. They hoped that a pilgrimage to the birthplace of Santiago Ramón y Cajal—born in 1852 and considered by many to be the founding father of neuroscience—would inspire humility in attendees as they tackled a more than century-old debate: how to categorize a dizzyingly varied class of brain cells called interneurons. One of the building blocks of brain circuits, interneurons help keep brain activity in check and, when they malfunction, play a role in diseases such as epilepsy, schizophrenia, and autism.

Cajal was the first to identify what are now called interneurons—he called them "short-axon cells"—using a technique developed in 1872 by physician Camillo Golgi, which stains cells black by impregnating them with silver nitrate. So varied were the cells' structures in the human cortex—some look like stars, others like bird's nests, baskets, or spindles—that Cajal divided them into dozens of subtypes. He argued that the diversity of the cells, which he called the "butterflies of the soul," was the key to higher cognition.

Many researchers followed in Cajal's footsteps, identifying scores of new interneuron types based on features such as the shape of a cell's branches or the neurotransmitters it secretes. They rarely applied quantitative measurements or consistent criteria, however. As a result, by the time of the 2005 meeting in Spain, the literature on interneurons had grown so cluttered with overlapping and contradictory terminology that it was practically unintelligible, says Giorgio Ascoli, a neuroinformaticist at George Mason University in Virginia, who attended the conference. "It was like the Tower of Babel."

Does a neuron's shape determine its identity, or its behavior? Neurons in the mouse visual cortex, colored by firing speed: Magenta cells are slow-firing, yellow medium, blue fast.

Ascoli and many of the other neuroscientists who gathered in Cajal's hometown were confident that they could tidy up the problem by the end of the long weekend. "We thought, we are all rational people, we all believe in data, we will create labels and codes and agree to them, and make the Babel tower issue go away." But by lunchtime on the first day, he says, it was clear that the task with which they were charged was "simply put, impossible."

Neuronal classification—not just of interneurons, but of cells throughout the brain and the overall nervous system—is one of the oldest and most controversial problems in neuroscience. With the notable exception of the retina, in which roughly 60 neuronal types have been identified, and the 302 neurons of a worm called *Caenorhabditis elegans*, scientists don't agree on how to identify most neurons. "To the embarrassment of thousands of neuroscientists, we still lack the basic knowledge

PHOTO: ALLEN INSTITUTE FOR BRAIN SCIENCE

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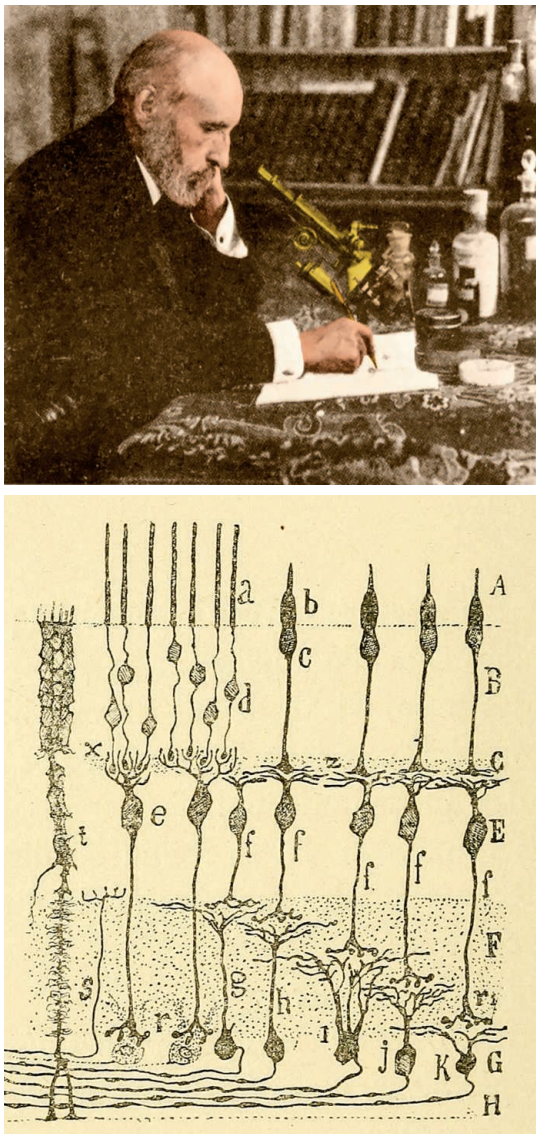
of how many types of neurons exist," says Peter Somogyi of the University of Oxford in the United Kingdom. That leaves them trying to make sense of the complex ecosystem of the central nervous system with only the most basic idea of the species making it up.

Lately, this problem has attracted a surge of attention from research efforts such as the U.S. Brain Research through Advancing Innovative Neurotechnologies (BRAIN) initiative, which, among other goals, aims to create a "census" of cells from brain tissue across different species. Without such a census, many researchers believe that BRAIN's primary goal of decoding complex cognitive processes by recording the activity of thousands to billions of neurons will be impossible, says Joshua Sanes, a molecular neurobiologist at Harvard University. "The recordings will be anonymous, and it will be a big mess," he says.

Scientists would also like to be able to pin down specific cell types that play a role in neurological disorders, or in the brain's responses to certain drugs. In a disease such as amyotrophic lateral sclerosis, for example, a subset of brain cells that helps control movement is obviously compromised. For other disorders, such as autism and schizophrenia, however, the neurons involved in producing symptoms are "utterly obscure," Sanes says.

He and many others now believe that a breakthrough in neuronal classification is imminent thanks to developments since the Spain meeting, such as new methods that rapidly sample gene activity from thousands of individual neurons, revealing patterns that distinguish cell types. Some see the quest to categorize as a fool's errand, however. "Evolution did not create cell types," but rather neurons whose gene activity adapts to their environment, argues Gordon Fishell, a neuroscientist at New York University (NYU) in New York City. But others say the dispute over whether neurons have well-defined identities is, as neuroscientist Amy Bernard of the Allen Institute for Brain Science in Seattle, Washington, puts it, "the kind of fight that happens when you don't have the right data."



Santiago Ramón y Cajal (top) considered the retina the most beautiful structure in the nervous system, and illustrated dozens of cellular subtypes within its layers (bottom).

THIS CONFUSION WAS ON FULL DISPLAY at the Petilla de Aragón meeting. After giving up hope of agreeing on a classification system for interneurons, Yuste says, attendees instead came up with 500 terms to describe the cells' various qualities, such as the length and sinuosity of their dendritic branches. "It was a very dry list," says György Buzsáki, a neuroscientist at NYU.

The group did not attempt to address a deeper rift, dating back to Cajal, between "lumpers," who tend to focus on commonalities between neurons, and "splitters," who tend to divide cells into many subcategories based on subtle differences. The most extreme lumpers argue that there are only two types of brain cells, aside from blood vessels: neurons and glia. Extreme splitters say that there are as many cell types as there are cells in the brain.

Lumpers have long accused classical neuroanatomists of being "like stamp collectors," who simply "want to collect as many stamps as they can," says Christof Koch, scientific director of the Allen Institute. Lumpers consider Cajal "a pathological splitter," says Richard Masland, a neuroscientist at Harvard Medical School in Boston.

Cajal never knew to what extent the cells he examined were representative of the broader populations of neurons, because of the haphazard nature of the Golgi technique, which only stains a few random neurons in every slice of brain tissue. As a result, many scientists are skeptical of his big, beautiful catalogs of neuronal classes. Today, Masland adds, the best evidence that Cajal was "not hallucinating" his many subtypes comes from the retina—the poster child for neuronal diversity. Of all the brain's structures, Cajal considered the retina the most beautiful, and published an entire book illustrating the cells within its five distinct layers and identifying dozens of cell subtypes.

Yet the conventional wisdom about the retina in the 1970s was that the structure was "relatively simple," Masland says. Most scientists believed that it had only five types of neuronal cells: rods and cones, which respond to different wavelengths of light; ganglion cells, which receive input from the rods and cones; bipolar cells, which connect the rods and cones to the ganglion cells; and amacrine cells, the interneurons of the retina.

"A huge turning point," Masland says, occurred in the 1970s, when researchers recording cells' electrical activity found that these basic cell classes could be subdivided by function. Among ganglion cells, for instance, a dozen or so groups could be distinguished based on their sensitivity to stimuli such as the direction of motion and changes in light intensity. In the 1980s, Masland and others found further evidence of diversity by showing that subpopulations of neurons expressed different neurotransmitters, such as acetylcholine and serotonin. Today, most neuroscientists accept that there are not just five, but at least 60 different neuronal cell types in the retina. "And that's the lower limit," Masland says.

Having at least some well-defined neuronal classes in the retina makes it "a great place to start" when testing new methods for cellular classification, Sanes says. In recent years, his group and many other labs have turned to what some consider the most promising marker of cellular identity: gene activity. In May, Sanes and his colleagues published in *Cell* their first test run of a method that many in the field hope make it possible to automatically classify neurons en masse.

PHOTOS: (TOP TO BOTTOM) SCIENCE SOURCE; OPEN KNOWLEDGE COMMONS/HARVARD MEDICAL SCHOOL

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Developed by geneticist Steven McCarroll and others at Harvard, the technique, called Drop-Seq, can quickly profile all the messenger RNA (mRNA), a reflection of which genes are being expressed, in thousands of individual brain cells. It starts by packaging each cell in a nano-sized oil droplet—a kind of nano test tube. After breaking open each cell and attaching a bead labeled with a unique barcode to all the RNA within, Drop-Seq sequences the genetic data in bulk.

The barcoded beads allow scientists to know from which cell each sequence came, Sanes notes. "Previously, if you wanted transcriptomic profiles for 10,000 cells, you'd have to use 10,000 test tubes." In the new study, Sanes and colleagues used the method to analyze the gene activity of more than 44,800 cells from a mouse retina. Using a machine-learning algorithm to sort through the cells' mRNA sequences, they identified 39 distinct subtypes within the 5 major classes of retinal cells. "It's not perfect yet—the computer tells you there are fewer cell types than we think are there—but it does get the main classes," Sanes says.

Other labs are performing similar analyses in lesser-known brain regions. This spring, neuroscientist Sten Linnarson of the Karolinska Institute in Sweden used a related but slower technique to analyze mRNA from more than 3000 individual cells within the mouse cortex, including interneurons and nonneuronal cells such as blood vessel and glia. After comparing the activity of 20,000 genes in each cell, he and his team identified 47 different subgroups (*Science*, 6 March, p. 1138). Single-cell RNA sequencing is "powerful and cheap enough to provide a definitive breakdown of the cell types that are in any given chunk of tissue all at once," says Linnarson, who expects the technique will be quickly adopted in "every little corner of the brain."

ALTHOUGH MASLAND CALLS RNA sequencing "probably the ultimate way to classify cells," he and others acknowledge that investigators don't know which genes are key to distinguishing neuronal type. One approach to identifying them is to trace a neuron's lineage from its birth onward, monitoring gene activity to see which ones turn on or off as the cell follows its developmental path.

At the Janelia Research Campus in Virginia, for example, neuroscientist Tzumin Lee is tracing the development of every neuron in the fly brain. Lee and his team label each of the larval fly's 100 neuroblasts, or neural stem cells, with a genetic marker that is transmitted to the next cell generation every time the cell divides. Then the team harvests RNA from developing flies at various points to track changes in gene activity as the insects' brains mature. Once the project is complete, the team will have a complete map of gene expression in the fly brain, including about 24,000 neurons. The approach "gets to the mechanism of how you create neuronal diversity," says neuroscientist Gerry Rubin, executive director of the Janelia Research Campus.

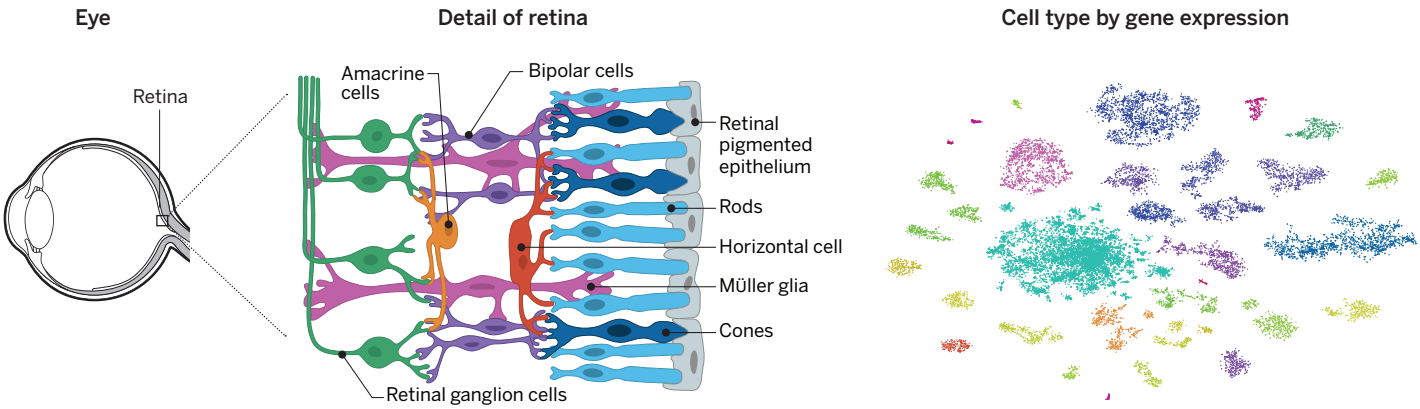
Masland, however, isn't persuaded that knowing the developmental trajectory of a cell will reveal how to classify it. "If you're trying to fix your car, you don't necessarily need to go back to the factory to understand its parts." And both RNA sequencing and analyses of cellular lineage miss what Somogyi calls the "most complex and beautiful issue" that defines a neuron's function: how its firing patterns change over time. Neuronal identity changes from moment to moment, Somogyi says, giving them "this incredible flexibility on the one hand, and reliability on the other."

Reliably classifying neurons will ultimately require multiple kinds of data—on cells' morphology, electrical behavior, and gene activity—says Andrea Beckel-Mitchener, director of the functional neurogenomics program at the National Institute of Mental Health in Bethesda, Maryland. The cell census-related grants the National Institutes of Health has begun funding on behalf of the BRAIN project aim for diversity: Just half of the 10 focus on RNA transcription, whereas the others explore variations in neuronal function. Similarly, the Allen Institute is collecting multiple kinds of data on cortical neurons—first electrophysiological and morphological measurements and ultimately single-cell genetic data. "The goal is to see whether every cell falls into one unambiguous class, like one of the 93 chemical elements," Koch says.

Ascoli and many other researchers also want to automate the process, using machine-learning algorithms to crunch through massive data sets without bias. Even if such algorithms can produce reliable cell classes, however, it's not clear what they'll ultimately tell us about how the brain works, Bernard says. Despite having a complete taxonomy of the neurons in *C. elegans*, scientists still don't have a full understanding of how the tiny worm's brain works, after all. That, Bernard notes, "doesn't lead me to believe this is an easy problem." But few would deny that knowing what makes up a brain is a good start. ■

Eying a way to ID neurons

The retina has five broad neuronal types—rods, cones, horizontal cells, bipolar cells, amacine cells, and ganglion cells (middle). Using a technique called Drop-Seq, researchers recorded gene activity in 44,800 individual cells from a mouse retina and, with the help of a machine-learning algorithm, sorted the cells into 39 distinct subtypes based on those data (right).



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